Patrick L. Iversen

Molecular Basis of Resilience

Adapting to a Changing Environment

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Preface

I spent over 40 years involved in diverse biological research projects. Each project focused my attention to explore subjects in greater depth. The diversity exemplified differences in ecological studies from molecular biology and studies at the organismal level. Each discipline is made of a community of talented investigators that create a distinct culture and inherent boundaries. My enthusiasm led to a desire to work across boundaries such as joining molecular biology with pharmacology and infectious disease with toxicology. Reflection has revealed a ribonucleic acid as a common feature naturally integrating core elements of multiple disciplines.

The bulk of my career involved RNA-based therapeutics. Initially, the concept was limited to disruption of the "Central Dogma" by interfering with translation of mRNA to protein. A compelling transition to oligonucleotide-induced exon-skipping expanded therapeutic options and paralleled the growing appreciation of the importance that transcription of most of our DNA genome to RNA that does not encode protein. However, once I observed oligonucleotide induced translation start-site shifting, I realized significant limitations in simply evaluating RNA expression. The consequences of shifting translation start-sites are opaque to technologies involving the transcriptome. I realized phenotype diversity is transient and extends far beyond counting genes in DNA sequence.

I would like to encourage integration of infectious disease into our evaluation of the environment. The potential for chemicals in the environment to influence the diversity of infectious disease perhaps driving creation of new emerging infectious agents but clearly influencing pathology. The appreciation that resilience is an essential part of host-pathogen interactions which are also responding to changing environmental influences.

My many fruitful interactions with colleagues have led to a desire to collect and integrate observations from my career into a book. The idea is to provide a novel perspective in the hope that scientists and curious people will build upon my observations to better understand the human as a part of the environment. I hope the concept of molecular resilience is not only informative but reveals optimism in biological adaptation.

I would like to acknowledge the inspirational contributions of Laurie, my wife, and family, the mentorship of Professors Barry Quinn, Martin Rechsteiner, Lester Partlow, Glen Hanson, Michael Franklin, and Edward Bresnick, and the instructional interactions with Drs. Ronald Hines, Pete Gannet, John Mata, John Desjardins, Brian Copple, Todd Page, Manuchair (Mike) Ebadi, Gerald Zon, Sina Bavari, Dan Mourich, Denis Burger, Dwight Weller, Dave Hindrichs, F. Joseph Daugherty, Larry Smith, Craig Marcus, and Andrew Annalora. I have also benefited from interactions with Thomas Stewart, Tommy (Smart Tom) Stewart, Alan Timmins, Dave Stein, Tim Geiser, Mark Arneson, Chris Dargon, and Dave Benneth.

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A	Adenine/Adenosine
AAV	Adeno-associated virus
Ach	Acetylcholine
AChR	Ach receptor
AChE	acetylcholinesterase
ADA	Anti-drug antibodies
ADD COMM	FDA advisory committee
ADE	Antibody-dependent enhancement
AHR	Ary hydrocarbon receptor
AICD	Activation induced cell death
AIDS	Acquired immunodeficiency syndrome
ALL	Acute lymphoblastic leukemia
AMKL	Acute megakaryoblastic leukemia
AML	Acute Myelogenous Leukemia
ANA	Anti nuclear antibodies, also anti DNA antibodies
APC	Antigen presenting cells
APOBEC	apolipoprotein B mRNA editing enzyme
ATM	Ataxia telangiectasia gene
ATRA	All trans retinoic acid
BCE	Before Christian Era
BCR	B-cell receptor
C	Cytidine/Cytosine
^{14}C	Radioactive isotope of Carbon
°C	Centigrade measure of temperature
CDC	Centers for Disease Control
CDER	Center for Drug Evaluation and Review
CEO	Chief Executive Officer
Ci	Curies, also mCI- milliCuries, μCi-microCuries; a measure of radioactivity
CML	Chronic myelogenous leukemia
CMV	Cytomegallovirus

CO	Carbon monoxide
CRADA	Cooroperative Research and Development Agreement
CRIPR	Clustered regulatory interspersed palindromic repeats
CVA	Coxsackievirus A
CVB	Coxsackievirus B
CYP	Cytochrome P450
DBD	DNA binding domain
DC	Dendritic cell
DCM	Dilated cardiomyopathy
DDT	Dichloro-diphenyl-trichloroethane
DEN	Dengue virus
DES	Diethylstilbesterol
DHF	Dengue hemorrhagic fever
DMBA	Dimethylbenzanthracine
DMD	Duchenne Muscular Dystrophy
DNA	Deoxyribonucleic Acid
DOD	Department of Defense
DSS	Dengue shock syndrome
EAV	Equine Arterivirus
EBV	Epstein Barr Virus
EctSC	Extodermal stem cell
EDC	Endocrine disrupting chemical
EID	Emerging Infectious Disease
EJC	Exon junctional complex
EPO	Erythropoietin
FDA	Food and Drug Administration
FeCV	Feline Calicivirus
FGF	Fibroblast growth factor
FLUA	Influenza A virus
FMDV	Foot and mouth disease virus
FMT	Fecal microbiota transplant
G	Guanine/Guanosine
GFP	Green fluorescent protein
GHI	Global Health Index
GR	Glucocorticoid receptor
GSHV	Ground Squirrel Hepatitis Virus
GST	Glutathione transferase
GVHD	Graft versus host disease
GWAS	Genome Wide Associative Studies
^{3}H	Tritium, radioactive isotope of Hydrogen
HAT	Hitone acetyl transferase
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
hCG	Human chorionic gonadotropin
HGPRT	Hypoxanthene Guanine Phosphorisobsyl Transferase

HGPS	Hutchinson-Guilford progeria syndrome
HGT	Horizontal gene transfer
HHV	Human Herpesvirus
HIV	Human Immunodeficiency Virus
HLA	Human Lymphocyte Antigen
HPLC	High pressure liquid chromatography
HPV	Human Papillomavirus
HRV	Human Rhinovirus
HSC	Hematopoietic stem cell
HSV	Herpes Simplex Virus
HTLV	Human T-cell Lymphotropic Virus
HUS	Hemolytic uremia syndrome
^{125}I	Radioactive isotope of Iodine
IARC	International Agency for Research on Cancer
IFN	Interferon; α-alpha, β-beta, and γ-gamma
IgG	Immunoglobin
IL	Interleukin
IND	Investigational New Drug Application
IOC	International Olympic Committee
IRES	Internal Ribosomal Entry Site
JEV	Japanese encephalitis virus
K	Potassium
LASV	Lassa virus
LCMV	Leukochoriomenigitis virus
LD	Lethal Dose, LD_{50} is a dose lethal to 50 percent of a population
LINE	Long interspersed nuclear elements
lncRNA	Long Noncoding Ribonucleic Acid
LSS	Life Span Study
LTR	Long terminal repeats
Mab	Monoclonal antibody
MARV	Lake Victoria Marburg Virus
MDR	Multiple drug resistant
MDS	Myelodysplastic syndrome
MEH	Microsomal epoxide hydrolase
MERS-CoV	Middle East Respiratory Syndrome coronavirus
MeV	Measles virus
MFO	Mixed function oxidase
mg	Milligram
MHV	Mouse hepatitis virus
MIC	Minimum inhibitor concentration
miRNA	Micro Ribonucleic Acid
mL	Milliliter measure of volume
MSC	Mesenchymal stem cell
MSTN	Myostatin gene
NA	Neuraminidase

Na	Sodium
NAT	N-acetyl transferase
NAS	National Academy of Science
NDM	New Delhi metallo-beta-lactamase
NHR	Nuclear hormone receptor
NIH	National Institutes of Health
NK	Natural killer T-cells
NMD	Nonsense Mediated Decay
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NO	Nitric oxide
NoV	Norovirus
NPC	Nasal Pharyngeal Carcinoma
NRTI	Nucleoside reverse transcriptase inhibitors
NSAID	Non-steroidal anti-inflammatory drugs
OSL	Oseltamivir- Tamiflu
ORF	Open Reading Frame
^{32}P	Radioactive isotope of phosphate
PAH	Polycyclic aromatic hydrocarbons
PAMP	Pathogen-associated molecular pattern
PB	Phenobarbital
PCR	Polymerase chain reaction
pfu	Plaque forming units
PhAC	Pharmaceutically active compounds
PMO	Phosphorodiamidate Morpholino Oligonucleotide
POTUS	President of the United States
PRR	Pattern recognition receptor
PSO	Phosphorothioate Oligonucleotide
PTEC	Proximal tubule epithelial cell
PRRSV	Porcine respiratory and reproductive virus
RABV	Rabies virus
RERF	Radiation Effects Research Foundation
RdRp	RNA dependent RNA polymerase
RIG-I	Retinoic acid-inducible gene 1
RNA	Ribonucleic Acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RotV	Ratavirus
RRM	RNA sequence recognition motiff
RVFV	Rift Valley Fever Virus
RXR	Retinoid X receptor
^{35}S	Radioactive isotope of sulfur
ssRNA	Single-stranded RNA; (+) positive strand, (-) negative strand
SA	Exon splice acceptor
SAD	Single ascending dose
SARM	Selective androgen receptor modulators

SAR	Structure activity relationship
SARS-CoV	Severe Acute Respiratory Distress Syndrome coronavirus
SD	Exon splice donor
SEP	Someone Else's Problem
SINE	Short interspersed nuclear elements
siRNA	Small interfering Ribonucleic Acid
SLE	Systemic Lupis Erythemotosis
SRSF	Serine arginine splicing factors
STEC	Shiga toxin producing Escherichia coli
SV40	Simian Virus 40
T	Thymine/Thymidine
T1D	Type 1 diabetes
TB	Tuberculosis casued by MTb- mycobacterium tuberculosis
TCR	T-cell receptor
TERT	Telomerase reverse transcriptase
TF	Transcription factors
TLR	Toll-like receptors
TMD	Transient myeloproliferative disorder
tRNA	transfer Ribonucleic Acid
U	Uridine/Uracil
^{235}U	Radioactive isotope of Uranium
U5MR	Under age 5 Mortality Rate
UNMC	University of Nebraska Medical Center
USAMRIID	United States Army Medical Research Institute for Infectious Diseases
UTR	Untranslated region
VDR	Vitamin D receptor
VEEV	Venezuelan Equine Encephalitis Virus
VOC	Volatile organic compound
VZV	Varicela-Zoster Virus
WARF	Wisconsin Alumni Research Fund
WHO	World Health Organization
WHV	Woodchuck Hepatitis Virus
WNV	West Nile Virus
XDR	Extensively drug-resistant
YFV	Yellow Fever Virus
ZEBOV	Zaire ebolavirus

Chapter 1
Prologue

Abstract All living things are associated with a boundary defined ecological niche. Steady state conditions are rarely constant but evolutionary adaptation is too slow to adapt to daily threats so a surrogate variation mechanism is necessary. The genome defines the most basic instructions for life so that a molecular biology perspective provides the foundation for understanding resilience. Variations in the expression of RNA offers rapid variation and this book proposes this is the basis of resilience. This book attempts to illuminate mechanisms of resilience beginning with elaborating threats leading to disruption in steady state conditions. Recognition of threats and defense systems are described followed by adaptive changes in gene expression that refine responses. Finally, environmental conditions are discussed that serve to dampen the adaptive response oscillator to disruptive threats at the level of RNA expression.

This prologue is intended to acquaint the reader with my background and the genesis of optimism for an idea that the benefit of transcriptome plasticity is resilience. I grew up in several National Parks, remote regions of the United States that are set aside to preserve natural environments. I attended 12 schools by the time I graduated from high school, a fact that forced me to develop personal resilience. My career path as a scientist followed a path from ecologist to pharmacologist to molecular biologist. I was a professor that transitioned to biotechnology ensuring research subjects involving very diverse in subject matter so I appreciate the value of plasticity.

Keywords Pharmacology · Drug metabolism · Chemical carcinogenesis · Molecular biology · Cytochrome P450 · Exon skipping · Resilience

Background

I have been a scientist as long as I can remember. Before starting school in Grand Canyon Arizona, I caught a horned lizard (*Phrynosoma*) and kept it in a box as a pet. These lizard's squirt a stream of blood from the corner of the eye as a novel defense strategy. I was in love. The park rangers visiting our house would tell stories of

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toads and ants in the region, they convinced me to let the lizard go free. A park naturalist showed me how to lure an ant lion from his sandy cone with a blade of grass. The enthusiasm for living things and excitement adults expressed over this special place established a foundation for my life in science.

My family moved to the Petrified Forest National Monument after I completed first grade. One of the first few days after arrival, I caught a snake in the desert just outside our house. I instinctively avoided the head so I carried the snake by its tail. I had no idea what kind of snake I had, in fact I did not know there were different kinds of snake. I brought the snake home to show to the family. A new discovery was that my brother expressed severe anxiety over the snake and my proximity to him, he ran. I then discovered my mother also shared a deep fear of snakes. They encouraged me to let the snake go free but the incident was not complete until I got a snake lecture from the park naturalist. The impromptu lecture was amazing; we looked at about a half-dozen different snakes in glass enclosures including the local rattlesnake. The concept of an animal capable of delivering a poison was mind expanding.

The years in the Painted Desert planted the seeds of the resilience of life. As I grew older, my journeys into the desert become longer with greater range from home. My parents concern for my explorations centered on my lack of social development. They began exploring boarding school options for my brother and I but we stayed to attend our one-room school. Monsoon-like rains arrived late in the spring leaving pools of water in the barren clay soil. My brother and I found a muddy pool filled with hundreds of tadpoles, possibly the canyon treefrog (*Hyla arenicolor*). We watched these tadpoles mature over a two-week period and all were gone, buried in the mud, when the pool dried up. The desert provides numerous examples unique life and resilience strategies.

I had freshwater aquariums from sixth grade. The aquarium experience included routine cleaning and removal of algae from the glass at times. I added catfish to the aquarium to reduce the algae but they did not completely remove the green haze from the glass. I added snails and more living plants and found the algae problem solved. An early lesson in the living interplay, ecology. The 9th grade scientist in me and availability of a plastic sphere inspired an idea of a self-contained ecological system. Gravel in the bottom, living plants, snails, and two fish were added to the fresh water system. I cut a hole in the top so a rubber stopper with a glass pipe could allow for pressure equalization. Those fish were still alive in an algae free plastic sphere after two years when I had to take the experiment apart. I see it is now possible to purchase self-sustained aquaria on the internet, confirmation that this is routinely accomplished. These self-sustained systems represent a robust demonstration of the balance of nature.

Hiking in the high desert of Arches National Park near Moab Utah, I observed hanging desert gardens suspended in cliffs hundreds of feet above the valleys below. Juniper trees and sage brush growing in desert sand in a physically isolated space. Access available to birds and insects carried by the wind but a system in balance for dozens of hundreds of years. The message is that life will find a way. How do living things prepare or adapt to isolated environments?

My first real world research position was with Gradient Modelling, Inc. in Glacier National Park for the summer of 1975. Gradient Modelling was the creation of Stephen R. Kassel, a recent Cornell University Ph.D. under the direction of R. H. Whittaker. Steve wanted to exploit the marriage of computer science and ecology. He divided the environment of Glacier National Park into six continuous "gradients" including: elevation, topographical-moisture and aspect, primary succession (soil development following glacial retreat), watershed location, alpine wind-snow exposure, and time since the last forest fire (Kessell 1976). My job was as part of the Northern Forest Fire Lab for data collection to measure species diversity and fire fuel loading. The data from several teams including mine supported the computer model, which would predict forest-fire burn area, anticipate resulting plant and animal communities, and support fire management decisions in the park.

Forest fires create diverse, mosaic, and resilient forests because the fires arise from lightning strikes that occur during rainstorms and are inherently limited to small regions. Human interactions involve fire suppression measures that support maintenance of large climax forests, vulnerable to large catastrophic fires and diminished ecoregion diversity. The objective of the project was to support a "let burn" policy implemented in Glacier but limitations included stakeholders with interests at the park boundaries; the Flathead Indian Reservation, the Blackfoot Indian Reservation, Canada, and the Bob Much Wilderness Area. The job involved weekly backpacking trips to most regions of Glacier National Park and measuring plant diversity, an obligate lesson in taxonomy. The greater my capacity to identify genus and species of animals, trees, shrubs, and herbaceous plants, the more the appreciation of the impact of the environment on species diversity.

I helped write a "Student Originated Studies (SOS)" grant to the National Science Foundation (NSF), which was funded. The project evaluated the ecology around Strawberry Reservoir during the summer after graduation (Worley et al. 1977). The objective was to use aquatic invertebrates as indicator species including: caddisfly (rock rollers), stonefly nymphs, mayflies, amphipods, damselflies, midges and worms as indicators of pollution in the area. We repeatedly sampled the creeks and streams leading into (Trout, Co-op, Indian, Horse, Clyde, and Soldier Creek) and out of (Strawberry River) Strawberry Reservoir to assess the impact of recreational and agricultural uses of the area. The reservoir had been managed for 50 years by the Strawberry Water Users Association and significant problems developed. Fishing villages and campsites were established with variations of pit toilets within feet of the shoreline and these sites were to be inundated by an enlarged Strawberry Reservoir. The reservoir filled to capacity in 1975 when a series of wet years led to the first spill over since 1952 and high water inundated many trailers, outdoor toilets, and other facilities. The flood problems were associated with water quality concerns for undesirably high nutrient levels. The project supported plans to improve management of the area to ensure water quality and future safe recreational use of Strawberry Reservoir. The lessons from undergraduate research clearly pointed to humans as part of the environment and our influence on ecology. This raises a contemplative question, Are we to live in harmony with the environment or are we adversaries?

Analysis of indicator species remains an active area of environmental research. These indicator species offer a time averaged measure of an organism's adaptive response to the environment, which provides immediate practical value to environmental monitoring. Clearly, the most interesting aspect of indicator species is the boundary they define and the potential for hyper-adapted species at the boundary. Boundaries raise a question, are the adaptations a function of the survival of the fittest organisms driving evolution or can fluctuations in populations of RNA in the transcriptome provide a faster adaptive responses?

After completing my undergraduate degree, I landed a position as a biology research technician in the laboratory of Dr. Martin Rechsteiner at the University of Utah. Marty's lab developed a red blood cell preparation to deliver macromolecules including tRNA into cultured eukaryotic cells. I was fortunate to experience this laboratory atmosphere and the cellular and molecular biology environment in the Biology Department which included respected molecular biologists; Drs. Gordon Lark (plant DNA expert), David Wolstenholm (mitochondrial DNA expert), Mario Capecci (Nobel Prize winner and gene transfer authority), and Baldimore Olivera (cone-shell toxin expert). My project involved SV40 virus and explored transformation in contrast to lytic infection in mammalian cells. The hypothesis was that mouse 3 T3 cells transformed with SV40 virus (SV3T3 cells) was due to failure of the virus to synthesize "late" proteins due to lack of specific iso-accepting tRNAs. I isolated tRNA from lytic TC7 African Green Monkey cells then loading the tRNA into red blood cells that were then fused to SV3T3 cells delivering tRNAs. We expected the SV3T3 cells to release SV40 virus into the medium but our hypothesis was incorrect (Schlegel et al. 1978). A second project involved using Sendai virus (a paramyxovirus) to fuse mouse 3 T3 cells with human HeLa cells to create mouse: human hybrid cells. I then observed the kinetics of chromosome loss as the hybrid cell line divided. I became expert at preparing metaphase spreads on microscope slides. The key observation was that mouse chromosomes, identified by their epicentric V shape, are maintained in favor of human chromosomes, identified by their metacentric X shape. The concept that the cell discards redundant genomic information is a novel rapid adaptation to changes in the cell.

I joined the laboratory of Dr. Lester Partlow in the Department of Pharmacology in the College of Medicine (1978–1979). The department was famous due to the Chairman emeritus, Dr. Louis Goodman, the author of the blue bible, "The Pharmacological Basis of Therapeutics." Utah and the Mormon culture offered a refuge for Goodman, a talented Jewish man, in the late 1930's when such ethnicity was discriminated against in the years leading up to World War II. Dr. Partlow was a cellular neuroscientist, a specialty of great personal interest. We dissected 12-day chicken embryos to remove sympathetic ganglia from adjacent to the spinal cord, a technique requiring steady hands and a stereo microscope in a sterile hood. Alternately, I removed the dorsal root ganglia rom the spinal cord to recover cholinergic tissue. Once the surgery was complete, the tissues was digested to produce a single cell suspension that was then subjected to intermittent vibration so the glial cells would attach to the culture dish leaving the neurons in suspension. I developed innovative strategies to encourage neurons to adhere to the culture dish enabling

studies of pure primary neuronal cultures (Iversen et al. 1981). I became expert at measuring acetylcholinesterase, butyrylcholinesterase, and carbonic anhydrase enzyme activities. I conducted experiments with the neurons separate from glial cell populations and then mixed them to evaluate neuronal-glial cell-cell interactions (Hanson et al. 1982).

Dr. Partlow supported my application to graduate school in the Department of Pharmacology, a highly competitive process as they accepted only 3 students from nearly 600 applicants. My intention was to stay in the Partlow laboratory investigating retrograde axonal transport. The project involved characterizing the movement of 1 micron polystyrene beads that attached to axonal membranes of neurons in culture. I set up a microscope with a heated stage and videotaped the culture for hours. The beads moved along the axon towards the cell body in a saltatory manner. I also used transmission electron microscopy and scanning electron microscopy to observe the interaction of the meads with the membrane. The techniques enabled visualization of cell-cell interactions.

I joined the laboratory of Dr. Franklin, younger energetic faculty member, and investigated the cytochrome P450 (CYP) enzyme system and drug metabolism. This family of enzymes were important to drug companies because they frequently inactivated drugs by oxidative metabolism. In addition, these enzymes metabolize a wide variety of foreign substrates, xenobiotics, from the environment and in some cases; the metabolic products are chemically reactive as radical cations. The reactive products form chemical bonds with cellular molecules including DNA, which link them to mutations and chemical carcinogenesis. The CYP enzymes reside in the membrane of the endoplasmic reticulum and their metabolic action transforms highly lipid soluble substrates capable of long residence in cellular membranes into more water-soluble products that are carried out of the cell and out of the body. The CYP oxidations represent an energy efficient reaction in contrast to reduction reactions, which highlight the need for cells to manage energy utilization. The cellular content of CYP enzymes is reduced when the cell goes through the cell cycle suggesting a functional connection between CYP and growth. My dissertation investigated the expression of multiple CYP enzymes in the rat liver as it regenerates following partial hepatectomy (Iversen et al. 1985). We knew there were multiple genes encoding CYP enzymes but we did not know how many and reagents were not available to evaluate specific CYP genes. Linking chemicals in the environment to metabolic enzymes provides a fertile area directly linking gene-expression to the environment.

Once I completed my dissertation, I joined the laboratory of Dr. Edward Bresnick at the Eppley Institute for Cancer Research in Omaha Nebraska. Dr. Bresnick was an aggressive scientist exploring the molecular biology of the CYP enzymes with interest in details of chemical carcinogenesis. I participated in a project to explore the molecular biology of mouse cytochrome P450c, now known as CYP1a1 (Hines et al. 1985). My project quickly turned using the mouse gene as a probe to find and clone the human version of CYP1A1. The group effort was successful in isolating the first gene encoding a human drug-metabolizing enzyme. I spent 9 months subcloning and sequencing this gene (Iversen et al. 1987), a task that would take hours

today. The molecular biology of CYP1A1 was exciting, rapidly expanding scientific questions that exceeded my capacity to conduct studies. Ed was an outstanding mentor but his responsibilities as director of the Eppley Institute diminished the time we could meet. Dr. Ron Hines was a new investigator at the institute having recently completed his postdoctoral fellowship with Ed and I relied on Ron as my mentor for the remainder of my postdoctoral fellowship. Reagents and expression vectors were rapidly becoming commercially available and served to expand perspectives in molecular biology and pharmacology. New techniques including the polymerase chain reaction (PCR) and automated DNA synthesizers and sequencers dramatically enhanced the pace of our research.

RNA Based Therapeutics

Dr. Manuchair (Mike) Ebadi invited me to join the faculty in the Department of Pharmacology at the University of Nebraska Medical Center (UNMC) in 1987. I combined the essence of molecular biology with pharmacology in the exploration of antisense inhibitors of gene expression. The concept that a short single-stranded DNA molecule could be synthesized to bind to its complementary single-stranded RNA transcript to inhibit translation had the potential to lead to a vast new area of pharmacology. My friend, Dr. Mario Stevenson, was growing the Human Immunodeficiency Virus-1 (HIV-1) in cell culture and we decided to collaborate on a project to use antisense oligonucleotides to inhibit HIV-1 viral growth (Stevenson and Iversen 1989). I then worked with a group at the NIH to reveal the sulfur modified DNA oligomer inhibited the HIV-1 reverse transcriptase activity independent of the oligomer sequence (Egan et al. 1991). An emerging belief that antisense oligomers could be RNA-based drugs became the focus of my future research.

I wanted to see if a synthetic oligonucleotide would have drug-like properties so I developed a method for synthesis of a radioactive phosphorothioate synthetic DNA molecule and then injected it into rats to estimate various pharmacokinetic properties (Iversen 1991; Iversen et al. 1994). I was convinced a synthetic oligonucleotide would distribute into multiple organs but would it inhibit targeted gene expression? I investigated multiple gene targets for inhibition in animal models including a mouse tumor model of chronic myelogenous leukemia (Skorski et al. 1994)) and rat models of CYP2B1 (Desjardins et al. 1995) and CYP3A2 (Desjardins and Iversen 1995). With feasibility and *in vivo* efficacy completed, I explored the potential therapeutic limitations; finding a cardiovascular toxicity (Cornish et al. 1993) and reactive metabolite adduct formation from small molecule therapeutics (Copple et al. 1995). These limitations define a fulcrum in the risk to benefit balance for a patient with a lethal leukemia. I worked with a group at UNMC to investigate an antisense inhibitor of p53, an overexpressed gene in acute myelogenous leukemia (AML; (Bayever et al. 1993a)). I directed the effort to prepare an investigational new drug (IND) application to the US Food and Drug Administration (FDA) and gained permission to proceed to conduct the first evaluation of a systemically administered synthetic oligonucleotides in humans (Bayever et al. 1993b). The first-

in-human milestone with antisense led to an international press release, which aired, on CNN International in 1992. The appearance of my name in the interview process led to a request from King Hussain of Jordan to treat his lymphoma as well as hundreds of requests to treat patients with this emerging technology. The international recognition of my accomplishment supported my promotion to full professor, resulted in numerous invitations to present these first-ever clinical observations, and a position on the national board of directors of the Leukemia Society of America. I participated in the creation of a new biotechnology company, Lynx, as a spin off from Applied Biosystems and prepared IND documents for antisense targeting *bcr-abl* in collaboration with Bruno Callebretta at Thomas Jefferson University, *c-myc* with Andrew Zalewski at Thomas Jefferson University, and *c-myb* in collaboration with Alan Gewertz at the University of Pennsylvania. My research effort for each of these projects involved firsthand experience with the concept of translating synthetic DNA into a drug product by exploiting the questions of dose, dose interval, route of administration, and dose limitations by conducting the first pharmacokinetic and preclinical toxicology studies.

I identified numerous sequence-independent limitations associated with phosphorothioate (PSO) chemistry including: activation of complement, inhibition of coagulation, inhibition of cell surface receptors, stimulation of immune responses, and the generation of radical oxygen in cells producing 8-oxo-guanine adducts in RNA, DNA and the oligonucleotide. I looked at the radical oxygen production in a V79 cell line selecting for cells capable of growing in 6-thioguanine ($HGPRT^{-/-}$) as well as oubain ($Na/K\ ATPase^{-/-}$) and identified enhanced mutagenesis frequency. Later studies with phosphorodiamidate morpholino oligomers (PMO) compounds did not produce this radical oxygen response. While I felt publication of this mutagenesis activity was important, I was not able to convince our regulatory affairs people to explore this further for fear of finding something we did not want to know. An extension of this limitation was in the role of GLP studies in which contract research organizations do not recommend non-traditional mutagenesis assays in part due to lack of validated V79 cell lines. Mutagenesis assays are generally done with no intent to understand the process and the FDA does not demand much of these assays since they have some difficulty determining how to interpret the data. Basically, don't ask and don't tell until something arises in treated humans. While these off-target effects might be acceptable in the treatment of life threatening disease, the PSO as DNA chemistry would not be appropriate for the broad evaluation of genome function.

My first NIH RO1 research grant involved using synthetic oligonucleotides to inhibit the expression of cytochrome P450 enzymes (CYP). The observed phenotype was prolonged midazolam-induced sleep in rats as CYP3A2 is the only enzyme responsible for metabolism (clearance) of midazolam. The grant compared the *in vivo* efficacy, pharmacokinetics, and toxicity of a variety of oligomer chemistries including phosphorothioate oligoribonucleic acids, ribozymes (Desjardins et al. 1996), and PMO (Arora et al. 2000). The PMO are steric blockers and the hypothesis was that they would be less potent due to their lack of a catalytic mechanism of action such as RNAse H, RNAse P, or RISC. The PMO were significantly more potent inhibitors and more sequence selective. The research finding led to my

leaving the University to join AVI BioPharma, a biotechnology company in Corvallis Oregon where the PMO chemistry had been invented. In retrospect, the diverse actions of antisense oligomers illuminated the emerging sophistication of RNA in a living cell.

The first research project at AVI BioPharma used PMO to replace the PSO approach used by Lynx to targeting *c-myc* (Hudziak et al. 2000) for the prevention of restenosis of coronary arteries following balloon angioplasty (Kipshidze et al. 2002). This product, AVI-4126, was licensed to Medtronic and then to COOK Cardiology where phase II studies were completed and while the results were positive, AVI-4126 (Kipshidze et al. 2007) was not as effective as rapamycin coated stents in competing clinical trials.

A New Paradigm

The idea that tumors arise when the rate of new cells exceeds the rate cells die led to interest in genes that regulate apoptosis. A simplistic view that an imbalance between genes promoting apoptosis like BAX and genes that prevent apoptosis like BCL-2. Reminiscent of inhibiting p53 to induce leukemia cell death, I targeted BCL-2 to inhibit anti-apoptosis and enhance BAX dominance to kill tumor cells. I boarded a jet in Eugene Oregon to San Francisco to meet my connecting flight 92 to Newark. It was September 11, 2001; my flight never left the runway in Eugene and the plane I planned to board in San Francisco had crashed in southern Pennsylvania. I was stunned, walking in a stupor out of the airport like a zombie. As I returned to my home, I could only think of the terrorists next steps. I resolved to investigate rapid development of anti-infectious disease agents with our antisense technology. I established a collaboration with Dr. Alvin Smith at Oregon State University to explore inhibition of Calicivirus in cell culture (Stein et al. 2001). I also engaged Dr. Bruce Geller to investigate the feasibility of targeting bacteria (Geller et al. 2003). Our anti-viral studies expanded to include SARS (Neuman et al. 2004), Dengue (Kinney et al. 2005), and West Nile Virus (Deas et al. 2005). The feasibility that an antisense molecule could be quickly designed with antiviral activity began to reshape the directions of AVI BioPharma.

Monday, February 9, 2004 I left my home in Corvallis at 5:00 am to check in for a flight from Portland (PDX) to Dulles (IAD) leaving at 8:00 am. I arrived at 4:00 pm (EST) collected my luggage and took a taxi to Fredrick Maryland in time for a 7:00 pm dinner meeting with Sina Bavari. We met Tuesday morning to discuss a letter of collaboration, begin preparing a CRADA (cooperative research and development agreement), and I gave a seminar describing AVI BioPharma technology to Sina's research group. I met with Dr. Javad Aman at 2:00 pm to discuss Ebola VP40 as a potential antisense target and a supplement to ongoing vaccine efforts. I met with Rekha Panchal at 4:00 to explore Anthrax bacterium as a project and then back to Sina at 5:00 pm to discuss targeting orthopox viruses. I had a short meeting with Dr. Kelly Warfield on Wednesday morning to discuss strategies for Ebola and

Marburg viruses and possible animal models for early studies. I left USAMRIID before lunch *en route* to Dulles for my flight back to PDX. Around 5:30 pm on that Wednesday, February 11, I was settling in on my flight Kelly Warfield was in the BSL-4 lab experiencing an accidental needle stick with a syringe that had been used to inject Ebola infected mice. On Thursday I met with Dr. David Hinrichs to discuss an immunology project involving c-FLIP and then on Friday, February 13th I had invited Ben Newman to give a seminar on our collaborative project involving SARS. Tom Stewart came to Corvallis for the seminar and to meet Ben. The morning was busy with plans for studies to evaluate the potential for SARS to become resistant to our antisense treatments.

Tom Stewart, Alan Timmins and I were in my office having returned from lunch when Sina Bavari called, "Would you guys do me a favor. Someone had an accident with Ebola virus in the BSL-4, a needle stick." "Can you make enough PMO to treat?" We agreed to consider the feasibility. We recruited Dr. Denis Burger, CEO, and Dr. Dwight Weller, VP of Chemistry and Manufacturing, and we all agreed, "We should at least try." This moment launched us into the hemorrhagic fever filovirus business. I felt two targets would be feasible and give us the best chance for success and proceeded to look at the GenBank sequence for Ebola genome. It was about 2:30 pm when I called Sina to confirm these would be a reasonable strategy. Next step was Dr. Dwight Weller and initiation of the effort for synthesis of two PMOs, one targeting the 3′-end of the viral genome and one targeting VP35. Synthesis was complete on Monday morning. I left Corvallis on Saturday to arrive in London Sunday morning to attend an international antisense meeting. On Wednesday at 8:00 pm I joined a conference call involving USAMRIID (Judy Pace, Sina Bavari, Dr. Mark Korterpeter, and Dr. James Martin), the FDA (Jeff O'Neil, Doug Throckmorton) and AVI BioPharma (Janet Christenson, Dwight Weller, Christina Fox, Doreen Weller, and Alan Timmins) to request compassionate use authorization to treat Kelly. The FDA requested endotoxin analysis for the substance, a search to ensure there are no human sequences targeted, an informed consent form, and an investigators brochure which we sent by FAX to Jeff O'Neil. We all agreed on a clinical event timeline predicated on a PCR positive sample from Kelly. Alan Timmins took a redeye flight to Dulles and hand delivered the two PMOs to USAMRIID on Wednesday morning. Elapsed time since the call from Sina, six days.

My group successfully targeted nearly every family of virus known to infect humans. We created AVI-4020 for the treatment of West Nile Virus and AVI-4065 for the treatment of hepatitis C virus (HCV) and both were evaluated in phase I clinical trials. Three more advanced examples include AVI-7537 for the treatment of Ebola Zaire, AVI-7288 for the treatment of Lake Victoria Marburgvirus (Warren et al. 2010), and AVI-7100 for the treatment of influenza A. The more advanced examples have completed single and multiple dose escalation human phase I trials (Heald et al. 2014, 2015). The use of viral genome sequence in the design of antiviral therapeutics led to the prospect of rapid response antiviral therapeutics for the development of countermeasures for emerging infectious disease. While these projects are currently dormant as there is no current business model to drive this work

forward. My research interest is in finding a workable model with either improved oligomer synthesis and or a global antiviral based on targeting host genes. Alternately, research to identify therapeutics to high value infections such as HIV, HBV, and HCV and to a lesser extent EBV, CMV, and HPV which would likely represent therapeutics to be used in combination with current and emerging standards of care.

Induced Exon Skipping

We discovered AVI-4126 induced a downstream shift in the exon 2 splice acceptor of *c-myc*. The downstream site was downstream of the AUG translation start site and led to in-frame initiation of translation even further downstream resulting in a dominant negative variant of *c-myc*. I wanted to explore the utility of oligomer-induced manipulation of exon use. The first project involved collaboration with Steve Wilton in Perth Australia which led to the discovery and development of Eteplirsen, designed to induce skipping of exon 51 in the dystrophin transcript in boys with Duchenne Muscular Dystrophy (DMD). This project led Sarepta Therapeutics to the first FDA approved drug for the treatment of DMD, ExonDys 51. This research area is leading the way to finding solutions to an extensive array of rare genetic diseases by the pharmaceutical industry. I feel we have only scratched the surface of oligomer induced exon skipping studies. Our recent studies have exploited "alternate exon inclusion" to induce ligand independent receptors including the T cell co-receptor CTLA4 and ligand independent nuclear hormone receptors including the vitamin D receptor and the glucocorticoid receptor.

Viruses with RNA Genomes

I spent years developing therapeutics for emerging RNA viruses (Ge et al. 2006; Iversen et al. 2012; Kinney et al. 2005; Lai et al. 2008; Neuman et al. 2005; Paessler et al. 2008; Stone et al. 2008). These viral genomes are unstable producing thousands of defective virions and a few successful infectious particles. This property results in inevitable resistance to antiviral therapeutics but demonstrates an important capacity to adapt. I joined Dr. Craig Marcus in the Department of Environmental & Molecular Toxicology at Oregon State University in 2012. We recruited Dr. Andrew Annalora as a research assistant professor to add structural biology expertise to our effort. The core lab effort centers on the cytochrome P450 enzymes and their interactions with nuclear hormone receptors. We explored transcript variants in the cytochrome P450 and nuclear hormone receptor families of genes finding unappreciated diversity in functional transcript variants (Annalora et al. 2017). An emerging hypothesis is that like RNA viruses, these genes express multiple transcript variants to enhance human adaptation to a changing environment. A key

distinction is that RNA provides the adaptive response to the environment and not alterations in DNA.

I now wish to integrate ideas, methods, technology, and emerging hypotheses. The early notions of a relationship between expression of CYP enzymes and the cell cycle have become more interesting. The CYP enzymes are generally suppressed during infection, a response that is rapid and observed after viral, bacterial, fungal, or parasitic infection. The hypothesis is that an endogenous CYP substrate is an inhibitor of DNA and RNA polymerases and suppression of CYP activity means elevated concentrations of endogenous inhibitor- a protective response. Aphidicolin, a fungal diterpenoid compound, is a potent inhibitor of DNA polymerase and a potential valuable anti-cancer agent but it is rapidly metabolized by CYP enzymes. The hypothesis is that various polymerases are linked to biological molecules in the environment and the CYP family modulate that activity. The diterpenes are chemically diverse with broad biological activity. Are these activities modulated by CYP expression? Can environmental exposure to chemical congeners disrupt the modulation? Alternately, the chemical carcinogenesis process I studies 20 years ago restricted interest to chemical adducts forming on DNA with disregard for the chemical congener RNA.

Dark Matter of the Genome

Historically, RNA sequencing (RNASeq) was focused on the transcriptome, or the population of RNA with a poly (A) tail. However, recent improvements in our understanding of the genome have revealed that over 80 percent of the genome is transcribed into RNA, focusing new attention several emergent classes of non-coding RNA. Hence, much of the key information from GWAS has been overlooked inadvertently, as many variants are found in noncoding regions or unannotated regions of the genome (St. Laurent et al. 2014). Non-coding RNA (Table 1.1) can be divided into long non-coding RNA, lncRNA, and small non-coding RNA. The small ncRNA include: (1) miRNAs that are 18–21 nt in length and are involved in regulation of gene expression, (2) siRNA that are 21 nt segments that regulate gene expression and transposon activity, (3) rasiRNA are 24–27 nt that help form centromeres and orient heterochromatin, (4) piRNA or piwi-interacting RNA are 26–30 nt molecules that regulate transposon activity and chromatin, (5) snoRNA are 60–300 nt in length that create 2'-O-methyl and pseudo uridylation modifications of other RNAs, and (6) snRNAs that are 100–300 nt which are involved in assembly of the spliceosome and nonsense mediated decay (NMD) of pre-mRNA (Dogini et al. 2014). lncRNA are a novel class of functional RNAs that impinge on gene regulation involving recruitment of epigenetic modifier proteins, control of mRNA decay and DNA sequestration of transcription factors. The human genome appears to produce over 60,000 lncRNA, the majority expressed at low levels. Early examples of trans-acting lncRNAs are inhibitory polycomb repressive complex (PRC2) and the activating Trithorax/MLL chromatin modifiers HOTAIR and HOTTIP. The lncRNome contains cis-acting enhancer elements (eRNAs) which control the expression of

Table 1.1 Expressed noncoding RNA

Name	Processing Mechanism	Function	Size
rRNA, ribosomal RNA	Exo- and endonuclease	mRNA translation	Large
tRNA, transfer RNA	RNAseP, RNaseZ, angiogenin	mRNA translation	90 nt
tRFs, tRNA fragments	Dicer, RNaseZ	Regulate translation initiation	13–30 nt
miRNA, micro RNA	Drosha and Dicer	Translation regulation	18-21 nt
siRNA, small interfering RNA	Dicer	RNA cleavage	21 nt
rasiRNA, repeat associated siRNA	Dicer	DNA/histone modifications	24–27 nt
piRNA, PIWI-interacting RNA	Ago3, aubergine, exo- and endonuclease	Regulate transposon activity	26–30 nt
snoRNA, small nucleolar RNA	5'- and 3'-exonuclease trimming	Pre-rRNA processing, modify tRNA, rRNA and snRNA bases	60–300 nt
sdRNA, sno-derived RNAs		Reulation of alternate splicing	
snRNA, small nuclear RNA	U1, U2, U4, U5: capping, U6-3'-end	Pre-mRNA splicing, nonsense mediated decay (NMD)	100-300 nt
lncRNA, long ncRNA	Transcription	Repression (PRC2), activation (HOTAIR), transcription enhancer (eRNA), alternate splicing (MALAT1), Staufen-mediated mRNA decay (SMD).	>200 nt
lincRNA, long intergenic ncRNA	Diverse	Regulation of splicing	>200 nt
vlincRNA, very long ncRNA	Transcription	Epigenetic regulation	>40 kb nt
Tel-sRNA, telomerase small RNA	Telomere transcript cleavage	Epigenetic regulation	
PARs, promoter-associated RNA	Transcription	Regulation of transcription	

neighboring protein coding genes. A lncRNA mediating alternative splicing via assembly of serine/arginine splicing factors within subnuclear components called speckles, MALAT1, is retained in the nucleus. A lncRNA called TINCR binds to the 3'-UTR and elicits Staufen-mediated decay (SMD; (Kornfeld and Bruning 2014)).

Life is a battle of enthalpy over entropy. The membrane barriers and cell walls found in virtually every living thing separate the greater enthalpic state from the randomness of non-life. Each challenge toward entropy, lack of order, threatens the persistence of life. Changes in the ordered state are obligate to adapt to drivers of disorder. Biologists study living things and living systems making them experts in adaptations capable of repelling entropy.

The purpose of this book is to explore adaptive mechanisms from a molecular biology perspective. Evolution is a subject that embraces changes in DNA leading to survival and expansion in the number of species. The challenges involve the interplay of features from living things to changes in the environment, which captures the study of Ecology. While separate, evolution and ecology overlap.

Evolution requires reproduction for the vertical transmission of traits. Changes or mutations in the genome sequence lead to separations between individuals regarding which is "fittest" in the face of a selection pressure, or driver of entropy. The accumulation of fit traits leads to an organism best suited to live in a specific ecology. In the case of humans, the accumulation will take numerous generations, which are roughly 20 years in duration. In the case of a virus, the accumulation will take hours. In this regard, viruses are hundreds of thousands of times more adaptable then humans. The co-existence of humans and viruses might suggest viruses will always adapt and given the race to repel entropy, viruses always win. Bacteria, fungi, and protozoa will follow quickly leaving humans to a short pulse of history in life on Earth.

Look at this premise more closely. Humans have a shorter time of existence but given the adaptability gap, we should already be gone. This points to an alternative, rapidly adaptive survival source in humans running in parallel with evolution (Fig. 1.1). That human adaptive mechanism relies of the exceptional variability in RNA. The purpose of this book is to reveal the origins of this highly tuned adaptive system.

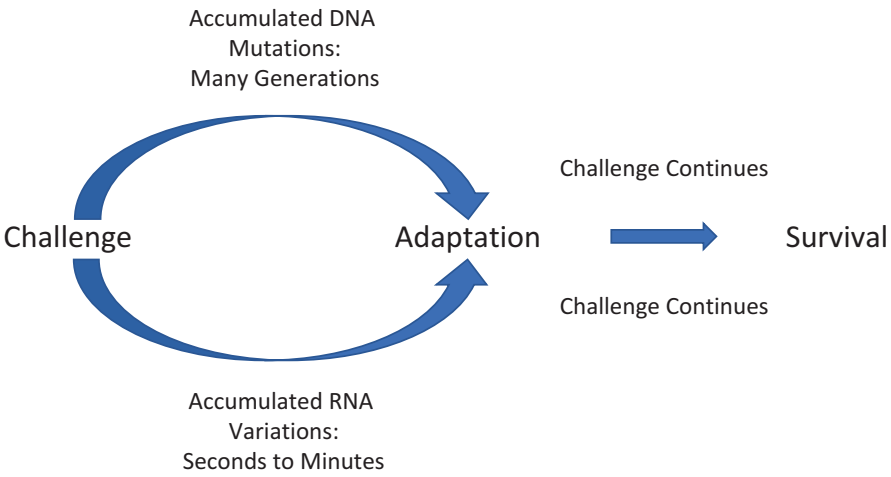

Fig. 1.1 RNA Resilience Hypothesis. DNA mutations accumulate over generations and are associated with adaption over multiple generations. RNA variations are expressed in seconds to minutes and are well suited to rapid adaptation, a plausible mechanism for resilience

When I was a graduate student only 35 years ago, we thought 2–3% of our genome made RNA. Over the past decade, we have observed an explosion in RNA information. While nearly all of our genome makes RNA, we do not know the function of all the diverse forms of RNA. Some have referred to this phenomenon as the "Dark Matter" of the genome. The potential for these non-coding RNAs to play a role in regulating transcriptome plasticity and resilience came to mind.

The approach to this question takes elaboration of RNA mechanisms of adaptability, beginning with single-stranded RNA viruses. As life becomes more complex, the mechanisms of adaptability become more elaborate as in bacteria. Mammalian complexity takes adaptability to the edge of chaos. While an RNA virus relies on RNA to adapt, humans integrate both DNA and RNA mechanisms to adapt. Multicellular organisms elaborated enthalpy in immune defenses and recognition of small molecules in the environment. The goal of this book is to build a logical bridge to the development of genomic enthalpy and explore integration of adaption to exposure to small molecules.

My time as a bench scientists appears to be behind me. The exponential gains in scientific information force us to focus our attention to more refined subject matter. I have taken time at this late stage of my career to reflect and study a bigger research picture. The larger the subject net, the more information gems will be missed. A book that pushes thought boundaries should be more interesting to read. In addition, by laying down a few paving stones of thought, the hope is for future scientists to push these boundaries of understanding into workable models of adaption. These models may one day prove insightful in solving pressing problems or even a rationale to leave some problems alone.

Overview

The first six chapters elaborate threats to human health. The Chap. 2 looks at how human populations spontaneously expand into niche boundaries exposing us to threats that drive the resilience process. The inevitable expansion beyond boundaries is termed "social entropy" since seeking life beyond boundaries is a fact of life. Resilience means life is often threatened but it is not fragile. Chapter 3 focuses on infectious disease, particularly emerging infectious single-stranded RNA viruses, as the most significant threat to human health. I review antiviral drugs including personal experiences with antiviral drug discovery and development. Viral genomes are sensitive to environmental conditions and have become resilience experts and evolution is mediated by RNA sequence plasticity. Chapter 4 describes "nonlinear anomalies" to highlight limitations in predicting the human outcome based on research studies with cells in culture and animal models. A personal experience with development of medical countermeasures for Ebola and Marburg viruses and interactions with the US FDA exploring the "Animal Rule" for drug approval. Chapter 5 focuses on bacterial infections and their diverse threat to human health. I include a brief review of antibacterial drugs and personal experience with bacterial resilience mediated by horizontal gene transfer. Chapter 6 shifts focus to cancer as a threat to

human health. Personal experience in discovery of novel therapeutics for the treatment of leukemia forms the body of the chapter. The spontaneous resolution of AML in children with Down syndrome highlights human resilience and the foundation for optimism in finding a cure to leukemia. Chapter 7 is a review of chemicals in the environment as a threat to human health. Brief examples of chemical carcinogenesis are included to illustrate how chemicals disrupt genomes. Historic research ignored RNA damage as a transient an irrelevant consequence of chemically induced nucleic acid damage. The emergence of important forms of RNA and their possible role in resilience is proposed.

The next section including Chaps. 8–10 discuss threat recognition and defense systems responding to improve resilience. Chapter 8 describes a key mediator of resilience, the immune response, as a threat recognition system and response via diverse RNA expression. Research into oligonucleotides designed to suppress specific RNA to manipulate the immune response including strategies to induce exon-skipping are described. Chapter 9 describes a parallel threat recognition and response system in the human cytochrome P450 (CYP) family of enzymes. The interesting observation that infections modulate CYP expression points to a role parallel to the immune response. CYP expression includes diverse splice variants that are largely uncharacterized. I propose metabolic clearance of small molecules as a metabolic companion to the immune system. Chapter 10 highlights the diversity in RNA expressed from a single gene. This represents a list of mechanisms for RNA transcriptome plasticity that provide the basis for resilience at the molecular level.

Chapter 11 is a personal account of the recently approved drug for the treatment of Duchenne muscular dystrophy, eteplirsen (Exondys 51). Based on observations of "spontaneous revertant muscle fibers" it was clear that we could design a drug to induce skipping of the dystrophin exon 51 to restore reading frame that would lead to expression of functional dystrophin. Controversy in the approval process highlights resistance to paradigm shifting technologies.

Chapter 12 is the summary. I address the question "what informs molecular mechanisms of resilience" that drives the limits to the adaptive response oscillation and provides boundaries for molecular resilience. I speculate that radical oxygen, epigenetic modifications, and ligands to nuclear hormone receptors play critical roles in regulating molecular resilience.

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Chapter 2
Social Entropy

Abstract We find ourselves in the "information age" with immediate access to limitless information but almost no reliable filter to distill quality from the contaminated volume. We are compelled to probe the limits of our existence often engaging in activities that threaten our health and survival. Central to the theme of this chapter an overview of health boundaries and the phenomenal biological adaptability rooted in the core of molecular biology. Genes are not digital switches in either zero or one position but are dynamic structures capable of responding to the smallest molecules in the environment. This instant genetic adaptability leads to cell physiology that is an ordered chaos leading to multiple possible outcomes following exposure to both subtle and extreme environments. Individuals are unintelligent, thoughtless creatures entrenched in a daily random walk but are capable of genius when taken together as populations. We are the collective result of interacting gene expression events, each of which adapts to a complex matrix of chemical signals, both endogenous and from the environs. Life is often threatened but is not that fragile.

Keywords Smoking · Radiation · Overpopulation · Olympics · Telomeres · Telomerase · Progeria

Introduction

Observations of human behavior suggest a prototype for perfect existence. Modern people seek a long life rich in fulfilment in which they have produced viable offspring under the umbrella of a loving God. Some strive to optimize their living conditions in order to ensure their perfect existence far into the future through healthy lifestyles, education, financial stability, insurance policies, and religion. Such aspirations lead to conflict and greater aspirations lead to greater conflict. A personalized approach could lead to a perfect house in a crime ridden, polluted, and corrupt neighborhood. Consider an approach centered at the individual, expanding to family and friends, and then to the environment and living populations. The anticipated outcome from actions should guide the selection of a single action from several possibilities.

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I have several friends that are engineers. They do not like to talk about their work in part because they must maintain confidentiality with their employer and in part, because the work involves intricate details they are certain no one outside their profession will understand. These same people do not ask me what I do in part because they know I would love to tell them and in part, because they know they would not understand the intricate details I would likely describe. However, I am often consulted when a health problem arises because I am not a physician but strive to understand what happens when health goes wrong. I am unique because I seek the understanding of the "white space" of medicine, the drugs that are not on the shelf or in the pharmacy. Most of the time their physician is a compassionate person with extensive knowledge of medicine. The process of medicine is delivered by a vast network of health professionals but often not as a team. They do not have the time or are not paid to dig into details of emerging infectious disease or rare genetic disease.

A troubling fact is that education of health care professionals is in such demand that the process is streamlined. Training is based on the natural history or course of a disease, outcome probabilities, and a sequence of approved procedures. Basic science in health professional training is limited by necessity. Sadly, most basic scientists are struggling to make a living and interactions with the health care complex are challenging. The only basic scientists that can afford to live in neighborhoods of health care professionals are the administrators, deans and department heads from universities. The daily content of science administrators is filled with acknowledging the financial gains of their institutions, the significance of money contributed by wealthy donors, and simplifying scientific accomplishments into jargon the general public can appreciate if not fully understand.

The bulk of highly trained scientists now find employment in industry. The incentive is financial reward but their efforts are limited to products in development. As a senior scientist in industry, my scientific interactions involved investors and members of the board of directors asking when I was going to make them rich. Unfortunately, I made these people very rich. The company re-organized as a prudent effort to accommodate success and those now wealthy people no longer felt the need to interact with me. I cannot complain, I was able to devote decades to the pursuit of medical white space. I am an expert at linking molecular biology events to changes health in a variety of medically relevant areas. Now I look back trying to synthesize this experience in a way that may streamline the interpretation of genome function into health and survival.

The flow of logic from molecular and cellular to the individual and family and then to populations and the environment is proposed. While extensive writings point to creating a healthy environment as a way to personal health and fulfilment (i.e. Rachel Carlson and the Silent Spring) less is widely known linking gene expression to health. All living things share DNA as a common source of instructions for living. DNA (RNA in some viruses) is the single chemical that defines heritability, which extends back in time over a billion years.

Challenging Lifestyles

Exceeding the limitations to adaptability is likely to result in death. “Smoking kills, on average, 1200 Americans. Every day. More people die every year from smoking than from murder, AIDS, suicide, drugs, car crashes, and alcohol, combined.” The surgeon general declared tobacco carcinogenic in 1964 followed by increasingly severe health warnings posted on each cigarette pack since 1966. People continued to smoke prompting the surgeon general to declare tobacco addictive in 1988, an acknowledgement of cigarette companies’ intentional cigarette design with sufficient nicotine to create and sustain addiction (Will 2017).

The curious persistence of the most highly taxed consumer product that causes harm if used as intended brings light to ineffective government regulations. A 1998, 46 states sued tobacco companies for $246 billion carving off $13 billion for trial lawyers. The past 50 years of collective non-profit and governmental actions has reduced the frequency of smokers from 50 percent to about 17 percent. That success in mind, smoking caused over five million global deaths a year from 1990 to 2015 (500,000 deaths in the United States each year) (Reitsma et al. 2017). However, smoking may provide a net financial gain for the government due to large tax revenue and smokers’ premature deaths limit their entitlement of benefits for the elderly including Social Security, Medicare, Medicaid, and costs associated with nursing homes. The US entitlement programs would fail (go bankrupt) if everyone stopped smoking. The outcomes analysis points to a conflict of interest in government regulators; money is more important than health.

Smoking essentially brings a poor air quality to the smoker leading to expected increases in the associated diabetes and heart disease. Smoking enhances the risk of Alzheimer’s disease and Crohn’s disease but will lower the prevalence of ulcerative colitis and Parkinson’s disease. A chemist friend related that smoking enhances the ability to smell arsenic, a helpful ability when using the toxic element in chemical synthesis reactions. Smoking has a long history of an association with weight loss typified by the 1968 introduction of the “Virginia Slims” brand of cigarettes by the Phillip Morris Company. The existence of smoking benefits is evidence of an adaptive response to smoking behavior.

The 80:20 percent rule is a superficial description of human behavior. A friend of mine uses the rule to describe how 80 percent of insurance sales come from 20 percent of the sales force. Some novelty stores are loaded with diverse items but 80 percent of sales come from 20 percent of the inventory on the shelf. Smokers represent about 20 percent of the population but will likely experience 80 percent of the health problems as they age (actually a bit less). What about the 20 percent of smokers that never experience health problems, the occasional 95 year old that states he smoked every day since he was 14? These individuals are exceptional by surviving. What adaptive response allows them to escape the probabilities of doom? Some might explain the escape artists by chaos theory that a unique set of circumstances were in place. It seems more plausible that biology provided for a survival solution, a resilience to smoke.

Both of my parents were smokers. I watched the rituals associated with their lighting of the cigarette. They would have a cigarette with coffee in the morning and one after each meal. The aroma in the first 5 sec of lighting was at times pleasant but the smoke after that was unpleasant. We took vacations by car in the summer and the worst part was their cigarette smoke in the car with the windows closed. In addition to their 30-year exposure, I inhaled their smoke for about 18 years. They each quit the habit "cold turkey" first my father and then my mother about a decade later. My parents are 93 and 84 years old and continue living with no evidence of smoking related disease. They beat the odds by quitting without help and not getting sick. Evidence of successful adaptation to environmental stress.

A pharmacopeia of medications are available to help people stop smoking. The FDA has approved five nicotine replacement therapies (NRT) that deliver nicotine in a form that does not involve smoking including transdermal nicotine patches, gum, lozenges, sprays and inhalers. The idea is to transfer the addiction from smoking to NRT and then slowly reduce nicotine exposure. The antidepressant, buprion, is a first line treatment for smoking cessation but other selective serotonin uptake inhibitors (SSRIs) can also be utilized. Varenicline, a high affinity partial agonist for the $\alpha_4\beta_2$ nicotinic acetylcholine receptor, causes dopamine to be released in the nucleus accumbens brain region helpful in reducing feelings of craving. Clonidine stimulates α_2 adrenergic receptors and was developed to treat high blood pressure but can now be used to help smokers quit. These medications clearly help but all carry off-target or side effects that can limit their use.

Behavior modification can include electronic cigarettes or eCigarettes (vaping). While these devices offer an alternative they also have health threats due to the vaporizing of oils and excipients like polyethylene glycol. Basic science research has identified serious concerns over eCigarettes based on nanoparticle distribution into the lung. However, there are those that prefer to wait over 10 years to count dead bodies so they can be sure this alternative is sufficiently risky.

The tobacco industry proved to be untrustworthy in their desire for sales over health concerns. The popular skepticism becomes associated with the medications to help quit smoking. Skeptics do not trust or can't afford smoking cessation medications but are sophisticated enough to avoid eCigarettes tend to seek out alternate approaches. Acupuncture, aromatherapy, hypnosis, herbal supplements, and pharmaceutical grade psilocybin attract those skeptics. Controlled studies of these alternatives have all failed to identify significant efficacy benefit compared to placebo but a placebo often has efficacy in this group of individuals.

Genome wide associative studies (GWAS) studies identified a variant in the α5 cholinergic receptor (CHRNA5) that is linked to smoking cessation and earlier emergence of lung cancer (Bierut et al. 2008). The variant is nonsynonymous (a mutation that changes an amino acid as opposed to synonymous- a mutation with no change in amino acid) and results in an amino acid change that alters nicotinic receptor conductance. The variant, known as rs16969968, is a single nucleotide polymorphism (SNP pronounced SNIP) in which the two alleles may be GG, GA, or AA. A meta-analysis of 24 published studies involving 29,072 individuals found the (A) allele was associated with a lower likelihood of smoking cessation and the

homozygous (AA) genotype was associated in a 4 year earlier median age of lung cancer diagnosis (Chen et al. 2015). The difference is that smoking longer can shift the time of lung cancer from 65 to 61 years old but these studies do not reveal the cancer driving mechanisms associated with smoking.

Half of all lung cancer is diagnosed in people over 70 years of age and less than 15 percent of those diagnosed will survive for more than 5 years (the world average life expectancy in 2014 was 71.5 years[1]). Candidate genes point to the basis of lung cancer. These genes are associated with carcinogen metabolism (CYP1A1, CYP2E1, GSTP1, GSTT1, NAT1, NAT2, MEH, and NQO1), nucleotide and base excision repair (ERCC2/XPD, XPF, XPA, XPC, XPG XRCC1, OGG1, APE, and XRCC3), cell cycle control (TP53, MDM2, and p21), inflammation (IL-10, IL-6, L-1B, and COX-2), telomere length, and tumor microenvironment (MMP1, MMP2, and MMP9; Marshall and Christiani 2013). Metabolically, inhaled carcinogens are "activated" by cytochrome P450 enzymes (CYP) which are often "inactivated" by a variety of enzymes such as the glutathione transferases (GST), acetyl transferases (NAT), epoxide hydrolase (MEH) and quinone oxidases (NQO1). Those activated carcinogens chemically react with the nucleotide bases in the genome that can lead to mutations but a collection of highly efficient repair enzymes remove the chemically damaged nucleotides. Those modified nucleotides that are not repaired often lead to mutations that influence the cell cycle and genes regulating programmed cell death resulting in a net increase in the number of cells, a tumor. The appearance of swelling leads to inflammation and an immune response that often removes the tumor. Those tumors that evade the inflammation associated immune response exploit their microenvironment to continue to grow and allow cells to exit the primary tumor site resulting in metastasis. In brief, our bodies actively fight the development of cancer at multiple levels but in rare cases cancer causing cells escape.

Cigarette smoking leads to a 3–5 fold increase in alveolar macrophages, cells responsible for gobbling up particles and dead cells in the lung. Activated macrophages in the lung are cleaning up the smoke but in the process release inflammatory cytokines like IL-1. These cytokines attract cells that mediate the immune response and enhanced release of oxygen radicals. The net effect over time appears to be diminished immune responses and greater susceptibility to infection. The reduced immune responses also mean greater likelihood that tumors will evade detection and hence grow to become full-blown cancers.

The human behavior of smoking is 7000 years old beginning as a shamanistic ritual. Over 1.1 billion people and up to one-third of all adults smoke. These smokers consume 4.5 trillion cigarettes a year littering our environment with 1.7 billion pounds of cigarette butts (Houston Chronicle 2014). Coastal cleanup projects find cigarette butts represent about one fourth of the pieces of garbage collected on the beach. A single smoked cigarette filter per liter of water is lethal (LD_{50} = 1.8) to marine smelt (*Atherinops affinis*; Slaughter et al. 2011). The small fish ingest cigarette butt fragments and the toxins bioaccumulate as larger fish eat the minnows.

[1] http://data.worldbank.org/indicator/SP.DYN.LE00.IN

This means that smokers and we get a second round of exposure when we eat certain seafood.

Smoking is part of our art, music, literature and daily culture. I do not think we will eradicate smoking in the near future if ever. I do not know how much smoking contributes to global climate change and I don't know if smoking was included in the Paris Accords, but it should be. Perhaps we should encourage younger smokers to continue and thus facilitate evolutionary adaptation. That the does not kill you makes you stronger.

Radiation Exposures

Exposure to radiation poses a human health risk. Global examples in which radiation exposure creates conflict begin with nuclear warfare. I experience conflict when politicians including the President of the United States (POTUS) use the term "nucular" which is not a word, ask your spell checker. The Manhattan Project brought together leading scientists to solve the problem of how to purify radioisotopes and to optimize conditions for bringing unique radioisotopes together so that an uncontrolled fission reaction would take place. Scientists frequently become scientists to solve problems resulting in a better life in the future so the work product of the Manhattan Project thrust many scientists into personal conflict. The application of their solution was an atomic bomb to kill humans in an attempt to end World War II. The conflict centered with the risk of human death, and suffering from the atomic explosion with the benefit of an end to human death, and suffering from a prolonged war. The motivation is to seek the lesser of two evils.

My laboratory at the Eppley Institute for Cancer Research employed several radioisotopes (^{3}H, ^{14}C, ^{32}P, ^{35}S, and ^{125}I) as molecular tracers to monitor cellular processes of biological synthesis and degradation and for probes to measure gene expression. The risk to human health is proportional to the exposure to radioactivity. The use of these radioisotopes is carefully regulated to protect the health of investigators such as myself and the key guidance was to keep exposure As Low As Reasonably Attainable (ALARA). Exposure is the product of the number of disintegrations per minute (dpm) and the energy associated with a disintegration. The unit of measure for dpm is Curies after Madam Curie, the female scientist that discovered radium. The unit of energy from a disintegration is million electron volts, mEV, and is different for each radioactive isotope. Most of our experiments used low energy beta emitters like tritium (^{3}H) and the amounts were microCuries (μCi) and were relatively safe but we used protective clothing and inserted shielding material between our bodies and the radioactive source to minimize exposure. However, every piece of equipment that touched the radioisotope had to be collected as radioactive waste, which was disposed by authorized handlers that carried it to designated landfill sites. The disposal process resulted in expense and limited the number of experiments.

The clinics, in rooms across the hall in some cases, used radioisotopes to treat patients. One example was ^{131}I (radioactive iodine) to treat an overactive thyroid. The medical use balances risk to benefit and the lower risk to a person from exposure to radioactive iodine than the health consequences of an overactive thyroid. A coworker, recently treated for an overactive thyroid, passed by me in the hall as I carried a film cassette containing a 10-day exposure of a film to a radioactive phosphate, ^{32}P, probe ruined my experiment because the radioactive iodine in her neck exposed my film. This highlights the relative exposure difference in an experiment compared to a medical treatment. The patient would use the restroom in the lab flushing several thousand times more radioactivity into the municipal sewage system than my lab would generate in a month. Safe management of radioactive exposure is not equal between research laboratories and medical clinics. The inequality is the result of different regulatory groups that have different objectives and safe is not the same safe between two rooms in the same building.

World War I fighting moved into the night and the cover of darkness. Luminous watch dials became essential to synchronize battle efforts. Radium paint provided the luminous numbers on the watch face painted by steady handed "Radium Girls." In order to maintain a sharp brush these women twirled the brush in their lips repeatedly dosing themselves with approximately 1.7 grams of radium paint per day. Eventually, entire factories became contaminated with radium exposing all personnel to the radioactive element. The industry grew to 120 factories and 2000 female dial painters. In 1922, 8 women died and 12 were seriously ill in a single luminous watch dial factory ushering a slow realization to the health risks of radium. The luminous dial technology shifted to tritium (^{3}H) but not until the 1980s and radium dials were in production until 1963. Numerous exposed individuals succumbed to radiation sickness during the decades of production but most of the workers did not. Perhaps chronic exposure can lead to a tolerance to low-level radiation.

On August 6, 1945 a ^{235}U device exploded over Hiroshima Japan and on August 9, 1945 a ^{239}Pu device exploded over Nagasaki Japan. Heat from gamma and neutron radiation killed 64,000 people immediately. Within 2–4 months, the deaths rose to 129,000 to 226,000 due to radiation sickness. In 1950, the Radiation Effects Research Foundation (RERF) initiated a Life Span Study (LSS) of 92,228 people within 10 kilometers of the blasts compared to 26,850 people that were not in either city at the time of the bombing. No significant increase in gallbladder, bone, pancreas, uterus, prostate or malignant lymphoma has been seen in the LSS to date. However, a significant increase in lung, liver and breast cancer have been observed (UNSCEAR 2000). The view of the cup that is 20 percent full is that some people survived extraordinary and sudden exposures to radiation. Can humans develop resilience to radiation?

Early in the twenty-first century, Japan derived 40 percent of its' electrical power from 54 nuclear reactors, all built near the coast of the Pacific Ocean. An overwhelming earthquake on March 11, 2011 and the resulting tsunami disabled emergency generators required to cool the reactors. The resulting meltdown registered as a 7 on the International Nuclear Event Scale (INES) acknowledging a major accident and nearly equivalent to the April 26, 1986 meltdown of the Chernobyl reactor

in Kiev, Ukraine. The immediate trauma of these events was lethal but less than expected. These reactors release short-lived ^{131}I, which will produce thyroid cancer in those exposed shortly after the release event. We wait and monitor as time progresses.

Uranium-235 (^{235}U) is the only naturally occurring fissile isotope making it a fuel of choice for nuclear reactors and some nuclear weapons. One kilogram of ^{235}U can produce as much energy as 1500 tons of coal, a significant potential to reduce release of CO_2 into our changing environment. Mining of yellowcake U_3O_8 uranium yields approximately 70,000 tons of primarily uranium-238 (99.27%) and uranium-235 (0.72%) is conducted in Kazakhstan, Canada, Australia, Niger, Namibia, and Russia. About 3 percent of global uranium is mined in the United States. The price of uranium rose from $7/lb. in 2001 to $138/lb. in 2007 stimulating an overly exuberant increase in mining. The exuberant mining practices and the Fukushima nuclear disaster in 2011 led to surplus supply over demand and the uranium prices fell closing mines and mills in the Paradox Valley of Colorado creating near ghost towns of Uravan, Nucla, and Naturita. As uranium prices return, conflict has arisen between Western Colorado miners and environmentalists over concern for uranium's immediate danger to health and exposure to decay products such as radon, strontium-90, and iodine-131. These heavy metals have been found in drinking water in areas surrounding the mines. Leaching uranium from the mined ores involves halide acids (hydrofluoric acid) and creation of highly toxic uranyl fluoride. The enriched uranium powder is flammable and small grains will spontaneously ignite. Some Colorado miners got sick but others did not once again pointing to possible resilient people. The larger environmental conflict posses global warming from coal fired power plants versus toxic metal contamination and radioactive waste disposal from uranium.

There are 449 nuclear reactors operating in 30 countries worldwide with 60 new plants under construction in 15 countries. Nuclear plants provided 11 percent of the world's electricity.[2] Each new plant incorporates improved safety features based upon lessons learned from aging reactors and next generation plants produce less radioactive waste per watt of electricity. That said, natural disasters, human error, and terrorism continue to threaten our safety. In addition, the world has approximately 15,000 nuclear weapons of which 9400 are in military arsenals.[3] There are 65 known incidents from 1945 to 1989 in which US nuclear weapons were lost, destroyed, or damaged (Mahaffey 2014). It is time to investigate and embrace the exceptional potential for adaptability to radiation.

Extremophiles are organisms that have adapted to extremely harsh environments and the first line of investigation to identify survival mechanisms that may be exploited in humans. Most extremophiles are single celled organisms but the tardigrade is a 0.1–1.2 mm multicellular creature with exceptional tolerance to temperature (low of −273 to high of 100 °C; Hengherr et al. 2009), pressure (7.5 GPa, Ono et al. 2008), and radiation (Horikawa et al. 2006). The median lethal dose of

[2] https://www.nei.org/Knowledge-Center/Nuclear-Statistics/World-Statistics

[3] www.cnn.com/2017/09/23/politics/nuclear-weapons-over-time-y.../index.html

radiation in humans is 4 Gy (Hall and Giaccia 2012) but the bacteria in the human gut, *Esherichia coli,* can tolerate 50 Gy. A notable radiotolerant bacterium, *Deinococcus radiodurans*, can survive 5000 Gy of gamma rays by exploiting an extensive DNA repair system. The median lethal dose of radiation in tardigrades ranges from 1000 to 6000 Gy (Hashimoto and Kunieda 2017) making these tiny animals 1000 times more radiotolerant than humans. The tardigrades have very novel protein, Dsup, which appears to shield their DNA from reactive oxygen species (ROS) and irradiation. Inserting Dsup into human cells enhances their tolerance to irradiation so perhaps therapeutic variants of the protein can be developed to help humans survive future atomic accidents.

My friend Denis Burger is an example of a resilient person to the sun's radiation. Denis went fishing on the shallow sandy reefs near Christmas Island. Denis stood calf-deep in the Pacific Ocean with fishing gear dressed in a bathing suit for a week. A colleague wearing long pants, long sleeved shirt, and hat with long brim and neck protection as well as zinc oxide on his face received second-degree burns on a half-inch region of an ear that was not shaded. What a striking example contrasting the response of two individuals exposed to equatorial sun on a shade less ocean. Denis's skin actually began to darken a few days before they left for the fishing trip, an anecdotal observation suggesting a role for the brain in radiation resilience.

Inevitable Overpopulation

I was involved in a citizens lobby group for hunger called RESULTS (Responsibility for Ending Starvation Using Legislation Trimtabbing and Support) from 1987 to 1991. The premise of this group was based on a study by the National Academies of Science (NAS) that found sufficient food is produced in the world so that no one needs to go hungry but what is lacking is the political will to distribute food to the hungry. I was inspired by quotes such as "The world doesn't just turn, someone has to push it" and "We are not passengers on this Earth but the captain and crew." I considered the fact that children are the most vulnerable people to food insecurity an unjust reality. I learned how to create a press conference in my hometown of Omaha Nebraska for the annual UNICEF report on the state of the world's children. My efforts culminated with creating a candlelight vigil for the world's children held at Boystown near Omaha. I am proud of my efforts and have always felt I received more than I gave in terms of good will.

The experiences were rich in so many aspects of food insecurity. A commonly held opposition belief was reminiscent of Ebenezer Scrooge- let the hungry die and reduce the population burden. It is true that in places where food is limited that death of children under 5 years old (U5MR) is also very high. On a global scale, family size increases with food insecurity. The belief that you need to have more children to make sure some survive. As regions improve food security, their family size decreases and so feeding people can "paradoxically" serve to reduce population growth.

One program of particular interest was the Women, Infants, and Children (WIC) federal feeding program. I volunteered to help at the local WIC office bolstered by the fact that every dollar spent on this program saves over 4 dollars in future healthcare costs. Trimtabbing means that you support programs where the returns far outweigh the expenditures, which also suggests sustainability. However, as I met families in the program, I found some families that depend on public assistance for three generations. A disturbing expectation by some of these families outside the employment grid that someone will always make sure their family is fed. Is this adaptive response sustainable?

The long range outcomes are that the world population is increasing exponentially and while progress in reducing hunger has happened, the world can still produce an abundance of starving people particularly children. Our world has always produced starving children and it may always repeat this tragedy. No matter how many times I put money in the red bucket during the holidays, that bucket is there again the next year. No matter how many refugees and children die from starvation each year, more appear the next year. I must face "losing my religion" as the inevitability of human suffering persists in spite of all the efforts of well-intended people of the world.

The NAS report that inspired so much of my optimism was prepared in 1985 when the world population was 4.85 billion but now the population has grown to over 7.4 billion. Estimates of the limit to a sustainable world population is 7.7 billion people (Christian Science Monitor 2008). The intense agriculture cultivation practices mean food prices increase with world oil prices (CNN Money 2011). Inevitable fuel price increases will translate into food riots (Buchanan 2008; Borger 2008). Soon all the efforts of well-intended people and policies seem doomed to fail. What will happen and when?

Most of the 795 million hungry people (1 in 9 of all people) live in developing nations.[4] Not surprising is that malnourished people cannot afford to buy enough food and farming supplies to produce their own food.[5] The observed effects of malnutrition include dizziness, headache, lack of concentration, irritability, and nausea. To the remaining 88 percent of the world's population this is "someone else's problem" or an SEP. Unfortunately, neglect is not an option or more directly, malnourished people threaten global health.

Everyone should be concerned by the outbreak genesis in malnourished populations. Infections cause 45 percent of global deaths in children under 5 years of age (Pelletier et al. 1995). Malnourished children are more likely to die from infections and once infected have a greater risk of dying (Christi et al. 2009). Impaired immune function is associated with enhanced susceptibility to infection and inability to clear infections in malnourished children. Their gut mucosa is atrophied which is accompanied by diminished secretory IgA so their barrier to infection is limited. Larger numbers of colonized bacteria alters the microbiome of malnourished children characterized by altered commensal flora. Immune responses are less effective typified

[4] www.foodaidfoundation.org/world-hunger-statistics.html

[5] www.thp.org/issues/hunger [the hunger project]

by less responsive granulocytes, effector T-cells, dendritic cells, and lymphocytes. Malnourished children do not respond as well to vaccinations and generally have lower antibody responses. Starvation leads to cytokines associated with Th2 (IL-4 and IL-10) responses are elevated while those associated with Th1 (IL-2, IL-12, and IFNγ) are diminished (Rytter et al. 2014). Combating viral infections relies on "cellular immunity" or Th1 responses consistent with problems surviving emerging viral infections.

A Global Hunger Index (GHI) has been developed to identify regions of the world where food assistance is necessary. The GHI values range from zero indicating no hunger to 100 representing the worst possible famine. Africa holds numerous countries with GHI values indicating significant food insecurity including the Central African Republic (CAR), Niger, Chad, Ethiopia, Sudan, Mozambique, Liberia, Madagascar, Sierra Leone, Burundi, and Zambia. The greater the GHI, the deeper the immune suppression and the lower the barrier to viruses leaving their animal reservoir and entering the human population in a process called zoonosis.

Examples of zoonotic viruses with origins in Africa include: Zika was isolated in 1947 at the Zika forest in Uganda from a Rhesus monkey with initial human cases in Uganda in 1952 and then in Nigeria in 1953. Cases outside Africa were observed in 1978 in Indonesia culminating with the 2015 outbreak in Northeast Brazil. West Nile Virus (WNV) was first described in 1937 from a febrile illness in Uganda. WNV outbreaks of increasing frequency and severity involving adults began in Bucharest in 1996, Volgograd Russia in 1999, and Israel in 2000. The virus crossed the Atlantic in 1999 with infections in New York City spreading to the contiguous United States, Mexico and Canada within 3 years (Chancey et al. 2015). Hepatitis C virus (HCV) is distributed globally but the Middle East and Africa have the highest prevalence worldwide (Chaabna et al. 2016). HCV infections represent 2.8 percent of the global population (185 million) and genotype 4 (15–18 million people) is prevalent in Northern and Equatorial Africa and the Middle East while genotype 5 and 6 are associated with South Africa and Hong Kong (Abdel-Ghaffar et al. 2015). Endemic canine rabies (*Rhabdoviridae)* causes 55,000 deaths annually localized to Asia and Africa. Additional viruses in the lyssavirus genus include Duvenhage and Mokola viruses with reservoir in shrews in Africa (Jackson and Johannsen 2010). Human rotaviruses cause diarrhea, primarily in newborns and children. More than half of the global rotavirus (*Reoviridae*) deaths (232,000) occur in Africa (Heylen et al. 2014). Acute lower respiratory infections (ALRI) typified by respiratory syncytial virus (RSV) are seen in 35 million episodes per year in sub-Saharan Africa resulting in 500,000 deaths (Rudan et al. 2013). A survey of children under 10 years of age from Tanzania with axillary tempertures over 38 °C from April through December of 2008 revealed 38% with either human rhinovirus (HRV) or human enterovirus (HEV) and co-infection was documented in 66% of the infected patients (L'Huillier et al. 2015). A unique enterovirus EV-A71 capable of causing poliomyelitis-like disease was found only in Central African Republic (CAR) in the stool of paralyzed children (Bessaud et al. 2012). O'nyong'nyong virus (ONNV) produced a 1959 epidemic in East Africa (Kenya, Uganda, Tanzania, Malawi, and Mozambique) affected over two million people. No other vertebrate host has been

identified but three strains restricted to Africa are known from Uganda, Senegal, and Ivory Coast (Lwande et al. 2015). Chikungunya (CHIKV family *Togaviridae*) was first recognized as epidemic in East Africa in 1952–1953 in what is now Tanzania (Robinson 1955). Since 2004 outbreaks associated with morbidity have occurred throughout the tropical and subtropical world. The virus is maintained in Africa by cycling from non-human primates to forest *Aedes spp*. mosquitos. Arenaviruses cause severe hemorrhagic fevers with fatality rates of up to 30 percent in those infected. Lassa virus (LASV), Lujo, and lymphocytic choriomeningitis virus (LCMV) are found in Africa and cause disease (Witkowske et al. 2015). Ebolavirus outbreaks in Africa are larger and more frequent since 1976.[6] The catastrophic 2014–2015 Ebola outbreak in West Africa infected over 24,000 individuals and was responsible for 11,000 deaths. Individuals from the highest poverty regions of Liberia were associated with the highest rates of Ebola transmission (Fallah et al. 2015). In addition, the outbreak reduced access to healthcare by 50 percent resulting in elevated deaths from malaria, HIV/AIDS, and tuberculosis by approximately 10,500 (Parpia et al. 2016). Viral infections from Marburg virus (MARV), monkeypox, and HIV-1 are also Africa centric in their origins. The brief and incomplete synopsis of known viral infections in malnourished regions of Africa describes a tangible liability to be diminished by bolstering the population immune system of these regions.

If the world has reached the limit of food security then perhaps strategic dietary supplementation can alleviate the worst aspects of starvation. Targeting deficiencies in key micronutrients is the goal of the United Nations Millennium Developmental Goal (MDG; Nair et al. 2016). Hematologist Lucy Wills discovered Marmite, a yeast extract, prevents and cures anemia observed in malnourished pregnant women in India in 1937. Examining the extract led to "Wills Factor" as the key ingredient in Marmite now known as folic acid (folate). Folic acid (vitamin B9) deficiency results in anemia due to bone marrow suppression because cells are not able to synthesize adenosine, guanine, and thymidine (Huether and McCance 2004). Sidney Farber exploited the relationship between folate deficiency and diminished growth of cells in the bone marrow to discover aminopterin in 1947, an anti-folate drug used to treat malignancies of the bone marrow (Farber et al. 1948). Aminopterin has been replaced by a closely related drug, methotrexate, and Farber is regarded as the father of modern cancer chemotherapy.

The human body needs vitamin A and D for maintenance of innate and adaptive immune functions (Mora et al. 2008). Frederick Gowland Hopkins found fat-soluble factors in milk necessary for growth of experimental rats in 1912 leading to a Nobel Prize in 1929 (Wolf 2001). Vitamin A is a fat-soluble yellow substance comprised of carotenes, retinol, retinal, and retinoic acid. Vitamin A deficiency is common affecting one-third of all children under 5 years old (WHO 2009), the cause of 670,000 deaths in children under 5 (Black et al. 2008). Vitamin A deficiency is the leading cause of preventable childhood blindness (UNICEF 2015), a condition prevented with less than 300 μg retinol per day. Vitamin A plays a major role in the

[6] cdc.gov

differentiation of T cell subsets, migration of T cells, and development of T cell dependent antibody responses (Ross 2012). Indeed, retinoic acid directs plasticity of T cells to shift Th2 (GATA3 expression) to Th1 (T-bet expression) enhancing IFNγ antiviral defenses while inhibiting Th17 (RORγt expression) induction and driving Treg (Foxp3 expression) development.

Elmer McCollum found that cod liver oil depleted of vitamin A could still treat rickets, discovering vitamin D in 1922. He called it vitamin D to reflect the fourth vitamin discovered. In 1923, Harry Steenbock reported foods irradiated with ultraviolet light had increased vitamin D content creating a lucrative Wisconsin Alumni Research Fund (WARF). Indeed, vitamin D is synthesized in the skin from cholesterol by sunlight and is not an essential dietary factor. Nearly 70 percent of the US population is vitamin D deficient based on a threshold level of 30 ng/mL 25 (OH) D. Vitamin D deficiency is associated with increases in pneumonia, Dengue fever, hepatitis B (HBV) and hepatitis C (HCV) viruses, as well as HIV (Borella et al. 2014). Vitamin D levels influence the spread of infectious disease (Kearns et al. 2015; Miragliotta and Miragliotta 2014). Vitamin D tends to activate the innate immune response while diminishing the adaptive immune response (Hewison 2011). Supplementation of diet with vitamin D is not universally supported based on conflicting reports of potential benefit and the suppressive effects on the adaptive immune system. Selecting a modest dose for supplement recommendations may balance the desire to improve survival while limiting potential harm.

Nonessential Competition

How can individuals continue to break Olympic world records? We celebrate human performance and exceptional physical acts in the Olympics every 2 years. Humans jump higher, run faster, swim faster, throw things farther, and jump farther at each Olympic interval. Young healthy individuals that trained extremely hard under the supervision of the best trainers created records from decades ago. What has changed to allow those records to be bested routinely? Clearly, every minute detail is exploited for optimal performance but can people continue to improve?

The athlete often probes the limits through enhanced diet, added vitamin supplements, and a variety of performance enhancing drugs. The International Olympic Committee has banned the use of performance enhancing drugs in an effort to focus on the human aspect of performance. The use of these drugs can help set new records but once everyone used them, the new records would once again converge to a new standard. Drug use comes with a penalty of future health threats so the beautiful form of the present becomes a broken form in the future.

Performance-enhancing substances offer insights into resilience mechanisms. The classical use of anabolic drugs including steroid hormones, human growth hormone, and anabolic steroids have been used to biuld up muscle mass. Selective androgen receptor modulators (SARM) such as MK-2866 (Endosarm) and RAD-140 represent nonsteroidal ligands for the androgen nuclear receptor are used to

improve bone density. The mechanism to be discussed later appears to drive resilience through activation of nuclear receptors. Other approaches use stimulants (dopaminergic, caffeine, ephedrine, methamphetamine, and amphetamine), antidepressants (bupropion-Wellbutrin), and nutritional supplements like creatine to improve muscle strength, decrease reaction time, and delay the onset of fatigue. Sedatives (ethanol and cannabis), beta-blockers such as propranolol, and anxiolytics including diazepam are used to limit agitation and calm nerves are used for a broad range of skilled tasks. Blood doping agents like human erythropoietin (EPO) and blood transfusion increase the oxygen-carrying capacity of blood for endurance sports such as long-distance cycling, running, and Nordic skiing. Finally, herbal medicines called "adaptogens" point to performance enhancing/resilience substances in plants. The molecular basis of these performance-enhancing substances is poorly understood and deserves in depth evaluation.

The term "sport" comes from chicken breeders to describe the odd baby chicks that stand out among the others. Most were malformations linking sport to a genetic abnormality. Some athletes do standout such as 7′4″ basketball players or 6′9″ 380-pound football players. The gender divisions are challenged, as transgender females have been successful against female from birth competitors. How will the limits of human performance be hacked in the future?

Perilipin is a protein encoded by the PLIN gene that sits on the boundary of a fat cell protecting the lipid inside from being used for energy. Fat in fat cells (adipocytes) is stored for future use and once the appropriate signal is provided, perilipin is modified to permit release of the fat for energy production. PLIN appears to be associated with human obesity (Soenen et al. 2009). I worked on a project to create a drug that would block the synthesis of perilipin. Our first successful candidate, "FatRat," was administered to an older rat with prominent inguinal fat pads. We observed reduction in body fat. We looked for potential undesirable effects such as elevated blood lipid levels. If the drug reduced lipid in fat throughout the body then we considered the possibility that it might appear in the blood. It did not. In fact, serum cholesterol levels were lower in the old animals treated with FatRat. The perilipin blocking drug did not progress in part because we were not certain of the pathway to gain FDA approval and in part because the cost of the drug would be high.

Myostatin (growth differentiation factor 8) encodes a protein that inhibits muscle cell growth and differentiation to protect the body from muscle overutilization of energy. The myostatin gene (MSTN) was knocked out in mice resulting in mice with twice the muscle mass compared to normal mice (McPherron et al. 1997). Once scientists knew what to look for they found cattle (Kambadur et al. 1997), sheep (Clop et al. 2006), whippets (Mosher et al. 2007) and humans (Schuelke et al. 2004) with naturally occurring deficiencies in myostatin and the associated robust muscle development. I explored development of inhibitors of MSTN synthesis as an adjunct approach to treatment of boys with Duchenne Muscular Dystrophy. While discovery was nearly complete, the project was put on hold while the company was focused on the dystrophin exon-skipping project, which successfully led to FDA approval. A monoclonal antibody was developed to bind myostatin as a competing

approach to muscular dystrophy (Kota et al. 2009). Pharmaceutical development of stramulumab, an antibody designed to neutralize myostatin is no longer in development. Advances in CRISPR technology offers a low cost approach to personalize performance but unapproved myostatin inhibitors for Olympic hopefuls. The International Olympic Committee (IOC) will need to investigate muscle genome sequencing as part of their screening measure for illicit performance enhancing drugs.

Perilipin and myostatin point to the existence of genes designed to regulate the distribution of energy between body compartments. The balance of these genes expression defines a possible zero sum game for total body energy. If you need muscle, muscle gains but at the expense of some other body system. The immune system can be very demanding of energy and in monkeys fighting an Ebola infection; we found a near complete depletion of all body fat, including pericardial fat. Hacking performance limits will likely lead to deficits in sensitive body organs.

Extending Lifespan

The pursuit of longevity permeates the known human history. From fictional fountains of youth to science fiction people capable of sucking the life force from young to rejuvenate their lives. Humans display a wide range in life expectancy with a current maximum of 122 years (not including examples from the Bible). Genetic differences in aging are associated with DNA repair, antioxidant defenses, energy metabolism, and recycling mechanisms (autophagy). The best efforts to extend lifespan include caloric restriction (Anderson et al. 2009), a collection of existing therapeutics including rapamycin (Harrison et al. 2009) and metformin, and dietary supplements including MitoQ, resveratrol and pterostilbene (Kaeberlin 2010). While interest is extremely high to confirm life extension, the research inherently takes a long time, which limits the rate of progress. Immortality mechanisms have shed light on novel anti-cancer strategies.

Muller coined the term telomere, Greek telos for end and meros for structures, in 1938 from studies in Drosophila indicating the ends of chromosomes are specialized structures (Muller 1938). The shortening of repeating telomeres is thought to be responsible for the "Hayflick limit" that cells have a defined replication limit and are not immortal (Hayflick and Moorehead 1961). The limit is 50–70 cell divisions at which time the cells become senescent and cell division stops. Human telomeric DNA is 5–12 kb of dsDNA (5′-TTAGGG-3′/3'-AATCCC-5′) followed by 150–300 nucleotides of single stranded 3′-overhang DNA. Telomeric DNA shortens by 50–200 bases during each S phase (DNA synthesis segment of the cell cycle) due to the inability of DNA polymerases to begin *de novo* synthesisin the 3′ to 5′ direction, known as the end replication problem described by Olovnikov (Olovnikov 1973) and Watson (Watson 1972).

Carol Greider, Elizabeth Blackburn, and Jack Szostak won the 2009 Nobel Prize in Physiology and Medicine for their 1984 discovery of telomerase (Greider and

Blackburn 1985). Telomerase is a reverse transcriptase synthesizing DNA from and RNA template carried by the enzyme (Sequence 3'-CCCAAUCCC-5′). Telomerase reverse transcriptase is a complex of two molecules each of telomerase reverse transcriptase (hTERT), telomerase RNA (TERC or hTR), and dyskerin (DKC1) that can overcome telomere loss through enzymatic telomere elongation adding repeats to the 3′-end of the chromosome. TERT is responsible for addition of 5′-d (TTAGGG)-3′ nucleotide segments to the ends of chromosomes which prevents degradation of chromosome ends. Loss of hTERT (chromosome 5) is associated with *Cri du chat* a rare genetic disorder 1 in 50,000 live births with greater incidence in females by a 4:3 ratio.

hTERT transcription is enhanced by c-myc, Sp1, HIF-1, and AP2 and transcription is suppressed by p53, WT1 and Menin. Shortened telomeres are associated with premature aging seen in Werner syndrome, Ataxia telangiectasia, Bloom syndrome, Fanconi anemia, and Nijmegen breakage syndrome (Blasco 2005). Telomere deficiency is linked to aging, cancers, dyskeratosis congenital, and *Cri du chat* while over-expression is associated with cancers and tumor formation. Telomerase is responsible for the self-renewal properties of stem cells. Dominant negative mutants of hTERT reduce telomerase activity within a cell leading to apoptotic cell death in cells with short telomeres. Some phytochemicals inhibit telomerase activity including isoprenoids, genistein, and curcumin.

Telomerase is expressed in more than 85 percent of cancer cells but not in somatic cells, pointing to expression as a nearly universal cancer marker. A noteworthy caveat being that telomerase is expressed in pluripotent stem cells and germline cells. The 10 percent of cancer cells not expressing hTERT are "mortal" or use the alternate lengthening of telomeres pathway (ALT). Telomere attrition leads to replicative senescence due to their inability to protect chromosome ends from DNA damage and repair mechanisms. Telomeres need to evade ATM and ATR checkpoint pathways, but telomere attrition triggers DNA damage responses and p53-mediated cell cycle arrest. Cells that inactivate the p53/p16 Rb checkpoint will go to crisis stage marked by genomic instability and aneuploidy and ultimately p53-independent apoptosis.

While hTERT is tumor cell centric, hTR is ubiquitously expressed in all human cells so telomerase activity is limited by the expression of hTERT. hTERT plus hTR are 280 kDa in size but the holoenzyme is 1.5 mDa due to associations with dyskerin and shelterin complex proteins. Telomerase activity is measured by the PCR-based TRAP assay which can be quantitative using an internal standard, ITAS (Buseman et al. 2012). Alternative splicing is one mechanism of regulation of telomerase activity. hTERT consists of 16 exons and 15 introns spanning 35 kbp. The alpha isoform is a dominant negative inhibitor of telomerase lacking part of exon 6 (alternate 3′ splice acceptor 36 bp into exon 6). A major splice form beta skips exons 7 and 8 but is out of frame and degraded by NMD. A low abundance protein, a cell has 50–100 telomerase molecules produced from 20 mRNA molecules of which only a fraction are full length transcripts (Wong et al. 2013). 9 of 14 splice variants of hTERT introduce a premature termination codon (PTC) in the reading frame and are degraded by NMD. SRSF3 is the major splice recognition

snRNP for hTERT and skipping exons 7 and 8 can be translated due to polysome binding and low NMD activity. Alternate splicing is regulated by SRSF11 (overexpression upregulated beta splicing), hnRNPL and hnRNPH2 (overexpression suppresses beta splicing). The beta deletion binds hTR (RNA recognition) so sequesters this from active hTERT protein. Cancer cells may express high levels of catalytically inactive hTERT splice variant to enhance anti-apoptotic activity at the mitochondria as exemplified in cis-platin resistance. Alternately hTERT overexpression enhances genomic stability and DNA repair (Listerman et al. 2013). Telomerase appears to be a resilience gene.

Targeting telomerase is a broad-spectrum anticancer strategy: telomeres are critically short in most tumors compared to normal tissues, telomerase activity is exceptionally high. Telomerase protein component, hTERT, and RNA component, hTR, as well as the template are targets. We found phosphorothioate DNA oligomers (PSO) with the telomere repeat sequence, 5′-d(TTAGGG)-3′, we called TAG6 induced apoptosis in Burkitt's lymphoma cells in culture and blunted the growth of xenograft lymphomas in mouse (Mata et al. 1997). Urinary excretion is the primary route of oligomer clearance and administration of a fluorescein conjugated TAG6, 5'-FITC-d(TTAGGG)-3′, to tumor bearing mice resulted in molecules with TAG6 plus multiples of 6 DNA extensions that were recovered in the urine indicating the 5′-d(TTAGGG)-3′ molecule is a substrate for telomerase. This observation also became a very popular *in vivo* assay for telomerase activity.[7] The PSO 5′-d(TTAGGG)-3′ molecules could slow cell doubling, induce death in OMA-BL1 (Burkitt's lymphoma) cells, and inhibit telomerase. These observations led to a small SAR project leading us to find the most potent PSO telomerase inhibitors contained 3–4 consecutive guanines free at the 3′-end of and oligomer. Further, while longer repeats such as 5′-d$(TTAGGG)_2$–3′, 5′-d$(TTAGGG)_3$–3′, 5′-d$(TTAGGG)_4$–3′ are more potent inhibitors, they can form G4 secondary structures that are no longer telomerase substrates. This was confirmed using a 5′-d$(TTAG\mathit{deazaG}G)_3$–3′ which cannot form G4 structures and found this molecule to be a dose-dependent, potent inhibitor with a CC_{50} in OMA-BL-1 cells of 0.2 uM (Page et al. 1999). The PSO SAR studies identified linear telomerase substrates in which the 3′- end must have at least 3-consecutive guanines. We next questioned if simple telomerase recognition is sufficient or would the optimal inhibitor need to engage telomerase reverse transcriptase activity. To test this we used a rat 70 percent partial hepatectomy model to measure recovery of liver weight/regenerate as an *in vivo* measure of cell division. We employed a phosphorodiamidate morpholino oligomer (PMO) chemistry with the 5′-(TTAGGG)-3′ sequence that is not a substrate for reverse transcriptase to test the hypothesis. The PMO did not influence cell doubling or liver cell growth indicating the reverse transcriptase must be active to be inhibited. The research project did not progress to clinical trials due to concerns over the PSO chemistry off target effects.

BIBR1532 (2-[€-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid) is a small molecule inhibitor of telomerase (IC50 = 93 nM) but not other RNA polymerases or

[7] Personal communication with CalBiochem, a company involved in sales of the oligomer.

DNA polymerases (Damm et al. 2001). It is a non-competitive inhibitor for binding to the DNA primer with IC50 of ~100 nM which is distinct from the mode of inhibition by nucleoside antisense approaches. The inhibition is in the elongation step with mixed non-competitive inhibition kinetics (Pascolo et al. 2002). It prevents telomere elongation in leukemia cells and causes cell death with IC50 of 30 to 80 uM but no activity in normal CD34+ cells (El-Daly et al. 2006).

Telomestatin is a natural product isolated from *Streptomyces anulatus* and has been shown to be a potent telomerase inhibitor (IC_{50} = 5 nM) due to G-quadruplex interaction. Telomestatin binds intramolecular guanine four stranded structurres (G4) and will stabilize G4 structures in the absence of monovalent cations (Kim et al. 2003). TMPyP4 binds intermolecular G4 structures and suppresses replication of ALT-positive cells as well as telomerase positive cells. Critically short human telomeres cannot form secondary structures and induce senescence by activating p53 or inducing p16/RB which initiates growth arrest and cell death. Compounds that inhibit telomerase activity also lead to telomeric disruption, formation of anaphase bridges, apoptosis, and anti-tumor activity in mouse xenograft models. Telomestatin can induce gel mobility shift for $d(TTAGGG)_4$ in the absence of monovalent Na + or K+.

The 2,4,6-triamino-1,3,5-triazine compound 12,459 selectively binds G-quartets and inhibits telomerase. hTERT is alternately spliced with 4 insertions at exons 4, 11, 14, and 15 as well as deletions in exon 5 (−alpha) or exons 7 and 8 (− beta). They find 12,459 reduces telomerase activity through alteration of hTERT splicing (making –beta transcripts; Δ7,8) by interacting with G4 motiffs in intron 6 (Gomez et al. 2004).

Sergei Gryazinov, a collaborator at Applied Biosystems, went to Geron with the pnDNA chemistry and investigated telomerase inhibitors with Gerry Shay. GRN163L is an N3'-P5' oligomer (5'-Palm-TAGGGTTAGACAA-NH_2–3′) targeting telomerase as a 12-mer with phosphorothioate linkage (Shea-Herhert et al. 2002) at an allosteric telomerase site (Pruzan et al. 2002) and conjugated to a lipid, palmate, which enhanced cell uptake (Herbert et al. 2005). Because it is an allosteric inhibitor, it does not target the active site of telomerase and is named Imetelstat (not an antisense naming convention). Inhibition (IC_{50}) of telomerase as defined by the TRAP assay is 50–250 nM. Cells with short telomeres will enter apoptosis when treated but A549 cells with $p53^{wt}$ and long telomeres are not easily killed (Dikmen et al. 2005). A human dose of 9.4 mg/kg is utilized which puts this compound at the limit of phosphorothioate tolerable doses- some extension provided by the short length of the compound (Thompson et al. 2013). Imetelstat has revealed efficacy against breast cancer (Goldblatt et al. 2009), non-small cell lung cancer (NSCLC; Chiappori et al. 2015), multiple myeloma (clinicaltrials.gov), and pancreatic cancer (Burchett et al. 2014). The continued clinical trials with Imetelstat represent the most advanced therapeutic designed to inhibit telomerase.

An innovative approach is driving suicide gene (TRAIL or Cas6) expression from the hTERT promoter. In yet another approach hTERT promoter is used to direct lytic replication of adenovirus, OBP-301 known as Telomelysin. hTERT pep-

tides have been (as of 1999) under investigation as cancer immunotherapy. A peptide vaccine, GRNVAC1 is now in development to induce immune responses to hTERT.

Telomeres are transcribed into lncRNA, telomeric repeat containing RNA (TERRA), which form DNA:RNA hybrids at chromosome ends that promote recombination. Dysfunctional telomeres trigger the DNA damage response (DDR). TERRA actively participates in telomere stability but can lead to DDR which threatens genomic integrity (Cusanelli and Chartrand 2015). TERRA engages ALT-mediated mechanisms through formation of R-loops and may act as a regulator of telomerase. TERRA is an attractive target for anticancer therapy.

The word progeria is Greek for "Pro" meaning premature, and "Geras" meaning old age. This very rare genetic disorder was first described in 1886 by Jonathan Hutchinson (Hutchinson 1986) and again in 1897 by Hastings Gilford (Gilford and Shepherd 1904) and now is referred to as Hutchinson-Guilford progeria syndrome (HGPS). This is a rare genetic disease with incidence of 1 in four million births (Hennekam 2006) and few of those affected survive beyond the age of 13. Cleavage of prelamin A to lamin A is mutated so that lamin A cannot be produced and prelamin A then accumulates on the nuclear membrane resulting in symptoms of progeria (Scaffidi et al. 2006). The accelerated events in HGPS are observed in "normal" aging suggesting a solution to HGPS may provide life extension to all.

HGPS mutation creates a novel splice-site in the lamin A pre-mRNA that can be suppressed with an antisense oligonucleotide (Scaffidi and Misteli 2005). The mutation in codon 608 of the lamin A gene (LMCA) is synonymous (G608G: GGC > GGT) becomes an aberrant splice donor site in exon 11 joining to the correct splice acceptor site in exon 12 skipping over 150 nucleotides of exon 11 (loss of 50 amino acids). An oligonucleotide targeted to the aberrant splice donor site will shift splicing to the correct exon 11 splice donor. Efforts continue to develop a therapeutic oligonucleotide but significant obstacles exist. How can an oligonucleotide be delivered to all cells in every organ of the human body? Clinical trials to test efficacy will be challenging, as there are only approximately 100 known HGPS patients in the world. A broad application of this drug to people wishing not to age would have and extremely small chance of success but a therapeutic capable of reversing or preventing aging might generate trillion dollar revenues.

Environmental Adversary

Humans have developed an adversarial relationship with the environment exemplified by Rachel Carson's Silent Spring. Carson pleaded for unity, in what seems to be a "can't we just get along" plea. The premise that we place man as a part of the environment implores that all that needs to be done is to find our natural place in the environment. Indeed, Carson's book served as an anchor to an emerging environmental movement. The Native American Indians exemplified this philosophy in showing respect for the environment and living as a sustainable culture with the environment. Jarad Diamond observed the breakdown in this philosophy in his

book, “Collapse” describing deforestation practices prior to the collapse of ancient civilizations. Are humans destined to destroy their own environment?

The industrial revolution introduced air pollution and chemical discharges creating water pollution near the end of the nineteenth century. Britain created the Alkali Acts in 1863 to regulate release of muriatic acid (hydrochloric acid) into the air. The growing demand for agricultural products from India led to the Madras Board of Revenue in 1842 responsible for forest management. “Silent Spring” emboldened the environmental movement to ban DDT in 1972. Robert White-Stevens, a biochemist at American Cyanamid, disagreed citing the benefits of compounds like DDT. White-Stevens was not alone in opposing teachings of Carson, a 2012 Nature review (Dunn 2012) cited 60 to 80 million deaths resulted from a misguided fear based on poorly understood evidence.

Environmental conflict continues with hundreds of well-funded foundations and institutes supporting policy to limit organic chemical polluting practices such as the Audubon Naturalist Society, the Environmental Protection Agency, and the World Health Organization. The opposition is also large and well-funded supporting the beneficial use of chemical insecticides, herbicides, fungicides and pesticides represented by companies that produce these compounds. Is the movement losing the war?

Is the human population a natural adversary of the environment? The global population exceeds 7 billion, the demand for food production and energy is a force that will not change. The response to the environmental movement is that we now have 20,000 pesticide products, many sold in every store with a garden center in the United States. Life expectancy in 1962 was 70.12 years in the United States and during the pesticide onslaught life expectancy has risen to 78.74 years in 2015. Increases in life expectancy are observed in India and Japan rising from 42.42 to 68.35 years and from 68.6 to 83.84 years, respectively, over the same 1962 to 2015 interval. Does this mean humans are winning their battle against the environment?

My life began in Carlsbad New Mexico on April 13, 1955. My father, a ranger naturalist, routinely leading visitors down into the Carlsbad Caverns, an isolated environment dominated by darkness and bats. My father spent his entire career as a park ranger and was a naturalist at this World Heritage Site. The National Park Service was created in 1916 charged with the roles of preserving the ecological and historical integrity of 59 designated national parks and 358 other sites encompassing 84.4 million acres. My father chronicled his career in a recent book, “The Centennial of a Great Idea” (Iversen 2016) describing our life in seven different parks and monuments as well as two cities where his role was more administrative than park management.

My work for Gradient Modelling, Inc. in Glacier National Park for the summer of 1975 supported a computer model, which would predict forest-fire burn-area, predict resulting plant and animal communities, and support fire management decisions. Natural forest fires are ignited by lightning strikes that occur during rainstorms, and are inherently limited to small regions as the rain extinguishes the fire. The result is a diverse, mosaic forest. Human interactions involve fire suppression measures resulting in large climax forests that are vulnerable to large catastrophic

fires and diminished ecoregion diversity. The objective of the project was to support a "let burn" policy implemented in Glacier National Park but fire boundaries need to be limited to the park boundaries. The Flathead Indian Reservation, the Blackfoot Indian Reservation, Canada, and the Bob Much Wilderness Area manage the land on the other side of park boundaries. My job involved weekly backpacking trips to most regions of Glacier National Park and measuring plant diversity, an obligate lesson in taxonomy. The greater my capacity to identify genus and species of animals, trees, shrubs, and herbaceous plants, the more apparent the limitations in defining a species. Even with genomic sequence, defining a species remains challenging.

Forest fires have been particularly devastating recently. When efficient fire suppression is implemented, a heavy accumulation of dead trees and branches add to a thickening duff layer. These conditions tend to limit plant and animal diversity leading to a more genetically homogenous forest that is sensitive to devastating infestations. The accumulation is an enhanced fuel loading and when fires are ignited in a forest with accumulated load, the fire is more intense and spreads to greater areas. A fire that started in Washington State in the 1920s spread across Idaho and all of Montana with spread rates exceeding 60 miles per hour at times. Fighting these large fires can be costly in lives and money but the efforts are often palliative, as the fire will continue to burn as long as the fuel lasts. Ecological systems must change in order to survive exemplified by the Ponderosa pine forest where fires remove underbrush every 3 to 5 years (Blotkin 2017). The Ponderosa tree bark is exceptionally thick and fire resistant while the pinecones require fire to melt the seal that holds seeds that are distributed after a ground fire. The environmental management lesson points to less intervention is better and can be less expensive. The ground fire clears overgrowth and prepares the soil for new Ponderosa pine trees.

Conclusion

We understand simple concepts like offense and defense. We need a game plan! A workable plan identifies threats and prioritizes each for the likelihood of "attack." The game plan requires both a defense: countermeasures for infection, infestation, pollution, and competition for limiting resources and an offense: plans to ensure sufficient food, water, and habitation. This will place sustainability as a big picture concept in our fight to survive over a test for each individual element of a plan. One aspect of countermeasures is the adaptability in living things.

Scientists rely on comparisons of conditions to identify significant differences. It is easier of see differences between a standard and an extreme condition because the differences are greater. We all seem to be scientists when we explore the limits of the human condition and seek ways to reach beyond those limits. Reaching limits leads to contradiction. We seek lifestyles that can pose health risk. We pursue energy production from atoms that can threaten populations. The current growth in world population may be reaching a limit for adequate food production. We thrive on

competing, which can reveal paragons of the human form while at the same time exposing cheaters. We seek longer lifespans but a drug capable of extending life's limits will disrupt the world's economy. Finally, we exploit our environment for food and financial gain while at the same time preserving wilderness.

Some people hack limits in ways I find excessive. Miami Beach offers beautiful sandy beaches where swimming and sunbathing have been the standard. I visited Miami Beach recently and found people that were not content to sit in the sun or swim. Rather, they required jet skis to thrash about in the surf often while wearing headphones to ensure uninterrupted entertainment. Their noise and frenetic behavior typified environmental greed. The people of the United States have an opulent standard of living. While people can join each other to enjoy exchanging ideas in pleasant conversation, I find most seek distractions of recreational drugs and the isolation of the internet. Rather than evaluating the merit of a philosophy of life by judicious reflection, people resort to shrill and extravagant rhetoric what George Will refers to as "clamorous politics and the survival of the shrillest" and his reference to Eric Hoffer's "Rudeness is the weak man's imitation of strength."

Why do we seek to push the limits of our existence? One likely explanation is "social entropy"- the law of thermodynamics that states boundaries are always increasing. The converse of "social enthalpy"- the role of social order, which indeed sounds a bit authoritarian. Given this is a law of social thermodynamics, we can be sure that seeking life beyond the boundaries is a fact of life.

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Chapter 3
The Threat from Viruses

Abstract Infectious disease represent the most significant threat to human health. Significant geologic cataclysmic events have caused the extinction of countless species, but these "Wrath of God" events predate the emergence of Homo sapiens. Pandemic infections have accompanied the rise of human civilization frequently re-occurring leaving a lasting imprint on human history punctuated by profound loss of life. Emerging infections become endemic and are here to stay marking their presence with an annual death toll. Each decade brings a new onslaught of emerging infectious agents. We are surprised again and again but are never prepared. The long-term consequences often remain unrecognized and are always inconvenient including cancer, cardiovascular disease and immune associated diseases that threaten our health. Reliance on clusters of clinical symptoms in the face of diverse and non-descriptive viral infection symptoms is a foolhardy form of crisis management. Viral success is based on rapid replication resulting in large numbers. Single-stranded RNA viruses with their high replication error rate represent a paradigm for resilience.

Keywords Emerging infectious disease · Endemic · Outbreak · Pandemic · Epidemic · Zoonotic · Epidemiologic

Introduction

Contemplating recent career paths, I reviewed a broad range of scientific questions. Most prominent was, why study an area of science that has no significant impact? In fact, why study anything that is not the most highly impactful area? This question demands a definition, what constitutes high impact? I decided to create a definition that impact and threat to life are related. Further, threat to human life is the highest impact and it is likely that things threatening human life may also threaten all life.

What presents the greatest threat to human life today? History should provide critical insights to answer this question. A comprehensive look would seek causes of mass extinctions over the past 3.5 billion years of life on earth. This perspective has been a trending subject in science with focus on five mass extinctions. There are

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Table 3.1 Mass extinctions

Name	Million years ago	Percent species extinct	Duration of event	Cause of extinctions
Ordovician-Silurian	446	65	4 M years	Unknown
Late Devonian	360	75	25 M years	Unknown
Permian-Triassic	250	85	7 M years	Volcanic eruptions
Triassic-Jurassic	202	62	<1 M years	Climate change
Cretaceous-Paleogene	66	75	0.07 M yrs	Asteroid impact

inherent biases in focusing on mass extinctions such as these events fail to appreciate small living things such as single celled organisms or bacteria. Another bias is in order to appreciate life in the past, the life form must occasionally produce a fossil when it dies. Those concerns aside- geologic events have threatened the survival of living things (Table 3.1).

The actual events causing these extinctions are frequently debated, for example, the asteroid impact 66 million years ago off the coast of Mexico was accompanied by a massive tsunami that was responsible for mass extinctions in Montana, the impact may have also triggered an enormous volcano in India resulting in ash and lava flow responsible for regional species extinctions, and the collective dust resulted in prolonged climate change which was probably responsible for even more species extinctions. The lesson remains the same, volcanic eruptions; climate change and asteroid impact threaten life in a highly significant way and are responsible for extensive selection pressure. Unfortunately, these events are unpredictable and so enormous little can be done about them. A mass extinction may not represent a legitimate threat or selection pressure because large numbers of species are completely eradicated leaving few survivors to drive evolution. Finally, outside of removing dinosaurs so that mammals could thrive, what impact did these events have on human life; humans were not yet on the earth when these events took place.

Refining the search to dramatic changes in human populations moves the period up to the last 100,000 years. Mass migrations took place about every 23,000 years, which is in line with the precession of the earth leading to a possible linkage between earth's precession and human migration. This may have been due to regional climate change like ice ages in the northern hemisphere. However, human population changes on this timescale are not easily documented so estimates of climate and human population dynamics will be limited. Time to refine the search.

Consider the exponential growth, outbreak, of tent caterpillars, *Malacosoma distria*, in Montana reported by David Quammen in his book Spillover (Quammen 2012). The extensive caterpillar population consumed all of the leaves from the local elm and cottonwood trees in the summer of 1993. The caterpillar activity produced a crackle sound, "like a distant brushfire." The city attacked these insects with a broad arsenal of modern countermeasures but to no effect. However, a nuclear polyhedrosis virus (NPV), uses the caterpillar host density like a critical mass in a nuclear weapon to explode destroying the caterpillars in an epic battle. The caterpillar population retreated to undetectable levels in a single season as a result of NPV.

Human populations changed over the past 20,000 years with particular emphasis on the most recent 7000 years. A human population curve over this segment of time shows an exponential growth period interrupted in the middle ages by the plague. Hypothesis: The plague and other pandemic infectious disease events appear to be the greatest threat to human life. Infectious disease kills 15 million people each year, 26 percent of the total 57 million annual deaths in the global population (Fauci and Morens 2012).

A brief review of pandemic infections over the past 3000 years illuminates several events that reduced human populations. The plague of Athens 430 BCE killed 25 percent of the human population (Table 3.2). Both bacterial and viral pandemic

Table 3.2 Brief overview of historical pandemics

Name	When	Infectious agent	Impact
Plague of Athens (Scientific American 2006)	430 BC	Typhus	Killed 25% of population in 4 years
Antonine Plague	165–180 AD 251–266 AD	Variola virus	~5 million killed
Plague of Cyprian			5000 deaths per day in Rome
Plague of Justinian	541–750	*Yersinia pestis*	Killed 50% of Europe's population
Black death	1347–1352	*Yersinia pestis*	75 million deaths
Smallpox	1518–1568	Smallpox virus	80% of children under 5 died
			Mexico's population ↓ 20 to 3 million
Plague of London	1665–1666	*Yersinia pestis*	100,000 killed; 20% of the population
Tuberculosis	1800's	Mycobacterium tuberculosis	25% of the adult population of Europe died
Cholera (Lee 2003)	1816–1826	Virbio cholerae	15 M in India; China, Indonesia
	1829–1851		23 M deaths in Russia 1865–1917
	1863–1875		30,000 of 90,000 Mecca Pilgrims in Africa
	1881–1896		50,000 deaths in America
	1899–1923		120 K in Spain; 90 K in Japan; 60 K in Persia
			800 K in India; 200 K in Phillipines
Influenza pandemic	1889–1890	H3N8/H2N2	Russian flu- 1 million deaths
	1918–1919	H1N1	Spanish flu- 50 M deaths; 2% world's pop
	1957–1958	H3N2	Asian flu- 2 M deaths
	1968–1969		Hong Kong flu- 1 M deaths

infections are significant human killers with bacteria like *Yersina pestis* killing half of the population of Europe and the smallpox virus killing most of the human population of Mexico. Pandemic infections are not restricted to history long before our time, the Spanish flu pandemic of 1918 killed 50 million people. My grandparents would tell stories of watching horse drawn hearses daily carrying the dead through their Indiana small village in 1919. It is safe to conclude pandemic infections are currently relevant and represent one of the most significant threats to human survival.

Yersinia pestis is a bacterium causing plague. Fleas can be infected with *Y pestis* which transmit the bacterium to rodents, the primary hosts. Changes in the environment may lead to the movement of rats into populated areas where humans become infected. Homer points to such an infection in the *Iliad* in his description of the Trojan War in 1190 BCE. Plague has returned several times since the Trojan War imposing enormous loss of human life (Table 3.2). The most recent plague epidemic killed over ten million people in India in the early 20th century. *Y pestis* is still out there ready for favorable conditions to pounce on human populations but outcomes are likely to be less dramatic due to understanding of sanitation practices, quarantine, and availability of antibiotics.

If infections are the greatest threat to human life, they should be critical drivers of evolution? Clearly infections pose selection pressure on the human populations. Origins of evolutionary thought did not include infection as Darwin established key evolution concepts on the Galapagos Islands. These islands are isolated and an unlikely place for the spread of infections. The concepts speciation point to geographical separation of populations so infections would most likely be restricted to isolated populations. In many cases the survival selection pressure is not identified or ascribed to insufficient sources of food. Unfortunately, common single-stranded RNA viruses are so unstable that there are limited data for a viral fossil record.

Pandemic infections remain a threat to human survival in the presence of the information revolution, daily medical breakthroughs, and global travel. The human retrovirus HIV currently a global infection that infects up to 25% of the population in southern and eastern Africa with a projected death toll of up to 100 million by 2025. Measles killed 200 million people in the last 150 years and the development of an effective vaccine in 1963 reduced concerns for this infection but there were 777,000 deaths in the year 2000. Vaccination programs are frequently disrupted due to complacence resulting from vaccine success, conflicts that shift healthcare focus, and social crisis such as the recent Ebola outbreak in West Africa.

Smallpox is also an ancient infection causing fever, skin lesions, and at times death. King Ramses V of Egypt is thought to have died from smallpox around 1200 BCE. Introduced into Mexico in 1520, smallpox killed 3.5 million Aztec Indians or about half of the population in a period of 2 years and then proceeded to decimate the population of South America. Variola is a highly infectious virus killing 300–500 million people during the 20th century inspiring the eradication campaign in 1967. Variola was eradicated by December of 1979 (De Cock 2001), a rare triumph of public health. The WHO deserves acknowledgment for this unprecedented accomplishment and proof of concept that human suffering is not inevita-

ble. However, variola is a DNA virus with limited rate of mutation and a narrow host range so that animal reservoirs do not exist. The eradication of other viral infections will be more challenging.

The world continues to confront a broad array of microbial threats. Progress and preparedness make our engagement a likely success for those microbes that resurface and infections for which we have experience. Medical and epidemiological uncertainties surround emerging infectious disease, those that challenge us with their novelty.

Emerging Infectious Disease (EID) Becomes Endemic

Pandemics dominate the infectious disease "fear factor" but each pandemic began as a much more frequent occurrence, an epidemic. Most but not all epidemics come from emerging infectious agents, the most significant problem facing life on earth today. The concept of emerging is a human centric term as most of these infections are endemic in an animal host that serves as a viral reservoir. A 2005 report from the University of Edinburgh identified 1407 human pathogens and 177 are emerging or re-emerging of which 75% are zoonotic, that is jump from an animal host to human. Numerous emerging infections caused by viral agents have imposed high impact on human survival (Table 3.3). All the viral agents in Table 3.3 have genomes based on single-strands of RNA except HBV which should focus scientific attention on RNA. There are numerous questions that strike investigators as they ponder a collection of viral agents like those in Table 3.3. The viral polymerase errors in replicating single-stranded RNA genomes are not corrected so the species are constantly changing. The apparent success of these viruses is that as they move from reservoir hosts to humans and as humans become immune to the initial infection, the population of diverse genomes offers multiple chances to adapt by finding a "fit" genome version which can propagate until the next transition requiring adaption.

Acquired Immunodeficiency Syndrome (AIDS) is caused by human immunodeficiency virus 1 (HIV-1), a retrovirus. These viruses have a single-stranded RNA genome that is converted into DNA, a paradigm shift in the flow of genetic information from DNA to RNA. The 36,000,000 human deaths caused by HIV-1 (Table 3.3) is accompanied by a spectrum of clinical signs; eg. fever, diarrhea, peripheral neuropathy, pelvic inflammatory disease, cervical cancer, cytomegalovirus retinitis, Kaposi's sarcoma, lymphoma, *Mycobacterium avium* infection, recurrent pneumonia, and wasting syndrome. HIV-1 genome diversity includes base substitution, insertion, deletion, recombination, and gain or loss of glycosylation sites which all arises from the limited fidelity of the viral reverse transcriptase. These mutations are found in clusters or hypervariable regions indicating the fit virus is selected for from vast numbers of less-fit genome sequences. HIV-1 emphasizes key observations: (1) EID is a significant contemporary concern, (2) we are not prepared for the novel characteristics introduced by EID, (3) clinical signs can be diverse and often mimic symptoms of other diseases, and (4) the replication mechanisms are often error prone resulting in an array of fit viruses.

Table 3.3 Emerging infections that are endemic

Agent	When	Origin	Impact
Dengue	1953	Manila	DHF/DSS 29,803 cases/year
	2010	USA	1986–1990 267,692 cases/year
		Worldwide	1,785,059 cases, 2,398 deaths
Hepatitis B (HBV)	1885	Bremen shipyard	191 cases
	2004	Worldwide	350,000,000 cases, associated with 600,000 deaths/year- WHO
Hepatitis C (HCV)	1987	Global	150,000,000 cases, associated with 350,000 deaths/year
Hepatitis E (HEV)	~1800	Central Asia; Global	20,000,000 infections/year, 3,000,000 cases and 57,000 deaths/year
Human immunodeficiency virus (HIV-1)	1981	Cameroon	35,000,000 people live with HIV, 36,000,000 deaths since 1981, 2,000,000 deaths/year
	As of 2013		
Rabies	2000 BCE	Mesopotamia	54,000 deaths in 1990
	1990	Global	
Influenza	2003	H5N1	13,000,000 cases, 390,000 deaths
	2009–2010	H1N1 pandemic	3 M cases, 250,000 deaths/yr.
	Annual	Global	
Lassa	1950s	Bornea, Nigeria	300,000 cases, 5,000 deaths/yr
Chickungunya	1952	Mozambique	1,250,000 cases
	2006	India	1,118,763 cases, 24,682 deaths
	2013–2014	Americas	
Ebola (Zaire EBOV)	1976	Zaire, DRC	318 cases, 280 deaths
	1994	Minkouka, Gabon	49 cases, 29 deaths
	1995	Kikwit, DRC	315 cases, 242 deaths
	1996	Mayibout, Gabon	31 cases, 21 deaths
(Sudan SUDV)	2007	Kasai DRC	264 cases, 187 deaths
	1976	Maridi, Sudan	284 cases, 151 deaths
	2000	Gulu, Uganda	425 cases, 224 deaths
(Bundibugyo BDBV)	2007	Bundibugyo, Uganda	149 cases, 37 deaths
(Gueckedou)	2013–2015	West Africa	28,616 cases, 11,310 deaths
Measles	500 AD	Persia	200,000,000 deaths
	1855–2005	Global	1,374,083 cases, 630,000 deaths
	1990	Global	
Deaths per year			3.98 Million

Dengue is a flavivirus with a positive sense single-stranded RNA (+ssRNA) genome carried by mosquitos to man. Dengue has been a tropical disease for hundreds of years, typically a disease of young children but in 1953 an emerging severity was recognized in Manila (Table 3.3), hemorrhagic fever (DHF) and dengue shock syndrome (DSS). More than 2 billion people are at risk of dengue infection but only a small fraction of those infected will develop DHF or DHS. Infected people develop antibodies that can lead to antibody-dependent enhancement (ADE) in subsequent infections and more severe DHF or DSS outcomes. It appears ADE events occur in people that produce immunoglobins (IgGs) with enhanced affinity to the activating Fc receptor due to the IgG1 subclass and lack of a fucose glycan modification of the IgG (Wang et al. 2017) *Aedes aegypti* is the primary vector transmitting dengue but a new vector, *Aedes albopictus*, now carries dengue to the southern United States. Emergence of dengue in the southern United States is likely due to used tires imported from Japan which provided a place for the Asian tiger mosquito to live. Outbreaks of dengue fever have become more numerous and more severe over the past three decades.

Viral "fitness" is constrained by the requirements imposed by the natural host so that it is a low probability event for a virus to move from the natural host to a human. While an insect frequently plays the role of vector carrying a virus from an animal reservoir to humans, several zoonotic viruses are transmitted by placing humans near rodents. Several arenaviruses, minus sense single-stranded RNA viruses, jump from their rodent natural hosts directly to humans through contact with rodent urine or saliva. Notable arenaviruses are named for the hemorrhagic fever (HF) region of their zoonosis; Bolivian HF is caused by Machupo (MACV), Argentine HF is caused by Junin (JUNV) and Venezuelan HF is caused by Guanarito (GOTV). These South American outbreaks are caused by new world arenaviruses in contrast to Lassa HF (LASV), an African or old world arenavirus. LASV is an endemic disease of West Africa (Table 3.3) and can be confused with DHF or Ebola infections.

Viruses that are transmitted by arthropod vectors are called arboviruses. In 1930, only yellow fever of six known arboviruses caused disease in humans. The discovery of arbovirus caused human disease expanded beginning in the late 1930s with western and eastern equine encephalomyelitis (WEEV, EEEV) and St. Louis encephalitis. Both Chickungunya and Zika (Table 3.4) are arboviruses that have emerged to become global infections in the twenty-first century. Zika not only became infamous for causing microcephaly in newborns of infected mothers and Guillain-Barre syndrome but is now sexually transmitted between humans.

I worked on a therapeutic for the treatment of Ebola infections from 2007 to 2013. The genome sequence recovered from each outbreak from 1976 to 2013 has been different. Studies were conducted at USAMRIID in their BSL4 facility by expert Ebola investigators. Rhesus monkeys (*Macaca mulatta*) were injected with 1000 plaque forming units (pfu) of Zaire ebolavirus (ZEBOV) Kikwit into their thigh muscle. All of the untreated control monkeys died between days 8 and 10 following viral injection. We measured viral burden by quantitative polymerase chain reaction (qRT PCR) a measure of genome copies per milliliter (copies/mL) of plasma and plaque formation a measure of infectious viruses per mL (pfu/mL) plasma. We found

Table 3.4 Emerging viral infectious diseases

Agent	When	Origin	Impact
Bovine spongiform encephalopathy (BSE- a prion disease)	1991	Unknown	vCJD 2.95 M exposed, ~60,000 cases (CJD), ~60 cases vCJD/yr
Crimean-Congo hemorrhagic virus (CCHV)	12th Century	Tajikistan	3,128 cases, 156 deaths
	2002–2008	Yozgat, Turkey	
Venezuelan Equine Encephalitis (VEEV)	1995	Venezuela and Columbia	11,390 cases, 16 deaths
HIV-2		Senegal, Cote d'Ivorie	Less pathogenic than HIV-1, tests for HIV-1 detect HIV-2
Human T-cell lymphotropic virus (HTLV-1)	1977	Japan	20–30 million infected
Human parvovirus B19	1975	Global	Epidemic every 4 years
Hendra virus	1995	Australia	Race horses; several deaths
Hantavirus	1950–1953	Korea	Soldiers 3,000 cases, ~300 deaths
(Oran, Laguna Negra)	1996	Andes	25–35% case fatality
(Sin Nombre)	1993	Southwest USA	24 cases, 12 deaths
Human herpesvirus 6 (HHV-6)	1986	Global	Roseola
Human papillomavirus (HPV)		Global	12% of all females infected
SARS CoV	2003	China	8089 infected, 774 Died; >$1B cost
MERS-CoV	2014	Middle East	929 cases, 372 deaths
Monkeypox	2003	Midwest USA	Imported African rodents
Japanese encephalitis		Asia	70,000 cases, >700 deaths/yr
La Crosse (LACV)	1965	Wisconsin	787 cases, 11 deaths
	2004–2013	United States	
Marburg (MARV)	1967	Entebbe, Uganda	31 cases, 7 deaths
	2004	Angola	252 cases, 227 deaths
Norwalk	1968	Norwalk, Ohio	18% of all acute gastroenteritis
Rift Valley Fever Virus (RVFV)	1918	Kenya	1977; 100,000 cases in Egypt
Ross River (RRV)	1928	New South Whales	Infects 5,000 people/year
Rotavirus	1943	USA pre-vaccine	2,700,000 cases, 37 deaths/year

(continued)

Table 3.4 (continued)

Agent	When	Origin	Impact
Yellow Fever Virus (YFV)	2000 BCE	Africa	10% of population died (~4000)
	1648	Yucatan	
	1793	Philadelphia	3400 cases, 452 deaths
	1905	New Orleans	127,000 cases, 45,000 deaths
	2013	Africa	
West Nile Virus (WNV)	1937	Uganda	39,557 cases, 1,668 deaths (neuroinvasive disease emerging)
	1999–2013	United States	
Zika	1947	Zika Forest, Uganda	
	2016	Global	111,333 cases, 10 deaths
	2017	USA	38,303 cases

an average of $1.7x10^8$ genome copies per mL on day 8 post infection and $1.2x10^6$ pfu/mL on day 8. Nearly 100 genomes present for each successful virus in a nonhuvwas not observed, the dominant population of viral genome sequence did not change from one individual to another or from one time to another within the same individual. The existence of defective viral genomes may offer potential for rapid change but in a stable host, change was not rapid. When one considers the magnitude of differences in conditions as a virus jumps from a reservoir host to a human, rapid adaptability is a great advantage. A virus that kills all the hosts is not a successful virus. Perhaps defective viral particles facilitate host immune responses giving them a chance to catch up to the rapidly proliferating virus.

The Ebola outbreak of 2014 illuminated the collateral damage that accompanies EID outbreaks. First, the first responders are local physicians and care givers are killed leaving a population lacking doctors and nurses. Even physicians trained to exercise appropriate caution when interacting with patients such as Dr. Sheik Humarr Khan died of the Ebola infection (Bausch et al. 2014). Second, women are the main caregivers in their families and 75 percent of Ebola deaths in the 2014 outbreak were women. This leaves families lacking traditional structure. Third, the outbreak disrupts production, labor markets and trade resulting in scarcity and inflation of food prices. Food security and nutrition are diminished which preferentially affects poor people as they spend 50 to 70% of their income on food. Finally, public health measures are discontinued. Before the 2014 outbreak, 97 percent of children in West Africa were receiving routine vaccinations but that figure fell below 27 percent. The West Africa loss of measles vaccinations was followed by measles out-

breaks in the United States, suggestive of a global response to a regional loss of public health.

Contemporary Emerging Infectious Disease

Coronaviruses are unique in their large single-stranded RNA genome (20,000 bases) and are often associated with mild disease, bronchitis and gastroenteritis. A 2003 outbreak of Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) in China rapidly spread to 8098 cases in 37 countries (Table 3.4). Retrospective analysis of this outbreak linked zoonosis to a culinary trend in which people sought out exotic animals to eat. These nontraditional food animals appeared in street markets in urban areas bringing people close to the reservoir host triggering the outbreak. The civet cat was identified as the source of the first human cases but this is not the natural host. The natural host is the fruit bat, which infects a variety of small mammals including the civet cat. A point of intervention has been removal of the civet cat from markets but the natural bat host remains in the environment maintaining the virus for the next outbreak.

A second coronavirus outbreak in 2014 of Middle East Respiratory Syndrome coronavirus (MERS CoV) rapidly involved 23 countries including Africa, Western Europe, and Southeast Asia. The number of cases is relatively low but the case fatality ratio is of great concern (Table 3.4). The camel is reservoir host in this case but it may not be the natural host since camels often get sick as well. Transition from animal-to-human transmission to efficient human-to-human transmission was observed quickly in the case of both SARS-CoV and MERS-CoV. This rapid adaptability of a highly infectious virus group should raise concern over endemic coronaviruses in companion animals since these viruses can clearly become pathogenic, can jump from animal to human and then become transmitted from human-to-human.

An ongoing outbreak of Yellow Fever in Africa (Table 3.4) is a striking reminder that EID can re-emerge into virtually naïve populations. Some people recover from the acute symptoms but liver damage causes yellow skin, the reason for the name for the disease. The liver damage leads to bleeding and kidney damage and occasionally death. Yellow Fever is a flavivirus that originated in Africa but was distributed to the world on barges and sailing ships to tropical ports. The virus arrived in the Americas on slave ships. This virus interrupted the building of the Panama Canal which attracted the attention of Walter Reed, the military physician responsible for demonstrating transmission by mosquitos. An effective vaccine is used worldwide with immunity lasting over 10 years. The limitation of this vaccine like most is that populations need to be vaccinated regularly which in a world constantly changing due to political and economic drivers leads to vaccination gaps.

Tumor Viruses

Cancer is one of the leading causes of human death but estimates are that 20 percent of all human cancers are directly caused by viruses (Table 3.5) (Morales-Sanchez and Fuentes-Panana 2014). The first tumor virus was identified by Peyton Rous in 1911. He took cell lysates from a chicken sarcoma which he passed through a filter known to hold back bacteria and then injected the filtered lysate into chickens. A tumor developed at the injection site. The virus is now known as Rous Sarcoma Virus (RSV), a single-stranded RNA virus. Rous won a Nobel Prize in Physiology or Medicine for the discovery in 1966. RSV is a retrovirus that captured a tyrosine kinase, src gene, that triggers uncontrolled growth in infected host cells.

The Avian Leukemia Virus (ALV) was discovered in Denmark early in the twentieth century capable of causing disease in blood forming tissues of chickens. A strain of ALV, avian myeloblastosis virus (AMV) was described in 1952 that provided a convenient source of tissue for biochemical studies. During the 1930s a strain of inbred mice was found to develop leukemia between 6 and 18 months of

Table 3.5 Viruses associated with cancer

Virus	Cancer	Impact
Epstein Bar Virus- 1964	Burkitt Lymphoma	7800 Africa Endemic
[Human Herpesvirus 4-HHV4]	Gastric Carcinoma	933,900 (9% linked to EBV)
	Hodgkin Lymphoma (US)	62,400 (46% linked to EBV)
	Nasopharyngeal Carcinoma (China)	80,000 (98% linked to EBV)
	TOTAL	197,450
Human T-cell lymphotropic virus [HTLV-1] -1980	T-cell leukemia/lymphoma (ATLL)	1 of 1000 infected are symptomatic
	1980	1 in 1500 HTLV-1 carriers
Hepatitis B Virus (HBV)- 1965	Hepatocellular Carcinoma (HCC) 0.5% of HBV infections	60–80% of the worlds HCCs, HCC is the 6th most frequent cancer,
	3.5 M * 0.005 = 1.75 M HCCs	5% of all cancers worldwide
Human Papillomavirus (HPV)-1985	Cervical Carcinoma (HPV-16,18)	528,000 cases, 266,000 deaths
	[75-90% caused by HPV]	12,900 cases, 4,100 deaths USA
Hepatitis C Virus (HCV)- 1989	Hepatocellular Carcinoma (HCC)	HBV and HCV lead to:
		720,000 deaths cirrhosis/year
		420,000 deaths from HCC/year
Kaposi's Sarcoma Virus (KSHV)	Kaposi Sarcoma	rare
[Human Herpesvirus 8-HHV8]		
Merkel Cell Virus (MCV)-2008	Merkel Cell Carcinoma	1700 cases/yr USA
Polyomaviruses: SV40, BK, JC	Solid Tumors	

age were cultivated at Cold Spring Harbor. A murine leukemia virus (MLV)was isolated in 1951 from these mice extending tumor viruses into mammals. Soon a feline leukemia virus (FeLV) was isolated in 1967 followed shortly by isolation of a feline sarcoma virus (FeSV). These viruses established the cancer virus concept.

Epstein Bar Virus (EBV) Denis Burkitt identified an unusual tumor in Uganda in 1957 at the Mulago Hospital in a 5-year-old boy with swelling in his jaws (Burkitt's Lymphoma 2014). After that he saw a second case at the Jinja district hospital on the shores of Lake Victoria this became more than a curiosity. A review of hospital case notes confirmed the prevalence of these tumors of the jaw and they were accompanied by swellings in kidneys, ovaries, adrenal glands and liver. He assembled data from 38 patients and histological examination led to description of "lymphoma syndrome" or the "African Lymphoma."

Burkitt received a grant in 1961 for £250 to visit 57 hospitals in eight African countries; Uganda, Kenya, Tanzania, Malawi, Mozambique, Zimbabwe, Zambia, and Republic of South Africa to investigate African lymphoma. The tumor was found everywhere at the equator in areas where year-round temperature was above 60 °C but not in areas over 5000 feet in elevation. Burkitt further determined the tumors only occurred where the annual rainfall was above 20 inches and not seen in the dry savannah of Nigeria. Burkitt contacted doctors in Papua New Guinea to discover these tumors were the most common childhood tumor in that country but only in wet coastal regions and not in the dry highland areas.

Burkitt's working hypothesis was that an insect transmitted virus infection was responsible for the lymphoma syndrome. He then observed adults with the lymphoma but only in individuals that moved into the "African lymphoma belt." These tumors were then observed in the United States and Europe at a rate of 1–3 per million or about 100X less frequently than in Africa. Biopsy samples did produce viruses but none that came from insects so the working hypothesis was abandoned.

A new hypothesis emerged between 1963 and 1966 in which *P falciparum* (a parasite causing malaria) was the causative agent for the lymphoma. It is believed that severe malaria does play a role in the development of Burkitt's lymphoma. At the advice of Peter Clifford, Burkitt administered methotrexate to treat patients which produced encouraging results. In 1961 Burkitt gave a lecture at Middlesex Hospital, "The Commonest Children's Cancer in Tropical Africa: A Hitherto Unrecognized Syndrome." Anthony Epstein attended the lecture which changed his life and provides a punctuation mark in the history of science.

Epstein introduced himself after the lecture and the two sat down for tea. Burkitt agreed to send tumor specimens to Epstein and Epstein received a grant from the US National Institutes of Health in 1963 for $45,000 which allowed him to employ two research assistants. Yvonne Barr was one of the research assistants that was able to propagate a virus after 26 tries with tumor specimens. The other research assistant was Bert Achong, an electron microscopist, who was first to identify the virus as a member of the Herpes Virus family. The virus bears the name Epstein Barr Virus or EBV and is a causative agent of Burkitt's Lymphoma.

An interesting historical note involves Werner and Gertrude Henle at the Children's Hospital of Philadelphia. They created antibodies to EBV infected cells and their survey of blood samples from American adults revealed over 90% to be EBV positive in 1966. They also showed that normal human lymphocytes could be made immortal by infecting them with EBV in 1967. We now know EBV silently infects children in the Western World but in those infected a bit later in life become susceptible to infectious mononucleosis (the kissing disease) as adolescents.

In 1972, a collaboration between George Klein and the Manolovs led to a discovery of a chromosome change that was specific to Burkitt's lymphoma. This chromosome change was a translocation of 8:14 and rarely 8:2 or 8:22 (Zech et al. 1976). The break points in chromosomes 2, 14, and 22 contained immunoglobin genes that become active in B lymphocytes as they produce antibodies during infection. A Nobel Prize in 1989 to Michael Bishop and Harold Varmus provided the significance to the break point in chromosome 8, bringing active immunoglobin genes near the oncogene *c-myc*.

In 1970, a second cancer common in North and East Africa as well as Southern China was found to be EBV positive, carcinoma of the post-nasal space (Nasal Pharyngeal Carcinoma or NPC). The tumor is observed in epithelial cells not B-cells and is not geographically linked to endemic malaria regions. In this case, it appears that aerosol exposure to environmental carcinogens (possibly in preserved fish) may introduce mutations in nasal epithelial cells. The NPC may be the result of silent EBV infection, carcinogen induced cellular mutations, and people carrying HLA A*0207 and B*46 antigens.

In 1975, several reports describing "fatal infectious mononucleosis" appeared. David Purtilo described X-linked proliferative syndrome (XLP) based on an international registry of boys with fatal EBV infections (Purtilo et al. 1977). Using genetic marker analysis from the international registry they narrowed XLP to a three million base pair segment on the X-chromosome. In 1998, a 384-base pair segment encoding the XLP protein of 128 amino acids was found to be lost or damaged in al XLP patients (Nichols et al. 1998). Curiously, the XLP protein loss leads to a defect in NK and T lymphocytes which are necessary for recognition of EBV infected B lymphocytes. These individuals cannot make antibodies to EBV because their B cells require help from T lymphocytes.

The development of potent immune suppressing drugs like cyclosporine A was linked to lymphoma after organ transplantation. Two girls with acute lymphoblastic leukemia received bone marrow transplants from their HLA-match brothers. The grafts were successful but they developed lymphoblastic leukemia from the transplant (male cells). These lymphomas do not present chromosomal translocation like Burkitt lymphomas revealing a new path from EBV to lymphoma. The immune suppression upsets the EBV host homeostasis crippling T-cells that are required for keeping EBV under control. Another situation where the virus can replicate without control is in HIV/AIDS patients and AIDS-lymphoma is yet another EBV induced casualty.

EBV provides key insights into tumors associated with viral infections. First, the same virus is linked to multiple tumors in different populations including Burkitt's

lymphoma, Hodgkin lymphoma, and nasopharyngeal carcinoma. Second, tumorigenesis involves more than one mechanistic pathway but interest has focused on the latent membrane protein-1 (LMP1), EBV nuclear antigen 1 (EBNA1), and BamH1-A reading frame-1 (BARF1) genes. Third, dissecting the association between infection with EBV and cancer has unfolded over a period of 30 years and led to a greater understanding of cancer, the immune system, and methods for immortalizing cells to study specific cell clonal populations. EBV has been a boon to scientific research while imposing a challenge to human survival.

Hepatitis B Virus (HBV) Approximately 2 billion people have been infected with HBV and 360 million suffer from chronic infection. Acute HBV infections are usually self-limiting associated with 5.2 million cases a year. Chronic HBV infections lead to complications in 15–40 percent of cases resulting in 0.5 to 1.2 million deaths each year. HB V causes 60–80 percent of the world HCC cases which represents 5 percent of all cancers in the world. The HBx gene is considered key to oncogenesis. Vaccination programs are effective in reducing mortality in infants and have reduced emerging prevalence of the disease. Dietary exposure to aflatoxin enhances HBV-related HCC (Sun et al. 1999) as well as co-infection with HCV and excessive alcohol consumption.

Hepatitis B Virus (HBV) was named due to differences in transmission: hepatitis A type virus follows fecal-oral transmission while B type virus is parenteral. The genome is circular DNA that is not fully double-stranded with the end of the full-length strand linked to DNA polymerase. The genome is 3020–3320 nucleotides long (full length strand) and 1700–2800 nucleotides for the short strand. Four genes are encoded including: (1) C the core protein (HBcAg) synthesized by uORF AUG to make pre-core protein, (2) P is the DNA polymerase, (3) S is the surface antigen (HBsAg) which has three AUG start sites that divide the gene into pre-S1, pre-S2, and S, and (4) X is a gene that is not fully understood but may be a transcriptional transactivator. Non-coding RNA include HBV PREalpha, HBV PREbeta, and HBV RNA encapsidation signal epsilon. There are 10 known genotypes which differ by 8 percent in sequence with distinct geographical distributions and are labeled A to J and at least 24 subtypes. Type F is the most divergent form and found in Central and South America, predominantly Brazil.

Human HBV has a narrow host range infecting humans and higher primates, eg. Chimpanzees. In the 1970s a Woodchuck hepatitis virus (WHV) was discovered which will not infect rodents. A ground squirrel form was identified (GSHV) that is a distant relative of the marmot and woodchuck. Indeed, DHBV infects ducks but not all species of duck, grey herons are infected with HHBV, the Ross' goose with RGHBV, the snow goose with SGHBV, white storks with STHBV, and cranes with CHBV. There are no hepadnaviruses in arthropods or insects.

Human T-cell Lymphotropic Virus (HTLV-1) HTLV-1 is a single-stranded RNA retrovirus, defined by their use of reverse transcriptase, a polymerase, that makes a DNA copy of the RNA 7 kb viral genome. The DNA viral genome is integrated into the host genome where it is referred to as a provirus and is replicated along with the

host genome during cell division. Only 1 percent of infected individuals will develop leukemia and this is observed 20 to 30 years after asymptomatic infection. The HTLV-1 tax protein is likely to initiate cell transformation through interactions with transcription activators and cell cycle regulators.

Hepatitis C Virus (HCV) HCV has infected approximately 3 percent of the world's population (~210 million) but screening of the blood supply has reduced prevalence. HCV is a flavivirus composed of a 9.4 kb single-stranded, positive-sense RNA. HCV is characterized by a single serotype but at least 6 major genotypes. Genotype 1b is the most common genotype seen in the United States and Taiwan. HCV becomes a chronic infection by evading host immune defenses through a combination of: (1) high replication rate (10^{12} virions/day) and (2) lack of error proofreading by the viral polymerase leading to mutations in response to immune pressure. The genetic variability of HCV has limited efforts to design an effective vaccine.

Human Polyomaviruses The polyomaviruses are small (3.4 kbp), double-stranded DNA viruses. Early studies with simian virus 40 (SV40) led to identification of the large tumor antigen (large T; LT). LT is also found in BK and JC viruses which are more suspect human tumor viruses and Merkel cell polyomavirus (MCV) which is now well established as a human tumor virus (Feng et al. 2008). The N-terminus of LT contains an LXCXE motif that interacts with the retinoblastoma protein RB while the C-terminus contains an ATPase/DNA helicase domain that can inactivate p53. The LS-p53 complex activates insulin-like growth factor I (IGF-I) which alone is capable of cell transformation.

Cardiovascular Viruses

A recent report of morbidity and mortality reveals heart disease and cardiovascular events are the number one killer with neoplasia and infections following close behind. However, infections frequently cause neoplasia and cardiovascular disease leading to death. If we combine cardiovascular events and neoplasia caused by infection, then infectious disease is the most significant threat to human life and qualifies as the area of greatest impact.

Enterovirus The picornavirus family are small (pico-), single-stranded, positive sense RNA genome (7.4 kb) viruses that synthesize a single polyprotein that is cut into a small collection of functional proteins by virally encoded (2A and 3C) and cellular proteases. Poliovirus is a picornavirus that has served as the prototype for the viral family. The enteroviruses are a group of picornaviruses that have been associated with cardiac disease. Coxsackievirus B (CVB), Coxackievirus A (CVA), and echovirus infections lead to cardiac signs 3.2% of the time. About one-third of patients with acute cardiac disease (inflammation of the heart) are antibody positive

for enteroviruses. While acute myocarditis is often self-limiting, chronic cardiac disease often leads to dilated cardiomyopathy (DCM) which is present with no heart inflammation. DCM is associated with heart failure which can be lethal (Table 3.6). These chronic infections lead to 50 percent of all cardiac transplants worldwide. The enteroviral 2A protease can also degrade dystrophin in the heart leading to cardiac necrosis, reduced ejection fraction, and then to dilated cardiomyopathy. Transmission of these viruses is from contaminated food and water.

Cytomegalovirus (CMV) The human herpesviruses are large double-stranded DNA genome viruses. The human herpesvirus-5 (HHV5) or cytomegalovirus (CMV) has a 235 kbp genome encoding over two hundred genes. One problem in finding associations with CMV infections is that it is not an emerging disease, 50 to 99% of the population has been infected making comparisons to a control group challenging. Most infections are observed in children and in newborns serious clinical findings can be observed. Hence, most adults carry latent infections that are reactivated when individuals become immune suppressed following solid organ transplantation, malignant hematological disease, and AIDS. Reactivation in immune suppressed individuals is associated with increased mortality. The association with cardiovascular disease has been demonstrated in two recent studies. In one, CMV infections were detected in 14.5 percent of coronary artery samples from bypass operations compared to 4 percent in of patients who needed cardiac surgery for reasons other than atherosclerosis Hebar et al. 2015). In the other, CMV reactivation (viremia) was detected in 16.5% of immunocompetent patients admitted for major heart surgery (Roa et al. 2015). The incidence of coronary artery disease is the major contributor to death from heart disease and if 14 to 16.5% of this disease is associated with CMV infections, this infection is a significant human health hazard.

Finding an Emerging Infectious Disease

Recognizing an emerging infectious disease involves well established strategies of surveillance; (1) identify unusual clusters of disease, (2) evaluate the spread of an outbreak, (3) estimate the magnitude of the problem, and (4) if possible identify the

Table 3.6 Viruses associated with cardiovascular disease

Virus	Cardiovascular disease	Impact
Enterovirus Infections	Myocarditis	CVB3-34.6/1000; Mortality in newborns is 75%
Coxsackievirus		Chronic infections-dilated cardiomyopathy (DCM) cause 25% of 750,000 cases of heart failure, 250,000 deaths; Responsible for 50% of cardiac transplants worldwide.
Cytomegallovirus	Heart Disease	Atheroscleotic plaque

infectious agent. The strategy has proven valuable for known infectious and noninfectious diseases but has limited capacity to detect emerging infectious diseases. However, deviation from the traditional approach to surveillance is not likely to gain support.

Al Smith, a veterinary virologist, approached me after a seminar I presented in 1997 in the College of Veterinary Medicine at Oregon State University. I had just joined AVI BioPharma as the head of their research and development program. Al was a veterinarian and professor and had devoted his career to the *Caliciviridae* family of viruses dating back to his time in the naval research station in California. He had isolated the nonhuman vesivirus group of caliciviruses not only from suffering sea lions in the Channel Islands, but from reptiles in the San Diego zoo and whales held in captivity. Al and his capable technician, Doug Skilling successfully propagated the virus isolates in cell culture, an accomplishment not shared by other laboratories at the time. Al developed nucleic acid probes and induced antibodies to these viral isolates. Over decades of research he assembled an extensive collection of vesiviruses which were held in redundant −70° freezers. His singular vesivirus focus gave the appearance of a zealot but his ability to cultivate viral isolates, his extensive collection of isolates, and his one of a kind detection reagents made him a one of a kind virologist. Al was well beyond retirement age and was an "old school" virologist ready at a moment's notice to take his bag into the real world and collect swabs from ailing animals. He maintained careful records of the condition of his patients, their location, and the setting of the animal in the community. Every field trip led to work in the lab propagating virus from his swabs.

Al wanted to know if I would be interested in finding an antiviral agent for these viruses. My prime directive at AVI BioPharma was to explore the capabilities of our proprietary antisense technology and I had yet to investigate targeting a virus. The caliciviruses have positive sense single-stranded RNA genomes which express three genes from a single polyprotein. We identified an active agent following an investigation with small collection of candidates (Stein et al. 2001).

The vesivirus group of caliciviruses are considered animal only and are not believed to infect humans but Al began exploring human samples with his collection of detection reagents. After several years making incremental progress, we found an association between human blood samples seropositive for vesivirus and markers of liver disease (Smith et al. 2006). We felt this evidence of an emerging infectious disease in humans and its potential to cause liver disease would be welcomed by the medical community.

Al and I made a trip to Washington DC at our own expense to relate the findings in person. We meet with virologists at the National Institutes of Health but were met with judicious skepticism. Harvey Alter had been instrumental in the discovery of Hepatitis C Virus (HCV) and felt all viral liver disease is already accounted for by hepatitis viruses. Hence, no interest in our findings. We met with the American Red Cross blood banking group in Shady Grove Maryland and were met with concern. Blood supplies are scrutinized by the nucleic acid test (NAT) to eliminate viral contamination and any further elimination of blood samples would threaten an already limited inventory. Our findings simply add complication to the blood supply

business. They asked for more compelling and more extensive data to add robustness to our claims. The only problem is that they were not interested in providing financial support for the recommended studies. The conundrum is all too common, you need more data to convince granting agencies to support the work but there is no support to add to the limited data.

Life on the cutting edge is frequently discouraging. Our strategy took two paths: one to seek commercial support and the other to create proof of concept data for an antiviral solution. The most logical commercial solution was to meet with Michael Houghton at Chiron. He was the driving scientific force in finding HCV and Chiron might like adding to their reputation by finding another previously unrecognized hepatitis virus. Indeed, Dr. Houghton invited us to bay area to discuss the project. He reviewed our data and the quality of Al's detection reagents. Chiron provided modest support and their in-house laboratory help to confirm our observations, a glimmer of hope. Al created a company, Calicitech, so that he could accept support and license his reagent patents from the university.

Testing an antiviral agent in humans would be expensive but in the absence of natural history of the infection would make human proof of concept impossible. However, Al had been contacted by a cat rescue facility in Atlanta, Mommy Cat, describing an outbreak of feline calicivirus (FeCV) in their cattery. These cats had all been vaccinated with an approved FeCV vaccine which failed to protect these cats. A veterinarian can decide to use alternative medicines if there are no alternatives and the owner oc the cats can sign the equivalent of informed consent. We provided our FeCV specific antiviral to Mommy Cat along with a detailed protocol for treatment. The infected kittens we treated survived while those not treated died providing encouraging data. We then discovered a similar outbreak in the humane society facility in Eugene Oregon, our neighbors. Again, we reached an agreement to treat infected kittens and were successful (Smith et al. 2008). This was the first time we treated an infection based on symptoms in an "outbread" population of cats. With over 100 kitten patients and remarkable survival differences between treated and untreated, we had our proof of concept.

There is no disease cluster to link to human vesivirus. Norovirus is a calicivirus and is known to infect humans, in fact it is well known on cruise ships, day care centers, and extended care retirement facilities. Our efforts to have human vesivirus recognized as an emerging infectious disease have failed. Chiron was not impressed with our antiviral and no longer were interested in evaluating the detection reagents. If we are lucky, human vesivirus infections will remain mild clinical oddities. Al Smith has an interesting way of responding, "That virus is out there infecting humans. It won't go away."

Non–Pathogenic Viral Infections

The existence of non-pathogenic viral infections led to the emergence of the study of the immune system, vaccination, gene therapy, and concern for future pandemics. We carry evidence of ancient retroviral infections in our genome from integration events that became vertically transmitted making up as much as 8 percent of the human genome (Meyer et al. 2017). These genomic fossils are called endogenous retroviral genomes (ERV). Most ERV sequences accumulate sufficient mutations over evolutionary time that horizontal transmission is unlikely. These viral genome segments are trapped but can still be transcribed and encode some viral proteins. The significance of these integrated genomes is a current topic of investigation.

Adeno-associated Virus (AAV) is a single stranded DNA virus that infects humans but are not known to cause disease. The lack of pathology has led to their use as viral vectors for human gene therapy. AAV can infect dividing and quiescent cells and will persist extra chromosomally without integrating into the genome of the host cell. AAV is a member of the *Parvoviridae* family in the genus *Dependoparvovirus*.

Mopeia Virus (MV) is an Arenavirus closely related to Lassa Virus and shares reservoir hosts. MV is naturally attenuated and nonpathogenic in humans. Infection of humans with MV protects against lethal challenge with LV (Wulff et al. 1977).

GB Virus C (GBV-C)/Hepatitis G Virus (HGV) is a flavivirus (Pegivirus Flaviviridae) named after G. Barker, a surgeon, first identified in 1995. HGV infects one sixth of the world's population but does not cause human disease. A meta-analysis of 15 publications investigating GBV-C infections in HIV-positive individuals indicate coinfection with GBV-C slows the progression of HIV disease in individuals that have been seropositive for 2 years or more (Zhang et al. 2006a). Two of the studies investigated GBV-C 5 years after documented HIV seroconversion estimate the hazard ratio of 0.36 (95% CI).

Human spumaretrovirus (HFV; Spumavirus) is a retrovirus first identified in 1971 from a lymphoblastoid cell culture from a Kenyan patient with nasopharyngeal carcinoma. HFV is homologous to primate foamy viruses and is most closely related to the chimpanzee foamy virus (SFVcpz). Early studies raised alarm for association with autoimmune diseases but more extensive studies with more precise diagnostic reagents fail to find a disease associated with HFV. HFV is a rare human infection and concerns parallel SFV infections in humans.

Simian Foamy Virus (SFV; Spumavirus-retroviridae) is a retrovirus infecting most primates born in captivity and people making contact with infected primates can become infected. Human infections frequently occur in males probably requiring a bite from infected nonhuman primates but are harmless. The infected cells often fuse to form syncytia of giant foamy cells, which gives the virus its name. The

error rate in SFV genomes is exceptionally low, 1.7 x 10^{-8} substitutions per site per year, compared to HIV 10^{-3} substitutions per site per year. Since so-called cross-species, infections have only been observed for a little over a decade the long term consequences are not known. These infections are watch and wait for everything from a zoonotic epidemic to identified disease clusters. Perhaps this is exactly the sort of infection that will emerge as a significant human concern in the future.

Torque Teno Virus (TTV; Alphatorquevirus) is a single-stranded, positive sense DNA genome virus about 3.8 kb in size in the *Anelloviridae* family. Nearly 100 percent of even healthy individuals are infected in some countries. The virus was discovered in 1997 as the "transfusion transmitted virus (TTV)" in a Japanese patient. It is often found in patients with liver disease but does not cause hepatitis on its own. Closely related Torque Teno mini virus (TTMV) were isolated in 2007 and found to have smaller genomes of 2.8–2.9 kb. TTMV infections are also common but do not cause any described human disease.

Human Adenovirus Type 5 (rAd5) is used to create an Ebolavirus (EBOV) vaccine encoding Zaire ebolavirus glycoprotein failed to protect animals immune to Ad5. A replication defective chimpanzee-derived adenovirus (ChAd3-EBO-Z) provided protection against lethal EBOV challenge in macaques but protection wane over several months. They boosted with a modified vaccinia Ankara (MVA-BN-Filo) that led to durable protection (Stanley et al. 2014). This vaccine progressed through phase I, single-blind, randomized human trials in Mali between 2014 and 2015. A single dose of the ChAd3-EVOV-Z is efficient as a prime vaccine strategy followed by MVA-BN-Filo as a boost was well tolerated in humans Tapia et al. 2016).

WU polyomavirus (WUPyV; Polyomavirus) is a 5229 base double-stranded DNA virus infecting less than 5 percent of the human population. Wu, named after Washington University, is found as a co-infection in various respiratory infections but WU does not cause disease on its own. WU is closely related to KI virus that also is not known to cause clinical disease. However, related polyomaviruses that are clinically relevant include BK virus associated with nephropathy, JC virus associated with progressive multifocal leukoencephalopathy, SV40 virus associated with mesothelioma, and Merkel cell polyomavirus associated with cancer.

Vaccinia virus (Orthopoxvirus) is a large double stranded DNA virus closely related to Smallpox. Edward Jenner, the father of immunology, found the milkmaids exposed to cowpox (vaccinia) were immune to smallpox in 1798. This was the first vaccine (named after vaccinia) leading to the modern vaccine that has allowed for the eradication of smallpox.

Viral sequences are constantly mutating with no purpose other than seeking a survival/infectivity benefit. This means viruses with no current pathology represent a pre-mutation reservoir for the next catastrophic human pandemic. The popularity

of RNAseq is likely to expand our catalog of nonpathogenic viral infections. However, the management of such information is in question.

Antiviral Drugs

Technology used to counter viral infections has resulted in over 90 approved drugs for the treatment of nine different human viral infections in just 50 years (De Clercq and Li 2016). Several different antiviral drug groupings have been reported, but the following arise from review of the mechanisms of action of antiviral drugs assembled in Table 3.7: (1) Inhibition of viral attachment and entry, (2) Inhibition of viral uncoating, (3) Viral Polymerase Inhibitors, Nucleotide analogues (NTRTI) and Non-Nucleotide Reverse Transcriptase Inhibitors (NNRTI) and DNA Polymerase Inhibitors, Nucleic Acid Synthesis inhibitors and Nucleotide Pool Size Agents, (4) Latency Reversal Agents, (5) Integrase Inhibitors, (6) Protease Inhibitors for both HIV and HCV, (7) Neuraminidase Inhibitors, (8) Immune Response Modifiers, and (9) Antisense Inhibitors.

Table 3.7 Comprehensive list of antiviral mechanisms of action

Name	Trade name	MOA	Use	Approval date	Number Agents
Amantadine		Inhibit Viral Uncoating	Influenza A	1966	2
Podofilox	Condylox	Antimitotic Agent	HPV	1990	1
IFN-alpha-2b	Intron A	Immune Resp. Modifier	HBV	1986	5
Saquinavir	Fortavase	HIV Protease Inhibitors	HIV-1	1995	12
Idoxuridine	Dendrid	ntRTI/DNA pol Inhib	HSV	1963	34
Oseltamivir	Tamiflu	Neuraminidase Inhibitor	Influenza A	1999	4
VZIG	VZIG	Entry Inhibitor	VZV	1981	7
Fomivirsen	Vitravene	Antisense	CMV retinitis	2006	1
Boceprevir	Victrelis	HCV Protease Inhib	HCV	2011	5
Raltegravir	Isentress	Integrase Inhibitor	HIV-1	2007	5
Panobinostat[a]	Farydak	Latency Reversal Agents	HIV-1	2015	4
Nevirapine	Viramune	NNRTI	HIV-1	1996	5
Simeprevir	Olysio	NS5A RdRp	HCV	2013	10
Foscarnet	Foscavir	Nucleic Acid Synth Inhib	HSV, CMV	1991	1
Ribavirin	Copegus	Nucleotide Pool Size	HCV, Viral HF	1985	1
Imiquimod	Aldara	TLR7 Against	HPV	1997	1

[a]approved for Multiple Myeloma use as antiviral is experimental and off-label

Antibodies

Administration of hyperimmune sera from immunized animals or human donors was the first effective treatment for infectious diseases. The practice has limitations but is still used to treat bacterial toxins and viral infections caused by CMV, RSV, HAV, HBV, RABV, VZV and MEV (Keller and Stiehm 2000). The development of human or humanized monoclonal antibodies (HumAbs) has created a feasible way to rapidly generate novel antiviral therapeutics. HumAbs have advantages over serum therapy in that they are chemically defined reagents with minimal variability, greater activity per mass of protein, and they have no immunological consequences related to serum sickness. Several mAbs have been approved for treatment of infectious diseases including viral and bacterial pathogens (Table 3.8).

The antiviral mAb discovery field is exploding with activity particularly for HIV and HCV infections. A humanized mAb targeting lymphocyte CCR5 receptors called PRO140 has demonstrated potent and prolonged anti-HIV-1 activity and a large margin of safety (Jacobson et al. 2010a). Administration of PRO 140 by the subcutaneous route offers patients a way to self-administer the mAb but importantly the mAb is transported in the lymphatics providing enhanced access to binding to the cellular target (Jacobson et al. 2010b). The next generation of antiviral therapeutics are likely to be dominated by mAbs.

Vaccines

Perhaps the only way to clear a viral infection involves a host immune response (Table 3.9). The innate immune response is particularly effective centered on a type 1-interferon pathway. Unfortunately, many viruses carry mechanisms to evade host innate responses and innate immune effectors do not have the capacity for memory. The adaptive immune response can mitigate infection with antibodies, generally to surface antigens, which prevent the spread of the virus and T-lymphocytes, which can clear virus-infected cells.

Table 3.8 Approved monoclonal antibodies for antiviral passive immunotherapy

Agent	Trade name	Virus	Target	Year
Palivizumab	Synagis	RSV	F protein	1998
Raxibacumab	Raxibacumab	Bacillis anthracis	protective antigen	2012
Siltuximab[a]	Sylvant	HHV-8	IL-6	2014
Obiltoxaximab	Anthem	Bacillis anthracis	protective antigen	2016
Bezlotoxumab	Zinplava	Clostridium difficile	Toxin B	2016
Avelumabb	Bavenco	Polyomavirus	PD-1	2017

[a]Approved for treatment of Castlemen's disease
[b]Approved for treatment of Merkel Cell Carcinoma

Viruses Not Targeted by Antiviral Drugs, Monoclonal Antibodies nor Vaccines

The vast majority of viral infections have no treatment options, neither drug, antibody, nor vaccine. Attention is focused on emerging viruses such as Epstein-Barr virus (EBV), human parvovirus B19, Human norovirus, human rhinovirus, human herpesvirus 6, human coronaviruses (SARS and MERS CoV), human astrovirus, human sapovirus, chickungunya virus, dengue virus, West Nile virus (WNV), Hendra virus, Nipah virus, Venezuelan Equine Encephalitis (VEEV), Eastern Equine Encehalitis (EEEV), Western Equine Encephalitis (WEEV), Ebola virus (EBOV), Marburg virus (MARV), Bundibugyo, Lassa Virus, Junin Virus, Machupo virus and Zika virus.

The first strategy will be to use an existing drug designed for another virus off-label. This seems likely for antiviral drugs like Cidofovir, Foscarnet, and Ganciclovir

Table 3.9 Comprehensive list of viral vaccines

Vaccines	Virus	Trade names	Year	Efficacy	Recommendation
Adenovirus	ADV-4,7				Military recruits
Hepatitis A	HAV	Havrix, Vaqta, Epaxal	1995	95	2 doses IM by age 1
Hepatitis B	HBV	Sci-B-vac, Engenix-B,	1981	85–90	babies of mothers with HBV
Hepatitis E vaccine	HEV	Hecolin			China approved in 2012
Human Papillomavirus	HPV	Gardasil, Cervarix	2006	70	Women 9–25, Men 9–26
Influenza vaccine	IFV A	FluMist, Flozone, Influvac	1930	40–60	Yearly >6 mo, >65 y/o
Japanese Encephalitis	JEV	Ixiaro	1930	90	In areas with endemic JEV
MMR vaccine	MEV	Prionix, MMR II, ProQuad	1963	>75	Children age 1+4
MMR vaccine	Mumps	Prionix, MMR II, ProQuad	1967	>75	Children age 1+4
Polio Vaccine	PV	Kinrix, Pediarix, Ipol	1955	99	All children, 3 doses
Rabies Vaccine[a]	RABV	Imovax, RabAvert	1885		High risk areas
Rotavirus Vaccine	RotV	Rotarix, Rotateq	2006	45	Routine Vaccinations
MMR vaccine	RUBV	Prionix, MMR II, ProQuad	1969	>75	Children age 1+4
MMRV	VZV	Tetra	2005		All Children 1–2
Shingles Vaccine	VZV	Zostavax	2006	51	All adults >60
Smallpox Vaccine[b]	VARV	Dryvax, Imvanex	1796		Virus erradicated
Yellow Fever Vaccine	YFV	YF-VAX	1938	99	Routine where endemic

[a]Developed by Pasteur and Roux

[b]First recognized vaccine attributed to Edward Jenner

particularly for double-stranded DNA viruses like EBV, HPV, and HHV6. Ribaviran has been used for a number of single-stranded RNA viruses including polio, Junin, and Lassa Fever. Secondary strategies will require investment of time and effort beginning with vaccine development and creation of monoclonal antibodies.

Platform Technology RNA-based therapeutics offer rational design for an expansive number of new antiviral strategies. This advantage is superimposed on the theoretical advantages of selectivity, specificity and affinity provided by Watson-Crick base pairing. RNA-based therapeutics are expected to provide a substantially more narrow range of pharmacokinetic properties and toxicities thus are easier to compare to each other and ultimately combine into multi-agent cocktails. However, the mechanism of action may vary from RNAse H or RISC mediated degradation of the targeted RNA or steric inhibition of RNA function. The objectives of our antiviral program have been to exploit the broad understanding of RNA-based therapeutics for antiviral activity with a common chemical type, the phosphorodiamidate morpholino oliogmer (PMO) and their enhanced derivatives. In this way the mechanism of action is common to all agents, which is steric blockade of RNA function.

Studies reported by Zamecnik and Stevenson introduced the first approach to identification of an antisense antiviral agent (Zamecnik and Stephenson 1978; Stephenson and Zamecnik 1978). They used a 13-mer targeted to Rous sarcoma virus. Since the Rous sarcoma virus pioneering efforts, antiviral RNA based therapeutics have involved multiple oligomer chemistries with a variety of different mechanisms of action.

Chemical approaches to oligomers directed to HIV have been plentiful. A nonionic methylphosphonate oligonucleotide targeted to the splice acceptor site of HIV tat inhibited splicing of viral RNA (Sarin et al. 1988) inhibiting syncytia formation and p24 synthesis at 3uM concentration. Poor aqueous solubility limited the utility of the methylphosphonate chemistry. Phosphoramidate chemistry was investigated for inhibition of the splice-donor and splice-acceptor of HIV tat Agrawal et al. 1988) and were more potent but these agents were cytotoxic and poorly water soluble. Phosphorothioate chemistry targeting HIV-rev (Matsukura et al. 1987) and HIV-tat were shown to be effective in inhibiting HIV replication, were not cytotoxic and were very soluble. Further, the HIV-rev phosphorothioate oligodeoxynucleotide was stable *in vivo* with an acceptable pharmacokinetic profile (Iversen et al. 1994). A 25-mer phoshorothioate called GEM91 targeting the initiation site of HIV-gag was evaluated in clinical trials (Agrawal 1998) but the trials were discontinued. I focused on phosphorodiamidate morpholino oligomer chemistry which is both stable and net-neutral in charge at physiological pH.

Single Stranded RNA Viruses with Positive Sense (ssRNA(+)) These viruses are the most simple in terms of genome size, number of potential translated viral proteins, their genomes are all linear and they enter the cell ready for translation. The design of steric blocking RNA-based therapeutics involves preventing translation, disrupting RNA secondary structure and masking recognition sites for RNA dependent RNA polymerase (RdRp). The targeting of either the 5′-terminus and the first

ORF-AUG are active. Further, efficacy in animal challenge studies is observed with high fidelity when the most effective agent identified in vitro is employed.

The family *Astroviridae* with six different human astroviruses (HuAstV) responsible for 2–17% of all gastroenteritis. We found the 5′-terminus[1] to be the most effective site to target. This was also the optimal site for *Caliciviridae* including vesivirus (VeV; Martin-Alonso et al. 2005; Stein et al. 2001), Norovirus (NoV; Bok et al. 2008), and feline Calicivirus (FCV; Smith et al. 2008); the *Flaviviridae* including Dengue (DEN; Kinney et al. 2005; Holden et al. 2006), and West Nile Virus (WNV; Deas et al. 2007; Zhang et al. 2008), *Arteriviridae* including agriculturally important Equine Arterivirus (EAV; van den Born et al. 2005), and porcine respiratory and reproductive virus (PRRSV; Zhang et al. 2006a, b); and the *Togaviridae* exemplified by Venezuelan Equine Encephalitis Virus (VEEV; Paessler et al. 2008). The family *Coronaviridae* revealed a new active target site in the transcription regulatory sequence (5'-CGAAC-3′) in both mouse hepatitis virus (MHV; Neuman et al. 2004) and the Severe Acute Respiratory Syndrome virus (SARS; Neuman et al. 2005). The *Picornaviridae* active target site involved a highly conserved sequence in the internal ribosomal entry site (IRES) in polio virus (PV; Stone et al. 2008), foot and mouth disease virus (FMDV; Vagnozzi et al. 2007), and the coxsackievirus (CVB3; Yuan et al. 2006).

Single Stranded RNA Viruses Negative Sense (ssRNA(−)) These viruses are generally more complex with respect to genome size, number of potential translated viral proteins, and multiple genome segments. The genome must be replicated prior to translation of viral proteins. The design of steric blocking RNA-based therapeutics is similar to the positive sense RNA genomes in that targets involve preventing translation and masking recognition sites for RNA dependent RNA polymerase (RdRp).

We investigated 11 different targets in measles virus (MeV), a member of the *Paramyxoviridae,* finding the translation initiation start site of N the optimal target (Sleeman et al. 2009). Studies with the human respiratory syncytial virus (hRSV) found the translation site for L to be most active (Lai et al. 2008). The *Orthomyxoviridae* studies investigated influenza A virus probing each of the 8 viral segments finding translation of PB1 active as well as the 5'terminal of vNP for H3N2 (Ge et al. 2006) and H3N8 (Lupfer et al. 2008) but a combination of targets was required in animal studies with a high pathogenic viral H7N7 isolate (Gabriel et al. 2008). More extensive influenza A studies revealed a new target, the M-segment splice donor site. This target was evaluated in phase I clinical trials.

The antisense platform technology has limitations in targeting viral sequences. Inhibiting virally encoded proteins or blocking viral replication by interfering with the polymerase does not always work. The *Arenaviridae* family proved difficult. We

[1] Collaborative studies with Drs. David Matson and Anne Campbell at Eastern Virginia Medical School identified the most effective target which involves the highly conserved sequence at the 5′-end of the 5'-UTR.

experienced some success with Junin and Lymphochoriomeningitis virus (LCMV) in cell culture observing 4 $\log_{10}$ reductions in viral titer with a PMO targeting the highly conserved viral genome terminus. However, we failed to provide survival benefit to guinea pigs challenged with either Junin or Lassa virus. We then tried the mouse challenged with LCMV and failed again but we observed hemorrhagic disease in the mouse, a severe consequence of infection that mimics the worst aspects of arenaviral infection. We decided to try targeting host genes to mitigate disease in the mouse and found targeting IL-17 provided a survival benefit (Schnell et al. 2012). The success in the face of failure puts targeting host genes at the center of attention for dealing with emerging infectious diseases.

The antisense platform technology represents an excellent approach to rapid drug discovery for emerging infectious disease. Rapid discovery demonstrated repeatedly but of course, the cost of advanced development is daunting. The application is best for immediate treatment of index cases, close contacts, and healthcare workers.

An Unfavorable Climate for Antiviral Therapeutics

We have an 800-pound gorilla in the room and everyone in the room considers it someone else's problem. The current course is to ignore the majestic creature until it starts tearing limbs from people and then the consensus is to kill the gorilla. Agencies like the World Health Organization (WHO), the Centers for Disease Control (CDC) and the National Institutes of Health (NIH) can assemble highly skilled personnel and can confer with some of the greatest minds in the world. Unfortunately, they are all aware of the potential problem that an emerging pandemic is likely to take us by surprise. Significant speculation that a single-stranded RNA virus will emerge killing tens of million people, costing hundreds of billions of dollars, and changing the course of human history.

There is a high probability that the virus will be influenza A (H5N1 or H7N9) that will jump from a reservoir population of birds and establish human-to-human transmission. The pandemic will be a global event by the time an effective vaccine is available. Neuraminidase (NA) inhibitors as therapeutic and prophylactic agents in the setting of pandemic influenza A (FLUA) were called in to doubt in the past decade (Michiels et al. 2013; Jefferson et al. 2014). Indeed, 100% of isolates in the 2008/2009 A/H1N1 pandemic were found to be resistant to adamantanes, and resistance to oseltamivir (Tamiflu; OSL) was observed in virus recovered from individuals taking OSL therapeutically or prophylactically (Dharan et al. 2008; CDC MMWR 2009), while effect on duration of shedding was not impacted. A recent outbreak of an influenza A (H7N9) virus caused 137 cases and 45 deaths in China revealed a novel NA mutation R292K resulting in high level resistance to OSL (Wang et al. 2014). Therapeutic options for treatment of individuals with complicated influenza A are severely limited, perhaps no options. Pandemic strains such as A/H1N1pdm09 carried significant morbidity and mortality, particularly in those

who had not experienced H1-strain influenza in their lifetime. We are also witnessing more rapidly emerging highly pathogenic avian influenza strains that are resulting in human infection; some such as A/H5N1 and A/H7N9 appear to becoming more efficient in person-to-person transmission, and reports suggest OSL resistance develops during treatment (de Jong et al. 2005; Lam et al. 2015). We are not ready for an outbreak of avian flu or any other emerging single-stranded RNA virus. Why not?

An estimate for the time and cost to develop a new drug is 10 years and $1 billion. The commercial use of a drug for an emerging infection is hard to estimate since by definition when you start development the infection has not emerged. Most of us would be unlikely to use our retirement savings to invest in a drug development project with no reliable way to expect a return on our investment. It is a poor business model. When you consider there may be hundreds of emerging infectious diseases each times 10 years and $1 billion each the task is daunting. On which disease should we focus?

Consider the Ebola drug AVI-7537. The Ebola therapeutic project began in 2004 following a laboratory accident at USAMRIID. We identified three compounds each with activity and when combined we observed unprecedented efficacy in a lethal challenge primate animal model (Warfield et al. 2006). Hundreds of experiments over the next 3 years optimized these agents (Swenson et al. 2009). We were able to obtain research grants from the Transformational Medical Technologies (TMT) division of the Defense Threat Reduction Agency (DTRA) within the Department of Defense (DOD) and we completed proof of efficacy studies (Warren et al. 2010). This led to submission of an investigational new drug application (IND) to the Food and Drug Agency (FDA) and phase I safety and tolerability studies were conducted I healthy human volunteers (Heald et al. 2014). After 11 years of continuous effort, we completed key aspects related to the FDA approval process under the "Animal Rule" and we streamlined our treatment to a single agent, AVI-7537 (Warren et al. 2015). However, shortly before the Ebola outbreak in Western Africa, the US government "budget sequester" cancelled our project and the most advanced therapeutic on the planet was not deployed to treat those infected during the outbreak. As the outbreak continued, we found no viral resistance of AVI-7537 unlike the monoclonal antibody therapy in use (Khiabanian et al. 2014). AVI-7537 sits on a shelf, a political casualty and an unfavorable business model.

Environmental Viral Reservoirs

The global virosphere may contain up to 10^{31} virus/virus-like particles (Suttle 2005), the greatest reservoir of genetic diversity. The Earth's atmosphere transports viruses all over the planet. Viruses are found in soil at 1.5×10^8 to 6.4×10^8 particles per gram of dry soil (Kimura et al. 2008). The surface oceans carry approximately ten million viral particles in each milliliter of seawater, most of which are bacteriophages (phage). The small viral particles are easily carried into the upper

atmosphere by up drafting winds. Bacteria are deposited from the atmosphere at a rate of 0.3 to 8 x 10^7 per meter each day and viral deposition rates are 9–461 times greater (Reche et al. 2018). These phages influence bacterial lifecycles and play a role in natural energy and nutrient cycles fundamental to life on Earth. The dynamics of phage-bacterial evolution drive changes in photosynthesis, phosphate, and nitrogen balance (Breitbart 2012). Human accidental release of radioactive waste (discussed in Chap. 2) and disposal of chemicals including potent antiviral and antibacterial compounds (discussed in Chap. 7) may alter the eco-evolutionary dynamics producing unanticipated environmental consequences.

Conclusion

The objective of this chapter has been to provide convincing evidence that infectious disease is the most significant threat to human health. The focus has been on viral infections because they rely on host ribosomes to produce their proteins, recent emerging infections have been from single-stranded RNA genome viruses, and replication of RNA viruses is error prone. Pandemic infections have accompanied the rise of human civilization frequently re-occurring leaving a lasting imprint on human history punctuated by profound loss of life. Emerging infections become endemic with an annual death toll. Each decade brings a new onslaught of emerging infectious agents. We are surprised again and again but have never prepared for these inevitable catastrophies. The long-term consequences often remain unrecognized and are always inconvenient such as cancer, cardiovascular disease and immune associated diseases that threaten our health. Reliance on clusters of clinical symptoms in the face of diverse and non-descriptive viral infection symptoms is a foolhardy form of crisis management. Infectious disease will certainly continue to pose the most significant threat to human health in the age to cell phones, artificial intelligence, and global commerce.

Rapid replication of viral genomes combined with low fidelity polymerases provide the foundation for an unending source of new emerging infectious agents. These traits also make viral genomes sensitive to environmental contaminants in a way that may expand probabilities for zoonosis. Infectious disease as part of our environment is not appreciated. The study of infectious disease is not a part of the curricula of students in environmental science/management. Textbooks in in environmental studies do not include chapters in infectious disease. The integration of research at superfund sites focused on chemical contamination with infection and zoonosis would result in valuable insights into threat analysis.

Viruses with RNA genomes lack sequence proofreading quality control during replication. The cumulative mutations in their genomes limits the genome size to under 30,000 bases. Essentially, a larger genome would evolve out of existence, so called catastrophe evolution. The limited genome size makes these viruses exceptionally resilient to a changing environment. The virus must economize by combining functions. This means evolution and resilience are the same thing in the RNA genome viruses. A unique insight is that in human evolution is restricted to the DNA genome and resilience limited to RNA, as it is in the RNA genome viruses.

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Chapter 4
Nonlinear Anomalies

Abstract The cell is the smallest basic structural unit of all living organisms. Each cell maintains an individual genetic blueprint of life in their DNA genome. Humans are composed of 10 trillion (10^{13}) cells ranging in size from roughly 10 to 100 μm (10^{-5} meters). A linear path of thought holds that examination of each individual cell will predict the behavior of the intact human, a primary driver for the study of cell biology. The unappreciated lesson from cell biology is the extent of genetic and transcriptomic plasticity that defines our resilience.

Translation of observations from biochemistry to animal testing include obligate *in vitro* testing. Can we skip this step? Animal experimentation is an integral part of drug development but limitations and skepticism deserve consideration. No animal models exist for many emerging infectious disease. Should we skip this step and proceed to human clinical trials?

Keywords Cell culture · Stem cells · Microphysiological systems · Animal model · Animal rule

Studies in Cell Culture

Scientists often seek to simplify processes and isolate variables to better understand complex systems. The early methods of tissue culture originate with Ross Granville Harrison studies growing embryonic frog cells on clotted lymph in 1907. Cell culture techniques became sophisticated to grow pathogenic viruses like polio in the late 1940s. John Enders, Thomas Weller, and Frederick Robbins were awarded the 1954 Nobel Prize in Physiology and Medicine for their methods for growing polio virus in monkey kidney cell cultures.

Human cells follow the pattern of all eukaryotic cells with a plasma membrane establishing the boundary of the cellular unit of life. The DNA genome is segregated into 23 pairs of chromosomes stored in the cell nucleus, separated from the cytoplasm by a nuclear membrane. A smaller DNA genome is stored in thousands of mitochondria, an organelle with specialized function that drives respiration and energy production. A series of structures and organelles separate cellular functions

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from the endoplasmic reticulum where membrane bound ribosomes translate mRNA into protein, Golgi apparatus where proteins and lipids are processed and packaged often in preparation for secretion outside the cell. Lysosomes and peroxisomes digest aged organelles often merging with endosomes to clear the area of particles found outside the cell.

The source of human cells for culture is human tissues. White blood cells can be isolated from blood and grown in culture but cells from organs require disaggregation. The cell culture process disrupts cell contacts and orientation with respect to connective tissue limiting relationships to the original organ function. The individual cells are maintained at body temperature (37 °C) and a gas mixture of 95 percent air and 5 percent CO_2. The liquid culture medium is buffered to maintain neutral pH (7.4), glucose, growth factors, and other nutrients. Cells that originate from different tissues often require unique mixtures of nutrients for optimal growth. One common approach to save money for media is to supplement with fetal bovine serum. Supplements can contaminate cells with viruses, mycoplasma, and prions originating in the cow or other source of blood serum.

Most cells isolated from a tissue will have a limited number of cell divisions called the Hayflick limit. When the cells reach the limit, they stop dividing in a process called senescence. This process is likely a representation of aging and so is a gradual process. The potential for cells to subtly change with each division means experiments often suffer limited reproducibility. Cultivation of millions of cells to senescence can result in a few cells that become immortal. The immortalized cells become "cell lines" with the ability to proliferate indefinitely. Numerous cell lines are available, some very well known, are very different from their original source, for example a human cell should have 46 chromosomes but established cell lines have between 69 and 74 chromosomes. The differences in an established cell line compared to a human tissue invoke analogies such as comparing pickles to cucumbers.

A chromosome can be observed under a microscope as cells prepare to divide after DNA replication. Each chromosome is made of two chromatids held together by a centromere. Human chromosomes generally have centromeric centromeres so they resemble an X while mouse chromosomes have eccentric centromeres that resemble a V. One of my first cell biology projects involved fusing mouse cells with human cells creating a mouse:human hybrid, a phenomenon that can only be observed in cell culture. The hybrid cells divide about once a day and if we observed these hybrids over a period of a week or two the chromosomal content changes. We grew the hybrids in hypoxanthine, aminopterin, and thymidine (HAT) media. Aminopterin blocks DNA synthesis by preventing synthesis of nucleotides but thymidine and hypoxanthine provide raw materials that enable cells to evade the blockade by salvaging nucleotides for DNA synthesis. We used mouse cells lacking hypoxanthine phosphoribosyl transferase ($HPRT^-$) and human cells lacking thymidine kinase (TK^-) so the HAT medium will promote hybrid survival but not either of the human or mouse parental cells. My project involved collecting hybrid cells in mitosis, staining their chromosomes, and counting human X's and mouse V's. I observed fewer mouse and human chromosomes after each cell division after the

initial fusion event. The hybrid cell discarded redundant functions with loss of chromosomes as the resilient cells adjusted to a new hybrid genome. Each hybrid cell discarded chromosomes resulting in a diverse collection of hybrid cells with a different assortment of human and mouse chromosomes all capable of survival in HAT medium, a chaotic process. Ultimtely, the hybrid chromosome number stabilized and remaining mouse chromosomes dominated in number over human chromosomes.

Established cell lines are valuable for some applications. The study of infectious diseases relies on cell lines to grow bacteria and viruses. Cell culture has been adapted to manufacture complex products like vaccines. The established cell lines can be transformed with recombinant DNA technologies to manufacture enzymes, synthetic hormones, and monoclonal antibodies. The use of established cell lines is not well suited for predicting the cell functions to functions in a tissue or organ.

Cultured Cells Are Temperamental

Individual cells self-assemble and exhibit specialized form and function as an embryo develops into a mature being. The dividing cells of a fertilized oocyte clump together increasing cell-to-cell contact. Adhesion receptors hold the neighboring cells together in a solid ball and actin and myosin fibers originate at the contact sites forming a contractile cytoskeleton. Cell division begins to create daughter cells that are different from each other with cells at the edge of the ball taking on polar features in which sub-cellular anatomy differs from one side to the other. The daughter cell in the central region resembles the parent cell. Two populations of cells with different function arise. This assymetric division is regulated by cytoskeletal microtubules and contractile actin microfilaments that in turn position the mitotic spindle. The outer layer of cells, the epithelium, forms the trophoectoderm that will develop into layers of the placenta while the inner cell mass will differentiate into the epiblast and the endoderm.

Cells bind to the extracellular matrix (ECM) through membrane bound integrins. The cytoplasmic side of the integrins are linked to actin and myosin contractile fibers through vinculin so that forces from outside the cell directly communicate to the cytoskeleton contractility apparatus. In a similar manner, membrane bound cadherins hold neighboring cells together and are also linked to the actin and myosin contractile apparatus. Mechanical forces are transmitted through integrins and cadherins creating changes in the cytoplasmic microenviroment. Calcium ions pass through activated membrane channels and the resulting calcium flux triggers activation of phosphatidylinositol-3-OH kinase (PI3K) and rho. The mechanical activation of cytoplasmic kinases creates cell signals that are transmitted into the nucleus where they activate the transcription factor AP1 resulting in expression of several genes. Cell function, shape, and identity rely on mechanical forces imposed through the ECM. Cells in an intact human rely on the forces of gravity and body movement essential to muscle and bone physiology, the pulsatile pressures of blood define

functions of cells in the heart and blood vessels, and forces associated with breathing create healthy lungs (Mammoto et al. 2013).

A translocation of chromosome 9 in intron 2 of the ABL gene with chromosome 22 in the B-cell recombination region (BCR) creates the Philadelphia chromosome that is associated with chronic myelogenous leukemia (CML). BCR-ABL mRNAs are produced from splicing either the first, second, or third exon of the BCR gene to the second exon of ABL as b1a2, b2a2, and b3a2, respectively. The fusion of the two genes replaces exon 1 of ABL with BCR. Intact ABL is a tyrosine kinase involved in cell adhesion and when fused with BCR a constitutively active tyrosine kinase is formed altering cell adhesion signals resulting in a myeloid leukemia. The gain of a constitutive tyrosine kinase activity leads to uncontrolled cell growth. We used a phosphorothioate oligodeoxynucleotide to suppress expression of b2a2 in a cell line derived from a CML patient and the cells died (Skorski et al. 1994; Wu et al. 1995). Our studies demonstrated loss of the constitutively active tyrosine kinase (p210) leads to cellular activities that cannot sustain life. The oligonucleotide approach faced challenges due to complexity of manufacture and cost and so advanced development was never attempted. The therapeutic strategy shifted to a small molecule inhibitor of the p210 kinase, Gleevec, approved by the FDA for the treatment of CML in 2001.

Why would inhibition of p210 kinase lead to tumor cell death? The hypothesis was that inhibition of the novel kinase would simply mimic cell growth of the pre-tumor cells, prior to the chromosome translocation because we blocked the gene that induced tumor development. We observed programmed cell death, apoptosis, an outcome that is not consistent with simply reverting to the earlier pre-translocation state. This nonlinear outcome common in many types of tumors that originate from the activation of oncogenes including *c-myc*, *ras*, and *braf,* is a phenomenon referred to as oncogene addiction. The observation suggests a tumor cell can depend on a single genetic lesion for tumor maintenance and survival. Put another way, the tumor cell became resilient to p210 kinase but then require the translocation p210 kinase for survival.

MYC is causally involved in more than half of all human cancers (Boxer and Dang 2001). Control at level of transcription, rapid mRNA turnover, and short protein half-life tightly regulate the normal expression of MYC so disruptive events like viral infection and chromosomal translocation (Dalla-Favera et al. 1982) enhance MYC expression. The activation of the oncogene MYC leads to suppression of apoptosis, differentiation, and proliferation arrest mechanisms while stimulating metabolic reprogramming, angiogenesis, and proliferation that can lead to senescence, apoptosis, and tumorigenesis. Paradoxically, inhibition of MYC, even transient inhibition, leads to tumor cell apoptosis and senescence that drive sustained tumor regression (Hudziak et al. 2000; Knapp et al. 2003; Devi et al. 2005). These hallmarks of MYC addiction differ with tumor type; lymphomas die through cell apoptosis when MYC is suppressed, osteosarcomas differentiate with loss of MYC but die through apoptosis if MYC expression is restored, and hepatocellular carcinomas differentiate becoming dormant with MYC inhibition but the tumor will reoccur if MYC expression is restored (Li et al. 2014). Inhibition of MYC in a

coronary vessel damaged by balloon angioplasty does not lead to apoptosis highlighting a different outcome in an undifferentiated cells compared to a normal cell (Kipshidze et al. 2004). Hence, the cellular microenviroment, tissue architecture, state of differentiation, and context determine outcomes from changes in the expression of MYC.

Stem Cells

An embryo begins as a single cell that has the capacity to become any of the human tissues, a characteristic referred to as totipotent. Embryos grow structures from three regions; ectoderm, endoderm, and mesoderm. Each of these regions is made of pluripotent cells capable of making a number of tissues, for example, ectoderm will make nerve tissues, mesoderm will make muscle and fat, and endoderm will make blood. These cells hold the potential to repair, regenerate, and restore function to damaged tissues driving medical research to isolate stem cells for therapy.

Embryonic stem cells (ESC) are retrieved from the inner cell mass of an embryo and are totipotent. They have limited availability as they require destruction of a human embryo that is associated with ethical and regulatory concerns. Their totipotent benefit is associated with the potential to produce teratomas, rare cancers. Once the most sought after cells for research, ESCs are a research fad of the past.

Induced pluripotent stem cells (iPSC) are pluripotent cells created by taking differentiated cells and inducing stem cell character though transfer of a small collection of genes encoding transcription factors (Oct4, Sox2, Myc, and Kif4). The discovery led to awarding Shinya Yamanaka a Nobel Prize in Physiology or Medicine in 2012 for the discovery that mature cells can be reprogrammed to become pluripotent. While pluripotent is not as global as totipotent, these cells avoid the ethical concerns of ESCs and less concern for tumorigenesis. iPSCs are also easily obtained and offer a significant advantage of autologous cells for transplantation expanding the concept of personalized medicine.

Hematopoietic stem cells (HSC) are mesodermal in origin and are also pluripotent. HSC can be retrieved from bone marrow, peripheral blood or from the umbilical cord. These are the most easily purified using surface markers such as CD34. HSC are $CD150^{+}CD48^{-}CD244^{-}$ while multipotent progenitor cells (MPP) are $CD150^{-}CD48^{-}CD244^{+}$ and lineage restricted progenitor cells (LRP) are $CD150^{-}CD48^{+}CD244^{+}$ confirming different levels of differentiation can be identified using cell surface markers. Further, HSCs can be long-term repopulating, LT-HSC versus short-term repopulating, ST-HSC separating the extent of stem cell self-renewal abilities. Current limitations are the relatively small number of cells that can be obtained and they do not differentiate (transdifferentiate) into some tissues such as heart muscle.

Diabetic retinopathy (DR) occurs in almost all patients with type 1 diabetes and 75 percent of patients with type 2 diabetes within 15 years of the manifestation of diabetes (Paine et al. 2012). Over 12,000 diabetic patients become blind each year

due to ocular complications (American Diabetes Assoc.). $CD34^+$ HSC are capable of homing to vascular lesions in the eye mediating vascular repair (Otani et al. 2004). The use of autologous $CD34^+$ cells eliminates the significant complication of transplant rejection. However, diabetic $CD34^+$ cells are dysfunctional, contributing to the diabetic complication of DR (Kijimo et al. 2014). While $CD34^+$ cells from healthy subjects could repair retinal capillaries in streptozotocin induced diabetic mice, spontaneously diabetic obese BBZDR/Wor rats and neonatal mouse oxygen-induced retinopathy animal models $CD34^+$ cells from diabetic mice could not (Caballero et al. 2007). An ongoing project investigates transient inhibition of TGFβ1 to restore the regenerative ability of $CD34^+$ HSC in diabetics to treat DR (Bartelmez et al. 2016).

Mesenchymal stem cells (MSC) are multipotent meaning they have a smaller range of tissues they can create. Their popularity is based on the fact they can be recovered from fat and large numbers of cells can be isolated.

Ectodermal stem cells (EctSC) are multipotent cells recovered from skin. These cells are of interest for nerve cell regeneration but techniques for isolation and purification are challenging and limited numbers of cells can be recovered.

Somatic cell nuclear transfer involves moving the nucleus of a somatic cell into an egg that has had its nucleus removed followed by implantation and pregnancy is a strategy to clone dozens of animal species. The strategy creates a totipotent stem cell that is limited in use to cloning an individual. The technique gained familiarity with the creation of Dolly the Sheep in 1997. Recent improvements led to successful cloning of a macaque primate. The potential therapeutic benefit lies in creating animal models of disease and sources of donor organs for transplantation.

Microphysiological Systems

Advances in cell culture models to be used as surrogates for organs and tissues but cultured cells can fail to maintain differentiation and expression of tissue-specific functions. Growing cells in three dimensional ECM improves cellular organization but these cells fail to reconstitute structural and mechanical features of an intact organ. A biomimetic microsystem has been created to incorporate an alveolar-capillary interface and mechanical strain of breathing in a “lung-on-a-chip” microdevice. (Hu et al. 2010) This sophisticated cell culture device can mimic lung responses to nanoparticles, bacteria, and inflammatory cytokines. The expanded cell functions provide a relatively low cost lung surrogate that enables drug screening, evaluation, and toxicity assessment.

Additional organ-on-a-chip microdevices have been developed. A human gut-on-a-chip responds to mechanical strain (0.02 dyne/cm^2), fluid flow (30 μL/hr), and microbial microflora by recapitulating intestinal epithelial cell differentiation including villi-like structures, an enhanced intestinal barrier, and peristaltic motions. Evidence of epithelial cell differentiation include a polarized columnar cell that spontaneously fold into intestinal villi like structures (Kim et al. 2012). A

kidney-on-a-chip recapitulates functions of the proximal tubule that exhibits albumin transport, glucose reabsorption, and a brush border luminal surface (Jang et al. 2013).

An ongoing hypertension project involves primary proximal tubule epithelial cells (PTEC) recovered from human kidneys grown in the Nortis' "Kidney-on-a-Chip" that are viable for over 28 days. These PTEC demonstrate basolateral to luminal orientation, and maintain metabolic and transport functions (Alder et al. 2016). The critical kidney functions are divided into filtration of blood, which occurs in the glomerulus, and regulation of water and salt balance, taking place in the tubules. The proximal tubular segment functions to: (i) secrete waste products, (ii) reabsorb water, salts, and glucose, and (iii) bioactivate/degrade vitamin D hormones. The kidney-on-a-chip allows for a human cell derived preclinical model that will accelerate the drug development process. In addition to robust evaluation of the mechanistic actions of a drug in the proximal tubule of the kidney, renal cells from a diverse array of human donors can provide essential insights into the relationship between genotype and therapeutic efficacy, as well as an early measure of variability in drug response.

Animal Models

An animal model is intended to be a simple representation of a complex system. Simplicity is important to understanding data but means an animal disease model will not reproduce all aspects of a human disease. Animals are used for two separate purposes including the evaluation of a drug's efficacy as well as potential toxicity. Given the high stakes in human life and profit potential, failure of animal models to predict human responses is a subject of considerable attention.

A CD28 humanized monoclonal antibody (Mab), TGN1412, was created to treat B cell chronic lymphocytic leukemia (B-CLL) and rheumatoid arthritis. The Mab binds to the T-cell receptor through a B7 family ligand. The first in man, phase I clinical trials were initiated on March 13, 2006. Six healthy volunteers were hospitalized with four suffering from multiple organ dysfunction following a sub-clinical dose of 0.1 mg/kg. Those volunteers suffered caused a severe cytokine storm (Hansen and Leslie 2006; Stebbings et al. 2007) but this effect was not observed in non-human primates. Toxicity due to lack of concordance in activation of memory T-cells in humans but not in non-human primates. One 28-year old volunteer suffered a ballooned head described as similar to the Elephant Man and another volunteer lost fingers and toes as a result of the drug adverse event. While the volunteers were released from the hospital in a month, their immune systems may never recover from the event. The developing company, TeGenero Immuno Therapeutics went bankrupt.

Approximately one-third of published animal studies are confirmed in human clinical trials and one-tenth were ultimately approved for use in patients (van der Worp et al. 2010). A comprehensive look at the reliability elicits strong opinion,

"Relying on animal surrogates of human illnesses is a flawed approach to science (Langlet 2009)." Large randomized clinical trials fail for medications supported by substantial benefit in animal studies including enteral probiotics for prevention of complications of acute pancreatitis (Besselink et al. 2008), NXY-059 for acute ischemic stroke (Shuaib et al. 2007), and strategies to prevent lethal reperfusion injury in patients with acute myocardial infarction (Dirksen et al. 2007). Chimpanzee and macaque models fail to predict efficacy of vaccines against acquired immunodeficiency syndrome (AIDS) 100 percent of the time. Only 3 of 494 (0.6%) interventions for acute ischemic stroke that reported efficacy in animal models led to a convincing effect in patients (Sena et al. 2007). The translational failure can be linked to clinical trial design that may be underpowered or mismatch between human studies and animal model studies, inadequate animal data or overly optimistic conclusions of animal efficacy, the animal model may not reflect the disease in humans, and ineffective animal studies are not published.

Preventing new blood vessel growth should reduce cancer metastasis from an established tumor. A drug in development to prevent anti-angiogenesis, sunitinib, led to increased metastasis following short-term application in animal models but sustained therapy led to encouraging reduction in metastasis (Ebos et al. 2009).

When reviewing animal model data look for four critical features. Randomization of animals into treatment groups to avoid selection bias. Blinding of investigators to eliminate performance bias in treatment care between groups. Blinding of investigators involved in assessing outcome to avoid detection bias. Finally, blinding in the intention to treat analysis particularly related to follow-up between treatment groups to avoid attrition bias. Further, selection of the number of animals in a treatment group can mean a study has no value if numbers are too small but a frivolous waste of animal lives if the groups are too large.

Different animal species reveal differences in organ toxicity. Which species should be evaluated and for which drugs? Toxic side effects resulting in cessation of drug development are observed in over one-third of drugs evaluated in human clinical trials. Clearly animal testing did not identify the severity of human adverse responses (Hartung 2013). Drug-induced liver injury is a common human toxicity leading to drug termination. There is poor concordance between animal and human toxicity with regard to liver function (Olson et al. 2000).

Improved animal models will include improving the quality of existing models, improving the way animal models are used in decision-making, and development of more sophisticated, clinically relevant, and predictive animals.

The Animal Rule

The Filovirus family has three genus, Marburgvirus, Ebolavirus, and Cuevavirus. These viruses are rare but cause severe disease often resulting in death. In 1976, novel viruses were isolated from patients from two large hemorrhagic fever outbreaks in Maridi, Sudan, and Yambuku, Zaire (now the Democratic Republic of the

Congo). These outbreaks resulted in the deaths of 431 or 602 infected people. The pathogens were characterized as two different subtypes of Ebola virus which referred to the Ebola river in Zaire. One was called Sudan ebolavirus (SEBOV) and the other Zaire ebolavirus (ZEBOV). Subsequent outbreaks in Africa and the Philippines have led to identification of Côte d'Ivoire ebolavirus (CIEBOV) and Reston ebolavirus (REBOV). Detailed analysis of these viruses revealed a close relationship between MARV and the ebolaviruses leading to their classification in the same family, *Filoviridae*.

The Soviet Union and other countries have conducted extensive covert research into the weaponization of filoviruses including ZEBOV-May (the Mayinga isolate). This has led to the classification of filoviruses as agents for biological warfare purposes by experts from around the world. As a result, the filoviruses have been ranked as highly dangerous, Category A Priority Pathogens, by NIAID and the CDC. Procedures for optimal lyophilization of ZEBOV-May were developed as well as long-term storage conditions and aerosol delivery are likely to have been developed in Russia and appears on the "List of Biological Agents for Export Control" by the Australia Group (an informal group of 39 member countries). While the risk of an attack with filoviral biological weapons is low, the recurring epizootics in wild animals may have a profound effect on the environment and the human population.

The concern over potential production of filovirus agents, including ZEBOV, for biological weapons development led to expanded filoviral research around the world. There are approximately 43 maximum containment facilities capable of working with category A pathogens around the world but fewer than half of those facilities work with filoviruses. Even with this level of containment there have been human exposures to filoviruses. Descriptions of cases of human infection at SPA are limited but one fatal accident with MARV-"U" occurred in 1988. A second fatal accident at SPA with Ebola occurred in 2004. I initiated a filovirus antiviral program after the February 19, 2004 accidental needle stick with Ebola virus in the BSL4 facility at USAMRIID in Frederick, Maryland.

This emerging infectious disease is not endemic to the United States but concerns for bioterrorism led congress to authorize the Animal Efficacy Rule, 21 CRF 314.600. Under this authorization, the FDA can approve a drug based on efficacy in a surrogate animal model. The requirements include a reasonably well understood mechanism of toxicity, efficacy demonstrated in one well-characterized animal species, the efficacy endpoint is clearly related to the desired benefit in humans, and data support the rational selection of an effective human dose. The FDA has approved nine drugs using efficacy data obtained from animal studies; (1) pyridostigmine bromide for prophylaxis against lethal Soman exposure in 2003, (2) hydroxocobalamin for treatment of cyanide toxicity in 2006, (3) levofloxacin for treatment of plague caused by *Yersinia pestis* in 2012, (4) raxibacumab for inhalational Bacillus anthracis in 2012, (5) ciprofloxacin for plague in 2015, (6) filgastim (Neupogen) to treat acute radiation syndrome in 2015, (7) moxifloxacin for plague in 2015, (8) pegfilgastrim (Neulasta) for acute radiation syndrome in 2015, and (9) obiltoxaximab for *B. anthracis* in 2016 (Snoy 2014). In addition, the FDA has

approved three biologics under the Animal Rule: (1) Botulism antitoxin heptavalent for treatment of botulism, (2) Anthrax Immune Globulin for treatment of inhalational anthrax, and (3) Anthrax Vacine for post-exposure prophylaxis for *B. anthracis* exposure. Pivotal studies must be conducted in compliance with Good Laboratory Practices (GLP), a challenge for studies conducted in high containment environment like a BSL4 laboratory.

The virus enters the body through accidental needle stick, small lesions in the skin, conjuctival exposure, via the upper respiratory tract or through erosions in mucous membranes of the body. It appears the virus replicates at the site of entry probably in a dendritic or Langerhans cells prior to gaining entry into the blood stream. The pre-symptomatic incubation period is 4 to 16 days in primary contacts and slightly longer in secondary contacts. The first symptoms include prostration, malaise, myalgia usually in the lumbar region of the back, pyrexia and severe headache frequently in the temporal and frontal regions (Kuhn 2008). The headache may be accompanied by vertigo and photophobia. Approximately 3 days after the onset of symptoms (days 6 to 10 post infection), a high fever of 39–40 °C is observed with complaints of burning or reddening of the conjunctiva. The fever is accompanied by easily detected viremia and a slight bradicardia. Five to seven days post onset of symptoms (days 8 to 14 post infection), a skin rash is observed beginning as pinpoint dark papules around hair roots on the face, buttocks, trunk and the outside of the proximal arms. One day later the skin develops a macropapular rash with red exanthemas on the soft palate. Female patients develop a non-itching exanthema of the labia majora and male patients develop scrotal dermatitis. The lymph nodes of the neck enlarge but no enlargement of the liver or spleen are generally detected. Severe nausea accompanied by vomiting and watery diarrhea is observed. Six to eight days post symptoms, ZEBOV is no longer detected in the blood but cutaneous erythema cover the entire body. A second less severe fever (38 °C) is observed and in fatal cases the fever is accompanied by tachycardia. Fatalities generally occur between days 8 and 16 after the onset of illness but any patient that survived to day 16 recovered.

The clinical course of ZEBOV is similar to MHF in humans, irrespective of the strain, and was originally described in Marburg, Germany in 1967. Necropsy studies of confirmed Marburg deaths reveal focal necrosis of the liver parenchyma. This coincides with high numbers of viral inclusion bodies within the infected hepatocytes. Further studies demonstrate that the primary targets of viral infection are antigen-presenting cells, primarily macrophages and dendritic cells (DC). Endothelial cells and hepatocytes are also primary infection targets. Therefore, it is not surprising that viral spread in the infected host entails all organs with high viral concentrations in liver, spleen, lymph nodes, kidney, lungs, and brain.

Many patients develop thrombocytopenia and severe hemorrhagic diatheses, including bleeding from the oral mucosa, gums, intestines and vagina. Patients did not hemorrhage from the fundus of the eye as has been observed for other viral hemorrhagic fevers, e.g. arenavirus infections. Clinical chemistry shows elevated serum glutamic-oxaloacetic transaminase (SGOT), serum-pyruvic transaminase (SGPT), glutamate dehydrogenase, sorbitol dehydrogenase and gamma-glutamyl

transpeptidase (GGT). These serum levels reach a maximum at day 7 or 8 post symptoms. Immature megakaryocytes eventually increase in bone marrow. Diminished clotting factors V and II and fibrin have been observed. Prolonged thrombin time greater than 4 min have been described indicating consumptive coagulopathy or DIC.

In addition to virus in the blood, ZEBOV has been detected in the urine and in throat swabs. IgM and IgG antibodies are detected in patient sera 4–7 days after disease onset with peak titers one to 2 weeks later. IgG titers tend to persist for 1–2 years after disease but transient seropositivity (e.g. IgG positive one week but IgG negative the next) has been described.

The consensus in the field is that the experimental non-human primate models of ebolavirus most closely mimic the natural disease observed in outbreaks of ebolavirus in humans. Experimental infections of non-human primates with human-derived ZEBOV from infected human blood lead to lethal consequence 100% of the time. All non-human primates develop febrile illness independent of the inoculated virus dose or the route of inoculation (Table 4.1). All other animal models (e.g., mouse and guinea pig) require adaptation in order for the virus to be virulent. It is for this reason that the non-human primate models are considered the "gold standard" in assessing potential drugs and vaccines against ebolavirus. Finally, the pathogenicity of guinea pig adapted ZEBOV-Mayinga-8mc has been shown to produce lethal human infection based on the 2004 infections in Russia that resulted from a laboratory accident.

Table 4.1 Comparing Ebola infections in animal models

Feature	Mouse adapted	Guinea pigs adapted	Non-human primates	Humans
Disease duration to death	4–55 days	6–12 days	5–10 days	2–21 days
Virulence	High	High	High	High
Fever	No	Yes	Yes	Yes
Peak Viremia	7.5×10^7–5.6×10^{11} pfu/mL	$>10^{5.2}$ pfu/mL	10^6–10^8 pfu/mL	$>10^{6.5}$ pfu/mL
Hemorrhages	Not profound	Rare	Depend on species	Occasionally
Maculopapular rash	No	No	Depend on species	Common (50% of cases)
DIC[a]	Not profound	Conflicting data	Yes	Yes
Liver enzymes	Elevated	Elevated	Elevated	Elevated
Lymphocytopenia	Controversial	Yes	Yes	Yes
Lymphocyte apoptosis	Probably	Probably	Yes	Yes
Thrombocytopenia	Yes	Yes	Yes	Yes
Cytokine response	Yes	Yes	Yes	Yes

[a]Disseminated Intravascular Coagulopathy

Guinea pigs infected with human-derived ZEBOV from infected human blood will develop a febrile illness lasting 3–7 days and then they recover. Serial passage of ZEBOV in cell cultures and animals has led to highly virulent guinea pig adapted virus (Table 4.1). Adapted virus causes animals to lose weight, develop pyrexia (41.1 °C) and an edematous face. Male guinea pigs develop enlarged testes and clotting times almost always increase. Clotting factors decrease, including II, V, VII, VIII and X and platelet counts fall around day 3 of the febrile period. Clotting times including PT and PTT are abnormal in the ZEBOV infected guinea pig. Guinea pig adapted ZEBOV challenge by the subcutaneous route always induces disease but challenge from the intranasal and conjunctival route only occasionally leads to disease. Infection has been shown to spread from infected to uninfected cage mates. The oral route of challenge does not result in disease and no viral transmission through semen can be demonstrated. Finally, viral spread in the infected guinea pig entails all organs with high viral concentrations in liver, spleen, lymph nodes, kidney, lungs, and brain.

A passage adapted Ebola virus, subtype Zaire (ZEBOV) to progressively older healthy BALB/c mice established a mouse-adapted virus (Bray et al. 1998). Serial sampling studies to characterize the pathology of the mouse-adapted ZEBOV revealed that the mouse-adapted ZEBOV model is similar to the guinea pig and non-human primate ZEBOV models (Table 4.1). Infection of BALB/c mice with mouse-adapted ZEBOV caused uncontrolled viremia and high viral titers in the liver, spleen, lymph node, and other organs; profound lymphopenia; destruction of lymphocytes within the spleen and lymph nodes; and marked liver damage.

We screened for active antiviral candidates in mouse and guinea pig models, publishing our results in per reviewed scientific journals (Warfield et al. 2006; Enterlein et al. 2006). We also revised our lead compounds from a simple PMO, to an enhanced delivery peptide conjugate (Swenson et al. 2009), ending in a chemically modified PMO called a PMOplus (Warren et al. 2010). Neither mouse or guinea pig models succumb to infection with wild-type or non-passage adapted ebolavirus excluding them from consideration under the Animal Rule. One aspect of our advanced development project involved characterizing the nonhuman primate model is sufficiently predictive of human disease.

We filed IND 69,012 to pursue clinical studies with AVI-6002 for immediate treatment of patients following documented or suspected exposure to Ebola Virus on November 24, 2008. The FDA completed their 30-day review of our application and concluded we may proceed with the proposed clinical investigations on December 23, 2008. The FDA included 46 tabulated comments requesting our timely response.

The submission of this IND and a parallel IND for Marburg represented a final milestone under a contract managed by the Transformative Medical Technologies Initiative (TMTI), a division of the Defense Threat Reduction Agency in the Department of Defense. The contract ended on November 30, 2008 so our INDs were submitted 6 days before the contract ended. We submitted an application for bridge funding to continue development that was awarded in 2009. We were prohibited from responding to FDA comments in their safe to proceed letter for over

6 months due to a funding gap period. The gap had a chilling effect on our relationship with the FDA as they expect a company that has submitted an IND intends to expedite developmental progress. A second funding gap period came at the end of the bridge funding period but ultimately we were awarded robust finding to take both drugs for Ebola and Marburg to approval under the Animal Rule.

We requested a Type C meeting with the FDA on March 22, 2011 to explore consensus in the application of 21 CFR 314, subpart I-Approval of New Drugs When Human Efficacy Studies Are Not Ethical of Feasible, the animal rule. These meetings involve submission of a series of questions probing guidance for development of our filovirus treatments. The meeting was held in Silver Spring Maryland on July 7, 2011 from 2:30 to 4:00 pm. The meeting was highly informative and demonstrated a common interest to collaborate on the best plan toward approval under the Animal Rule. Evidence of this mutual interest was a request to submit protocols for the nonhuman primate studies to the FDA to ensure each study would meet their ultimate review criteria including randomization and blinding procedures, both genders, and adequate numbers to ensure statistically meaningful observations. Inclusion of key collaborating USAMRIID personnel and DoD contract officials in the meeting usually restricted to FDA and Sponsor meant all stakeholders received guidance first hand.

Some aspects of FDA guidance proved challenging. The Animal Rule guidance placed high priority on pathogenesis including mortality, which is approximately 55 percent in humans. However, the FDA also demanded a viral dose in nonhuman primates that would result in 100 percent mortality. The viral challenge in humans could be from diverse viral "quasispecies" through mucous membranes, cracks in skin, inhalation, and possibly needle sticks. The FDA requested a plaque-purified virus (to limit quasispecies) from a lethal human infection that had not been passaged to inject into muscles of nonhuman primates. The request for a virus recovered from a lethal human infection was more complex than initially apparent. We used a Marburg virus isolate from a physician that survived infection through extraordinary medical interventions but FDA guidance recommended a virus isolated from a baby that had succumbed to infection. Further, the request for the viral isolate came after several years of studies of nonhuman primate studies.

The series of events required to conduct a lethal challenge nonhuman primate study became complex requiring teams of project managers. A detailed protocol was created in collaboration with the team of investigators at USAMRIID and our scientists. Once consensus was arrived at for the protocol, it was submitted to the FDA for a 30-day review. In parallel, the protocol was submitted to the USAMRIID institutional animal care and use committee. Feedback from these reviews lad to revisions and resubmission to both groups. Once approved, we submitted the protocol to the federal animal care and use committee for approval. Hence, a 4 to 6 month process to create a protocol before we could begin assembling material for each study. These studies meant killing nonhuman primates and putting highly skilled investigators in harm's way. While intricate, this procedure provided comfort in knowing we were likely to conduct the highest quality studies.

The culmination of years of effort arrived as we initiated a phase I clinical trial to evaluate safety, tolerability, and pharmacokinetics of our lead candidates in healthy human volunteers. The first of which were single ascending dose studies (SAD). The studies confirmed the safety of our PMOplus chemistry (Heald et al. 2014). After completing the phase I study for our drug candidates for both Ebola and Marburg viruses the federal government shutdown, later imposing the "sequester". The DoD was forced to cut our budget in half in 2012, selecting to drop support for the Ebola therapeutic while allowing work to continue with Marburg.

Key advancements in the Marburg drug development followed the sequester. First, we refined the drug candidate to a single gene target in nonhuman primate studies (Warren et al. 2015). We used innovative technologies involving "deep sequencing" to look for potential viral evasion that could lead to resistance. We found the filoviruses do not adopt evasive measures to our drug (Khiabanian et al. 2014). These studies provide strong support for our antiviral strategy while creating FDA compliant documentation for these first of a kind studies. We completed studies to reveal survival benefit could be observed when treatment was delayed for 4 days after nonhuman primates were infected (Warren et al. 2016). Finally, we completed the multiple ascending dose (MAD) studies in healthy human volunteers. Our candidate was well tolerated when administered in a manner proposed for treatment of patients. Further, comparison of the pharmacokinetics in the human with nonhuman primates enabled estimation of the effective dose and dose regimen to be used in infected people (Heald et al. 2015). We now had the most advanced filovirus therapeutic having satisfied the four key elements of the Animal Rule.

The Ebola outbreak of 2014 brought remorse to the selection to drop Ebola during the sequester. However, the company leadership breathed a sigh of relief as concerns for a small company engaging in multiple late stage projects meant infectious disease would be eliminated soon. The sequester provided an excuse not to engage in offering help during the outbreak. We had sufficient active pharmaceutical ingredient to treat four to six individuals and had an inventory of synthetic raw materials to make enough to treat another 20 people. While some of us scrambled to seek a pathway to help, the company pointed to lack of support as an excuse not to help, the DoD pointed to the sequester as a reason not to engage, and people from the NIH, WHO, and CDC excluded our agent from consideration in favor of monoclonal antibodies. Many lessons learned but there are still no FDA approved therapeutics to treat people infected with filoviruses.

Opposing Philosophies

30 million people in the United States suffer from over 7000 rare genetic diseases every day of their life. If each future treatment requires over 10 years and more than $1 billion. Genetic disease sufferers will need to wait and most likely will never see a therapeutic designed to treat their specific disease. Once unfavorable, drug discovery for rare disease, defined as under 200,000 patients, has become analogous to

"Money Ball" for the pharma industry. The financial cost for phase III clinical trials, the greatest expense in drug development, can be smaller. The drug price potential exceeds $200,000 per patient per year so companies can recover development costs with smaller demand on manufacturing and distribution. If 100,000 patients pay $200,000 a year for a drug then revenues will be $20 billion a year. Spend $1 billion to return $20 billion a year is an impressive return on investment (ROI). This magnitude of ROI will attract every board of director from every public company. With 7000 different diseases, business models will show strong upside potential for years to come.

Drug development for a disease focuses on the individual disease filled with good karma. If we are forced to think globally successful programs for 7000 diseases to treat 30 million people could lead to a greater than $6 trillion a year in drug costs. This singular effort will more than double the current health care expenses of $2.8 trillion. Expenditures for drugs to treat rare diseases will become more controversial, likely to be accompanied by demand for lower drug prices. However, lower prices removes the business incentive and a diminished incentive to market drugs for rare diseases. Thus, we can speculate recalibration will mean drugs will be developed for some but not all rare diseases. Can the marketplace lead to a fair and equitable selection of which rare diseases will gain drug therapies?

Emerging infectious diseases and growing multidrug resistance leave a growing population vulnerable to life threatening catastrophe capable of spreading worldwide. The time and cost associated with infectious disease treatments is falling behind their rate of emergence. People will just continue to die as the infectious problems will not simply go away if we ignore them. Once again, a drug with an inherently small market will demand higher drug prices. A striking juxtaposition of human life in opposition to higher drug prices. A plausible solution is to reduce the cost and time required for drug development leading to expeditious approval.

An aggressive development timeline may involve minimizing preclinical testing. However, forces of pharmacoeconomics, portfolio management, and return on investment will more likely define which potential solutions will move forward. Resilience comes in many forms; the need for personalized medicine attracts microentrepaneurs. Drugs for rare and emerging problems begin with small groups of scientists.

It is not ethical to test drugs in humans without careful vetting in preclinical models. The history of drug development has taught us to learn as much as possible before subjecting humans to new drugs. Skipping in vitro and animal model studies is reckless because even if they are not perfect predictors of human responses they add critical information. Among the risks of aggressive development timelines are legal liabilities and reduced confidence from the financial communities.

The proposition that all the preclinical studies are of little value in predicting human responses points to conducting the humans as soon as you can find a clinician wiling to conduct the studies. Participants in clinical studies have a right to informed consent, have a right to withdraw from the study, share in the decision-making process with the physician, and have a right to privacy. Clinical trial design involving mico-doses far below those required to lead to a biological effect can be

implemented to immediately eliminate toxic compounds and provide an early look at pharmacokinetic properties of the drug. Information in humans is immediately applicable to the intended human use so why attempt to predict human responses from preclinical studies.

The resolution of these opposing perspectives falls on an aggregate opinion of drug advocates interacting with drug regulators, patient advocates interacting with clinicians, and financial risk takers measuring their returns against risk averse investors.

The development of a new drug to treat hemorrhagic filovirus infections under the Animal Rule was not complete. We needed to treat hundreds of healthy volunteers to create a human safety database. This sort of database is easily provided for drugs that have already been approved and now seek retasking for use against biological threats. The purpose for the human safety database is to identify toxicities that occur less frequently. On one hand, a drug being developed to treat Ebola can be used to treat patients under an Emergency Use Authorization (EUA). The emergency would be declared by the Secretary of Health and Human Services (HHS) and if an Ebola outbreak ever appears in the United States it will be declared an emergency. In this case, a drug with no human safety database will be administered to treat patients. My conflict with the human safety database is why expose hundreds of healthy volunteers to a drug that cannot possibly provide benefit to them if that drug could be used under an EUA that would certainly accompany its intended use.

Raising this concern always leads to indignant opposition. "You just don't understand advanced drug development." Alternatively, "Just accept that a human safety database is necessary." Adding to the conflict is the actual calculation for just how many patients will be required for an acceptable human safety database. I asked an FDA administrator at a public meeting and he said, "It's a power calculation" referring to federal regulations regarding human safety databases. We asked the FDA in our questions in our Type C meeting. The reply was non-committal but definitely more than 50 but probably less than a thousand, likely to be around 350. This deepened my concern that a human safety database for a drug intended to treat Ebola is a fool's errand. Termination of the program means I will not need to concern myself over the ethics of a database.

The demand for new drugs for rare and emerging diseases will inspire scientific innovation. An exciting time for scientists urged by financial support to find solutions to novel problems. However, the education of health providers is increasingly streamlined. Some programs focus on protocols for health care delivery at the expense of training in biochemistry and pharmacokinetics. The concern is an emerging sophisticated drug boom will arrive at a time when health care providers are least prepared to understand how drugs they work around actually work. Deans and department heads of academic centers focus on greater enrollment creating "puppy mills" to train our future health care providers. I would not express such a controversial statement if I had not repeatedly observed the phenomenon.

Conclusion

Scientists seek solutions to problems that often do not have immediate human application. Like dropping flasks in the hope of finding a miracle cure, there are limitations to the interpretation of research studies. Predicting a human outcome from research studies involving cells in culture is not a prudent exercise, at least for now. Animal models often fail to provide predictions of safety and efficacy. After all, we cured cancer in mice two decades ago, so they say. Nevertheless, some experiments are better than others are and some animal models can predict human outcomes for certain conditions. The decision falls on critical review and skilled reviewers.

I was once asked to only conduct studies that created products, so called "killer apps." Reading the literature leads me to believe that about 80 percent of studies fail to provide key information. However, those studies are necessary to guide the design of the 20 percent of studies that drive our knowledge. I do not think anyone can ensure which studies must be done without making a mistake but deliberate review of the literature and previous work can definitely reduce the frequency of mistakes. In an age of information explosion, the diligence aspect of science has grown to become a significant challenge.

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Chapter 5
Bacterial Infectious Disease Threat

Abstract Human disease resulting from bacterial infection is a lottery. Outbreaks can spread though like wildfire through the global human population. Global bacterial diseases including syphilis and tuberculosis have been the drivers of modern medicine from drug discovery to objective clinical trials. Bacteria can create modular genetic segments in plasmids and exploit bacteriophage in transfer of genome segments among bacteria to enable resilience. Bacteria are also able to transfer genetic material with eukaryotes including humans through a process of horizontal gene transfer. We live in an age of effective antibacterial drugs in an effort to control infection and improve survival. Recent appreciation of bacteria living in our bodies, the microbiome, illuminates complex metabolome interactions with the human host. The microbiome and the accompanying metabolites can manipulate human resilience.

Keywords Microbiome · Syphilis · Tuberculosis · Horizontal gene transfer · Human chorionic gonadotropin · Antibiotics

Introduction

Bacteria were the first living cells on Earth and are. Fossilized microorganisms identified in a hydrothermal vent in Quebec Canada lived 4.28 billion years ago, shortly after the formation of the Earth, 4.54 billion years ago. Bacteria (called prokaryotes because they predate the origins of the nucleus) are part of the environment as their earthly biomass is greater than all plants and animals combined. The 20-million different species of marine bacteria account for 50–90 percent of the oceans biomass. Bacterial activities make our planet habitable as they condition the air, decompose dead things, and fix nitrogen facilitating plant growth. Indeed, the adult human body has ten times more bacterial cells than human cells so these small microorganisms are of significant interest. While most bacteria live outside our blood system separated by barriers like the intestinal wall, respiratory epithelium or skin, those bacteria inside barriers are kept in check by our immune system.

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The structure of the bacterial cell includes a phospholipid bilayer cell membrane like all living things. The bacterial genome is on the order one million base pairs of linear DNA wrapped in a circular chromosome that is attached to the cell membrane which is thought to facilitate segregation during cell division. Outside the cell membrane, bacteria frequently have a rigid cell wall with notable exceptions of mycoplasmas and *Chlamydia*. There are two structural forms of the cell wall, one found in gram negative bacteria, the more complex of the two with an outer membrane dominated by lipopolysaccharides and a periplasmic inner layer made of peptidoglycans, and the other in Gram positive bacteria, a simpler peptidoglycan with teichoic acids.

Bacteria reproduce by replicating their genome then dividing, entirely self-contained. However, sharing of genetic information between related species is an ongoing process involving three strategies: (1) transformation involves release of DNA into the environment by a donor cell and then uptake of that DNA by a recipient bacterium, (2) transduction in which DNA is transferred from donor to recipient by a bacteriophage (bacterial virus), and (3) conjugation in which bacteria make contact and transfer a plasmid DNA structure from the donor to the recipient. The DNA sharing between bacteria represents the earliest form of sex and offers substantial adaptability and survival benefit.

Bacteriophage genomes are about the same size as viruses that infect humans, a few thousand bases. Within the 19 known families of bacteriophage, 8 families have linear double-stranded DNA (ds-DNA), 6 have circular ds-DNA, 2 ss-DNA, 2 linear RNA, and 1 circular ss-DNA genomes. Bacteriophage are abundant, seawater contains up to 9 x 10^8 phage per milliliter. They are also important because they can carry genetic information to bacteria but also kill bacteria. The United States Food and Drug Administration has approved bacteriophage products that kill bacteria in meat and poultry as well as *Listeria monocytogenes,* bacteria found in some dairy products and processed meat.

Pathogenic Bacteria

The Earths biomass of bacteria browse the environment recycling the rest of life. Only a small fraction of bacteria develops the specialized characteristics that permit infection and pathology in humans. Antibiotics represented the man's control over infectious disease with a flurry of activity from the early 1930's into the twenty-first century. Antibiotic use has expanded dramatically from sixty-four patients in 1945 (Tillett et al. 1945) to 258 million courses of antibiotics in 2010 (Hicks et al. 2013). In an odd turn of events, 70–80 percent of all antibiotics sold in the United States are used at subtherapeutic concentrations for improved feed efficiency in cattle, chickens, turkeys, pigs, sheep, geese, ducks, and goats for agricultural purposes. Recognized as a generator of antibiotic resistance, agricultural uses are prohibited in Sweden and Europe. Pathogenic bacteria have been fighting back with our help

and their rapid genetic adaptability so that in the United States, over two million illnesses and 23,000 deaths are caused by antibiotic-resistant infections.

Bacterial Outbreaks

Consumption of contaminated food provides an anchoring event to bacterial outbreaks. These outbreaks precipitate robust news reports complete with the food source and a numeric tally of the number of infected people and subsequent lethality.

I collaborated with a distinguished research group and the Uniformed Services Hospital in response to an outbreak of Escherichia coli O104:H4 in Germany from May to July 22, 2011. The outbreak originated in a batch of contaminated sprouts resulting in 4075 cases and 53 deaths. Shiga toxin (Stx-) producing *E coli* (STEC) strains O157:H7 and O104:H4 ("O" refers to the cell wall antigen number; "H" refers to the flagella antigen number) that cause hemorrhagic colitis (HC) are transmitted through contaminated food and water. The 2011 STEC outbreak of *E coli* O104:H4 in Germany resulted in hemolytic uremia syndrome (HUS), characterized by hemolytic anemia, thrombocytopenia, renal failure, and in severe cases, death (~50% mortality). Similar outbreaks in the United States originating in ground beef, sprouts, and vegetables from O157:H7 strains account for 265,000 cases a year (CDC). STEC produces two types of Stx, Stx1 and Stx2, which are associated with HUS. Antibiotics like ciprofloxacin that cause DNA damage are contraindicated for the treatment of STEC because they lead to higher incidence of HUS. The Stxs are encoded on lysogenic bacteriophages within the STEC genome. The bacteriophage respond to potential "death" signals in their host bacteria which triggers the lytic cycle releasing the Stx producing bacteriophage. Antibiotics trigger the "death" signal which induce the stx-phage to enter the lytic cycle enhancing the expression of Stx and release the toxin. Patients often require treatment before determination of Stx expression so these patients are at risk of drug induced HUS. More troubling is that the potential for individuals to become STEC carriers are not known and not recorded. The concern is for recrudescence and bacterial shedding of the STEC from a patient that has been asymptomatic.

The observations of studies conducted in collaboration with the laboratory of Dr. O'Brien at the Uniformed Services University were presented at the annual ASM meeting. The project involved targeting the expression of acpP that is known to inhibit *E coli* growth (Tilley et al. 2007) a positive control for delivery and efficacy, recA, ecoQ, and stxA. The acpP PPMO effectively killed (4-log reduction) five different strains of *E coli* (both O157 and O104 strains) expressing stx at concentrations of 1.25 to 2.5 μM (MIC value). Neither the recA or ecoQ targets were effective which indicates blocking signals from the lytic cycle to stx expression is challenging and confirm the PPMO are not inherently active, i.e., experimental negative controls. The stx2A gene was effective in preventing the expression of the Shiga Toxin by up to 20-fold. Treating the bacteria with ciprofloxacin (7.5 ng/mL; 0.5

MIC) induced toxin expression by over 2500-fold and the stx2A PPMO reduced this induction to 0.0076 at 40 μM. The potential to use the stx2A inhibitor in combination with antibiotic therapy in suspect patients with bacterial diarrhea and those with history of toxin expressing *E coli* infections could drive therapeutic development.

Oncogenic Bacteria

Cancer caused by infectious disease was a concern in the 16th century when patients suffering from the disease were isolated from the healthy population. A variety of cancers associated with bacterial infections is described in Table 5.1.

Helicobacter pylori is a spiral-shaped bacterium that looks like a child's drawing of a seagull under the microscope. These bacteria live in the epithelial mucous layer of the stomach lining. *H. pylori* causes over 90 percent of duodenal ulcers and up to 80 percent of gastric ulcers. This is a common infection, two-thirds of the world's population is infected (4 billion people) and frequency increases with age in the United States. People infected with *H. pylori* have a 2- to six-fold increased risk of developing gastric cancer (Parsonnet et al. 1991; Nomura et al. 1991; Forman 1991; Talley et al. 1991). An adaptation to living in the stomach where acid and low pH degrade living things has been expression of urease an enzyme that degrades urea liberating CO_2 which scavenges excess H^+ to form bicarbonate. The infection is easily treated but emerging antibiotic resistance and poor patient compliance lead to treatment failure between 6 and 40 percent of the time.

I conducted the first studies with peptide conjugated PMO compounds to create potential antibacterial agents against *H pylori*. A peptide with ionic side chain amino acids at neutral pH that become neutral at lower pH was designed to trap the

Table 5.1 Bacteria associated with cancer

Bacteria	Cancer	Impact
Helicobacter pylori	Gastric cancer	952,000 cases; 65–80% linked to infection; CagA. 4th most common cancer, 3rd leading cause of cancer death
Chlamydia trachomatis	Cervical cancer	All infections- 29,056 deaths/year US
Neisseria gonorrhea	Bladder cancer	US 820,000 new infections/year; 170,000 deaths
Borrelia burgdorferi	MALT lymphoma	30,000 cases/year Lime disease US
Salmonella enterica	Gallbladder cancer	Rare tumor
Coxiella burnetii	B-cell lymphoma	1468 patients positive for Q-fever: 6-B-cell lymphoma and 1 follicular lymphoma
Mycoplasma pneumonia	Leukemia	Unknown

"cargo" in *H pylori* (Iversen et al. 1997a, b). The antisense cargo targeted genes encoding bacterial toxins (vacA-cytoplasmic vacuolation gene and cagA-cytotoxin-associated gene A), inflammation promoting genes (napA- neutrophil activating factor), and genes necessary for bacterial growth (urease-catalyzing urea to neutralize gastric acid, nixA-a nickel insertion into urease enzyme, and copA-copper transporting ATPase). The most profound efficacy involved conjugating the delivery peptide to chloramphenicol (Iversen et al. 1997c). Unfortunately, the grey baby syndrome associated with chloramphenicol that nearly toppled Park Davis also prevented development of the enhanced *H pylori* antibacterial.

Treatment to eradicate *H pylori* may not lead to benefit over risk in the long-term. Helicobacter infection may protect individuals from developing gastroesophageal reflux disease (Vicari et al. 1998), Barrett's esophagus (Vaezi et al. 2000), and esophageal adenocarcinoma (Anderson et al. 2008). The protective role of *H pylori* infection may extend to reduced development of asthma (Reibman et al. 2008; Chen and Blaser 2008). These responses appear to be due to the inflammatory interaction of the *H pylori* with dendritic cells (DC) which elaborate IL-18 that drives development of $CD25^{+}/Foxp3^{+}$ T-regulatory cells (Rad et al. 2006). Indeed, the protective/beneficial effect of *H pylori* also drives the pathogenic effects in driving peptic ulcer (Robinson et al. 2008) and perhaps stomach cancer. Hindsight points to treatment for older patients but not younger patients infected with *H pylori* infection but this is not a consensus suggestion.

Chlamydia trachomatis is the most common sexually transmitted bacteria in the United States estimated at 2.86 million infections per year. Treatment is highly effective (97 to 98% effective) but failure to clear the organism can lead to pelvic inflammatory disease (PID) and infertility. Some patients develop perihepatitis, Fitz-Hugh-Curtis Syndrome, and reactive arthritis (Reiter's Syndrome) which is accompanied by urethritis and conjunctivitis (can't pee, can't see, bad knees). People are frequently re-infected with *Chlamydia* which requires antibiotic treatment.

Neisseria gonorrhea is most commonly a sexually transmitted disease. Treatment has been complicated by emerging antibiotic resistance. Fluorquinolone resistance was recognized in 2007 leaving cephalosporins as the only remaining class available, preference to oral cefixime. In 2010, decreased susceptibility to cefixime appeared suggesting emerging resistance is near. Current treatment is a combination of ceftriaxone and azithromycin in the United States. Disseminated gonococcal infection (DGI) is frequent and can lead to septic arthritis, perihepatitis, endocarditis and meningitis.

Borrelia burgdorferi is the causative agent of Lyme disease and chronic inflammation which has been suspected of causing primary cutaneous B-cell lymphoma (PCBCL; Monari et al. 2007). However, a large retrospective study concludes *Borrelia* infection does not increase the risk of solid tumors (Chang et al. 2012). The CDC estimates ten times more people have Lyme disease than have been reported. Lyme is called the "new great imitator" because symptoms mimic so many other diseases.

Salmonella enerica serovar Typhi is gram negative bacterium the causative agent of typhoid fever. An infection of tremendous historical significance resulting in the plague of 430 BC that killed one-third of the population of Athens and killed 81,300 Union soldiers in the American Civil War- more than died from battle wounds. A positive association between *S typhi* and gallbladder cancer has been reported (Koshiol et al. 2016) several times but no mechanism has been put proposed.

Mycoplasma pneumonia is a pleuropneumonia-like organism, PPLO, "walking pneumonia" an atypical pneumonia that develops over a period of several days. Infection evades immune responses as it grows intracellularly where the organisms can persist producing chronic or latent infections. *M. pneumonia* is observed in electron micrographs of leukemic blood, bone marrow, and tissues suggesting a relationship between PPLO and leukemia. A childhood delayed first exposure to *M pneumonia* is associated with Acute Lymphoblastic Leukemia (ALL; Alexander 1997).

Syphilis

Ted was an elderly man in a McNabb Illinois where he generously donated his time as a little league baseball coach. He taught me how to throw a baseball in the summer of 1962 when I joined his team. I was visiting my grandparents for the summer and my grandfather was a devoted baseball fan. Ted exhibited characteristics of *Tabes Dorsalis* caused by damage to nerve cells of the spinal cord leading to stiffness of posture and gait. Ted used a cane in each hand to facilitate shuffling of his right foot followed by dragging the left behind. I was a carefree 7 year old and had no clue of the origins of Ted's challenge, even after my grandmother disclosed the origin as syphilis. While the tertiary stages of syphilis carried significant social concern, the economy of McNabb relied on farming, and people were happy to have a baseball coach for the youngest players.

Syphilis is a chronic systemic infection characterized by episodes of active disease interrupted by periods of latency. Considered a sexually transmitted disease (STD), among the most common of all infections in all societies, syphilis was historically incurable and lifelong. Infection triggers a multistage process: (1) an incubation period of approximately three weeks associated with a localized skin lesions (pustules) and swollen lymph nodes, (2) a second stage is a general systemic infection associated with fever, rash and malasise lasting a few weeks to months followed by a latent period that often lasts many years, and (3) a third stage characterized by progressive, destructive multi-organ disease including mucocutaneous, musculoskeletal, cardiovascular, and central nervous systems. The involvement of so many organ systems with potential for symptoms from any one, several or all means the spectrum of symptoms can vary between patients and within a single individual over time so that syphilis is called the great imitator.

The helically coiled (spirochete) *Treponema pallidum* are Gram-negative bacteria that cause syphilis. Origins of European syphilis appear in Barcelona in 1493,

arriving from the island of Hispaniola where Admiral Don Christopher Columbus had congress with native women. In 1494, dozens to hundreds of slaves captured in Hispaniola were brought to Spain following subsequent trips to the New World. King Charles VIII of France unknowingly enabled the spread of the disease by recruiting stricken Spanish soldiers into his army as he prepared for their campaign against Naples. In addition, many of Columbus's sailors and women captured from the New World were sent by Spain's King Ferdinand to assist Alfonso II of Naples to fight the French. Consequently, the first widespread reports of syphilis appear in Naples, Italy in 1494/1495. King Charles was the first of many monarchs to succumb during the outbreak. An early description of the "barbarian poison" identifies abundant pustules on the genitals accompanied by itching and pain in the joints. A fever and inflamed skin with revolting scabs followed by a "sanguine humor" that oozes from the swollen tubercles. The sickness did not last more than a year but left the skin covered in scars, especially in the private parts.

Some believed gonorrhea and syphilis were the same disease while others felt an organ can only be infected by a single pathogen at a time. John Hunter, a Scottish Surgeon and leading authority on venereal diseases, may have inoculated himself with both using a lancet used to recover infectious material from a prostitute between 1767 and 1786. He reported the chronology of the infection in "A Treatise on the venereal diseases in 1786. The details of this self-experimentation are disputed (Qvist 1977) suggesting the chronology was from a patient. Hunter died of a heart attack in 1793.

Mercury (Hg) was used for centuries by Arabs to treat leprosy and yaws, both infections caused by the genus *Treponema*, and in 1497 was used to treat syphilis. The alchemist Paracelsus (1493–1541) who sought a substance that would purify the body of disease amalgamated Mercury with gold. The philosophy was that gold was the color of the sun, the source of life and energy, and cinnabar ore carrying mercury is blood-red so the two should be combined. A toxic mercury salve called quicksilver or quacksilver marketed at the time as a speedy cure left us with the modern term "quack" when referring to an unskilled medical person.

Treatment for syphilis was restricted to mercury from 1497 to 1909. Mercury did not cure a single syphilitic patient and most likely accelerated the death of many. The proposed curative dose is almost the same as the lethal dose although a topical prophylactic application was very useful/effective. The side effects included new skin ulcers, paralysis, tremors, anorexia, gastric distress, diarrhea, nausea, and rotting teeth. The mechanism of action has not been described in detail but the potential for mercury to bind to sulfhydryl (SH) groups is likely to be involved and SH-activated enzymes of the spirochetes are essential for their viability suggesting a bactericidal effect.

Ironically, the mercury formulation appeared as a little blue pill, and now a different little blue pill generates billions of dollars for drug companies every year for treatment of erectile dysfunction. The advent of chemotherapy was associated with controversy: (1) only high dose mercury will cure versus (2) infinitesimal doses of mercury will cure syphilis. The founder of homeopathy, Samuel Hahnemann, proposed the latter view. The homeopathic dose ranged from about one millionth to a

30-fold dilution based on the stage of disease with the greatest dilution for patients with initial chancre still evident. This low dose strategy acknowledges the need to deliver drug deep into tissues and high doses produced severe off target effects.

Consumption of little blue pills is a critical attractant to historians investigating the association between syphilis and historical figures. Indeed, syphilis infected some notable individuals including Mary Todd and Abraham Lincoln. Abraham Lincoln consumed blue pills consisting of licorice root, rosewater, honey, sugar, 65 grams of elementary mercury, and dead rose petals. Mary Todd tried them in 1869, four years after her husband's death, but discontinued treatment due to a severe adverse reaction. Mary Todd was diagnosed with *tabes dorsalis*, a diagnosis closely associated with syphilis, before her death.

The desire for lower dose and less toxic therapy led to the use of more heavy elements. In 1821, Martin of Lubeck administered iodide (I) on a burned sponge to the ulcers of the throat. Wallace of Dublin improved topical iodide to potassium iodide (KI) in 1834. We still use KI crystals to treat questionable water when camping. KI is a good disinfectant.

A new philosophy emerged in which plant matter from the place of disease origin is used to treat syphilis. This led to infected patients drinking solutions of sawdust from the guaiac tree imported from Hispaniola. Little is reported regarding the efficacy of this strategy but an antibiotic agent, Chaulmoorgic Acid from the Kalow tree, was in use to treat leprosy in Burma from ancient times. Other drugs were discovered using plants and fungus from the disease origin philosophy. For example, malaria was known to come from swampy areas where willows are found. Aspirin (acetyl salicylic acid) comes from the bark of the willow, *Salix*.

A series of significant discoveries included identification of the spirochete, *Treponema pallidum*, in 1905 followed rapidly by the development of the Wasserman reaction, a rigorous diagnostic test, in 1906. These discoveries set the stage for the discovery of Salvarsan (an arsenic containing arsphenamine compound) in 1909 by Paul Ehrlich. The interest in finding a cure for syphilis introduced acceptance of germ theory, development of serological tests, and emergence of risk to benefit in therapeutics.

The University Hospital in Oslo, Norway withheld mercury treatment from 2181 patients with early syphilis between 1890 and 1910 based on the notion that mercury toxicity interfered with the body's healing power. In 1925, 473 patients who did not return to the clinic for treatment with Salvarsan were identified. These untreated patients were compared to a cohort from the same era that were treated with Salvarsan and these patients had four times the frequency of neurosyphilis and twenty-six times the incidence of bone and skin lesions. Salvarsan may not have cured patients but it improved aspects of disease progression.

Over 500 years of reports of syphilis suggests that roughly twenty percent of the pre-treatment era population become infected. Syphilis was endemic in Europe reaching a relatively steady infection rate of 15–25 percent of the population of France. In 1932, the Tuskegee Syphilis Study began in Macon County, Alabama where 40,000 people tested for syphilis identified an infection rate of 25 to 36 percent. Syphilis infected 5–10 percent of the population of the United States in the era

of World War II. The clinicians reported 500,000 new cases each year with 600,000 cases of advanced syphilis each year. Over 60,000 babies were born with congenital syphilis in the 1940's. The disease was responsible for 15 percent of all blindness and 10 percent of the clinical cases of insanity. Clearly, an unmet medical need of the past.

A second era in treating syphilis extended from 1909 to 1943 where mercury was replaced with arsenicals and bismuth initiated by Paul Ehrlich's magic bullet, arsphenamine (Salvarsan). Associated with the advent of a precise diagnostic endpoint for the disease, the Wassermann reaction. The search for improved versions of arsphenamine led to oxophenarsine in 1933. Bismuth was found to be of value to treatment of syphilis and was used as an adjuvant to arsphenamine until the early 1040's. The Cooperative Clinical Groups of the United States adopted a standard regimen of arsphenamine or oxophenarsine and bismuth for 12 to 18 months without rest periods. The two drugs were administered in alternating courses and never administered concomitantly. The adverse events were numerous and serious including early appearance of serious forms of late syphilis, which meant only about 5 percent of patients, ever completed a full course of therapy.

The October 1943 presentation of Dr. John F. Mahoney of the US Public Health Service Venereal Disease Research Laboratory of four patients treated with penicillin marked the new era of syphilis therapy. The supply of penicillin was limited but a governmental cooperative program led to parallel efforts to manufacture penicillin. An optimized protocol for syphilotherapy was reached by 1947 combined with improved formulations and treatment schedules. Penicillin was responsible for safe, durable, and inexpensive treatment of syphilis, gonorrhea, and chancroid. The clinical response to this transformative therapy relegated arsenic and heavy metal reagents, "to the domain of the dodo and the moa." (Goodman and Gilman 1955) Further, the management of venereal disease no longer required the syphilologist and moved to the general practitioner. The treatment of early syphilis involved 600,000 units of procaine penicillin G in aqueous suspension (2 mL) intramuscularly daily for 10 days.

The historical impact of syphilis includes torment for innumerable musicians, artists and poets (Hayden 2003). A fascinating account of Adolf Hitler's secret syphilis infection explains much of his seriously aberrant behavior. A Jewish prostitute in Vienna may have infected Hitler in 1908, which contributed to his desire to exterminate Jews. Syphilis incidence reached epidemic proportions after World War I leading some observers at the time to question the vitality of the human race. Hitler and a few unbalanced cohorts felt the Jews carried the disease hereditarily then spread it to the rest of the population. In addition to the chilling prospect that syphilis contributed to the holocaust, Hitler's tertiary syphilis manifested itself as psychosis, heart disease, and paranoia from 1937 to 1945. One can only imagine the, "How did I get wrapped up in this mess" felt by those close to Hitler as the end of World War II approached.

The history of syphilis is also the history of modern medicine. Long centuries of human suffering inspired great science and the advent of microbiology, immunology,

Table 5.2 Notable antibiotic commercial developments

Company	Antibiotic/origin source	Year
I.G. Farben in Germany	Salvarsan/Arsenicals	1909
	Prontosil/Analine dyes	1932
Parke Davis	Chloramphenichol/Streptomyces venezuale	1947
Eli Lilly and Company	Vancomycin/Amycolaptopsis orientalis	1952
	Cephalothin/Cephalosporium acremonium	1962
	Erythromycin/Saccharopolyspora erythraea	1949
Merck	Imipenem/Streptomyces cattleya	1979
Squibb	Aztreonam/Chromobacterium violaceum	1981
Rutgers-Schering-Plough	Streptomycin/Streptomyces griseus	1944
	Everninomicin B/Micromonospora	1964
Bristol/Myers	Chlortetracycline/Streptomyces aureofaciens	1945
Upjohn Co.	Lincomycin/Streptomyces lincolnensis	1962
Pfizer	Clindamycin/Streptomyces virginiaea	1967

and clinical science. The origins of many of today's recognizable pharmaceutical companies also follow the science devoted to syphilis (Table 5.2).

Syphilis is not just a disease of historical significance. Efforts to control/eradicate syphilis have not been successful (Hook et al. 2004). The World Health Organization (WHO) estimated a global 12 million new cases of syphilis in 1999 (WHO 2001). Recent, post penicillin, epidemics of syphilis occurred in Russia in the 1990s, China (Chen et al. 2007), United States, Canada and England in the first decade of the twenty-first century. The CDC recommended treatment for uncomplicated, early stage syphilis is a single dose of 2.4 million units of penicillin G administered intramuscularly. However, penicillin does not cross the blood brain barrier, is not indicated for use in pregnant women, and should not be used in patients with penicillin allergies. These limitations combined with the desire to administer therapy parenterally have led to use of second-line oral antibiotics including macrolides such as erythromycin and azithromycin, and tetracycline's such as tetracycline and doxycycline. Macrolide resistance has now emerged as the *Treponema* have accumulated mutations and methylation sites in the 23S rRNA (A2058G) target site (Stamm and Bergen 2000). This resistant strain shows cross-resistance to Clindamycin (another antibiotic class targeting 23S rRNA) and is currently present in the United States, Canada, Europe, and China. Rifampicin is an antibiotic targeting the DNA-dependent RNA-polymerase encoded by *rpoB* and all spirochetes tested are intrinsically resistant. Firm evidence of tetracycline resistance has not emerged for *T. pallidum* but resistance mutations in the 16S rRNA target site have been identified for *H. pylori* and *Propionibacterium spp.* pointing to high priority sites to monitor. Fortunately, *T. pallidum* has not developed resistance penicillin.

Most people alive today have never seen an individual with advanced syphilis which is in contrast to a century ago when everyone knew and feared this disease. Fewer have any idea of the impact syphilis has had in shaping modern medicine, our approach to antibiotic drug discovery, and the emergence of the pharmaceutical

industry. The lack of appreciation by today's society is a strong testimonial to the success in treating "The Great Impersonator, The French Disease, or The Barbarian Poison." Recent reports of increased frequency of syphilis cases should send cold shivers down the spine signaling an emerging fear. We should feel a "Ripple in the Force" but only an esoteric group interested in public health show concern for the impending social change.

Tuberculosis

My first encounter with *Mycobacterium tuberculosis* (MTb), the agent responsible for tuberculosis (TB), was in 1963. I was in the third grade at the Navajo Regional School in Sanders, Arizona. Attending this school required a long bus ride down Route 66 from my home in the Painted Desert within the Petrified Forest National Park. Ten to twelve students, children of park employees, represented the only Caucasian students in this elementary school of approximately 500 Navajo students. The Navajos, the largest tribe in the United States, are descendants of the Athabascan people that crossed the Bearing Sea over a thousand years ago. The Navajo (called the Dine people) branched off from the Athabascans between 1300 and 1400 as did the Apache a bit further to the east. Colonel Christopher "Kit" Carson accepted the Navajo surrender on July 20, 1863 then led the Navajo population of roughly 25,000 in 53 different forced marches in the "Long Walk" to Fort Sumner from 1864–1866 (Burnett 2005). The brutal treatment reduced the population to approximately 5000. The stay at Fort Sumner is likely to have infected the Navajo people with tuberculosis. The 27,425 square mile Navajo Nation was established to relocate the Navajo (larger than 10 of the 50 United States) with tribal lands in Arizona, New Mexico, and Utah. By 1912, ten percent of the Navajo population was infected with MTb called "*jei di*" meaning, disappearing heart, in the native language.

The Navajo Nation grew to number 85,000 by 1955 (173,667 by 2010 with 332,129 claiming Navajo ancestry). TB was an active disease in 2–3 percent of the population with 50–60 percent of children infected. There were 200 cases of TB on the Navajo Nation in 1955. In 1952 an active campaign launched by the first woman elected to tribal council, Annie Wauneka, led to disease reduction. By 1957, only 17 deaths from TB were recorded on the Navajo Nation (Denschle 1959). The incidence is now low and there were 17 cases in 2009 and 26 cases in 2010. The impact of diabetes, hypertension and suicide now exceeds that of TB on the Navajo Nation.

One component of the anti-TB campaign involved a screening survey of the Sanders Elementary School in 1963, which included my third grade class. The tuberculin skin test (TST) involves a cutaneous injection of MTb antigens on the inner forearm. If you have been exposed to MTb, an immune response referred to as a delayed hypersensitivity reaction, will take place and a swollen area will appear at the injection site as T-cells move to the area. Two to three days after administration of the TST, health care workers inspect the site. If the TST is positive, the person was infected with MTb and additional tests are recommended to assess whether the

individual has latent or active TB. Three days after our TST injections, a group of doctors and nurses arranged themselves outside our classroom after our lunch break. We waited in line while each of us was inspected for the telltale injection-site swelling. A Navajo girl behind me in line panicked as she had a two-inch diameter swollen area at the injection site. She showed this to me and two of her Navajo friends standing next to me in the line. Her two friends rushed her to the girl's bathroom as the line slowly progressed to the inspectors. After all the class assembled in the classroom and the inspectors departed, the girl refugee who happened to sit behind me returned from her evasive asylum in the bathroom. I thought it best to help the nurses find this girl so she could get medical attention and I told our teacher the girl had been missed. Navajo children believe the nurses take children away and kill them but the reality of separation from family and friends was likely. Consequently, my attempt at kindness was met with hatred expressed as a 35-man dodgeball game erupted at recess with only me at the center being pummeled by rubber balls. The harsh treatment only lasted a couple of weeks but I did not make any friends that year.

A critical lesson from the experience is cultural differences in the perception of public health. Mainstream Americans accepted "germ theory" nearly 100 years ago. Modern Navajo people have recently accepted the infectious nature of MTb but retain fondness for the medicine man version which posits the disease comes from contact with wood that has been struck by lightning and the disease persistence is due to incorrect technique in chanting traditional songs. Human behavior will play a central role in infectious disease and changing that behavior will be a challenge that must include respect for people's beliefs.

Tuberculosis is a multistage systemic infection and disease progression or, more commonly, reactivation after years of a latent, asymptomatic, period. The typical reactivation is a chronic pneumonia with fever, cough, bloody sputum, and weight loss. An individual with untreated MTb will unknowingly infect 10–15 other people each year. Symptoms are not specific to TB and include a cough persisting for over three weeks, extreme tiredness, fever, night sweats, loss of appetite, and weight loss. The weight loss overtime gave tuberculosis the name, "consumption," as patients begin to look like skeletons and the ribs stick out including their junction with the spine.

TB probably emerged in our early ancestors 15,000 to 20,000 years ago in east Africa. As we migrated and populated the world, we carried MTb. Estimates of one person in four died of tuberculosis in Europe and Asia in 1851 (Daniel 2011). In 1882, Robert Koch identified *Mycobacterium tuberculosis* as the infectious agent responsible for tuberculosis. A vaccine, Bacille Calmette-Guerin (BCG) based on attenuated *Mycobacterium bovis* began human testing in 1920 and in 1953 a survey revealed an 80 percent reduction in infection rate following BCG vaccination. The successful fight continued with introduction of antibiotics targeted to MTb with streptomycin in 1944, isoniazid in 1952, and the combination of streptomycin, para-aminosalicylic acid (PAS), and isoniazid in 1960. Drug-resistant MTb was reported in the United States in 1970.

The MTb organism grows slowly with generation times of 15–20 h and often exists in a dormant phase in patents, the latent phase. These features necessitate prolonged treatment courses which jeopardizes patient compliance. Treatment consists of three or four agents, which in concert, can eradicate organisms throughout the body and prevent the development of resistance. An initial treatment phase with rifampin (RIF), pyrazinamide (PZA), and isoniazid (INH) for 8 weeks is followed by a continuation phase with INH and RIF for 4 to 7 months. Alternate first line drugs include ethambutol (EMB), streptomycin (SM), oflxacin, levofloxacin, moxifloxacin, and rifabutin (RFB). First-line therapy can convert 95 percent of patients to culture-negative status within 3 months.

MTB infections are declining globally but an estimated 440,000 cases of multidrug-resistant tuberculosis (MDR-MTb) and 50,000 cases of extensively drug-resistant MTb (XDR-MTb) were observed in 2009. Resistance arises due to poor adherence to treatment regimens and a variety of provider errors. A collection of second-line drugs are now used for these drug resistant MTb including macrolides (streptomycin, kanamycin, amikacin and related capreomycin), fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin, and moxifloxacin), rifabutin, ethionamide, cycloserine, para-aminosalicylic acid (PAS), clofazimine, linezolid, imipenem, and meropenem-clavulanate. This represents the kitchen sink of antibiotics to be thrown against MDR and XDR-MTb revealing the near panic situation. The poor adherence to treatment regimens should be expected in that 3 to 4 drugs to be taken over 4 to 7 months is challenging. Consider the treatment regimens for MDR or XDR-MTb which involve even more drugs to be taken for up to 2 years. The prognosis is an MTb antibiotic death spiral.

Outbreaks of MDR-MTb are often associated with HIV infection. An outbreak of XDR-MTb in KwaZula-Natal Provence of South Africa focused attention to the dual epidemic. The dual infection rates have reached 60 to 80 percent of MTb cases with known HIV in some African countries as of 2008. Adding to the emerging challenge, in 2012 the Clinical Infectious Disease Journal reported cases of totally drug-resistant MTb (TDR-MTb) in India.

The problems with MTb drug-resistance and the lack of new FDA approved antibiotics for the treatment of MTb inspired a collaborative study with the Karolinska Institute in Sweden in 2010. The studies employed synthetic RNA analoges to inhibit specific gene expression by an antisense mechanism of action which we had observed to be effective against Gram negative bacteria (Geller et al. 2004; Geller et al. 2003; Geller et al. 2005;). We utilized cell-penetration peptides (CPP) conjugated to phosphorodiamidate morpholino oliogmers (PMO) referred to as peptide-PMO (PPMO) that we had observed to facilitate transport into other bacteria (Geller et al. 2010). Inhibition of genes expressed in MTb included targeting leuD, mgtC, pirG, pcaA and erp. In addition, inhibition of host genes considered essential to MTb survival included targeting IL-10, SOCS1, SOCS3, STAT3, IL-27, and TGFβ (Iversen 2012). This collection of genes was selected based on previously published studies of genes considered essential to MTb growth and survival. PPMO 11-mers targeting either leuD or mgtC were effective inhibitors of mycobacterial growth in 7H9 broth. Growth of BCG in BMM was reduced by over 1 log for PPMO

targeting mgtC, leuD and pirG. These observations provided reproducible evidence of robust inhibition of mycobacterial growth by PPMOs and support the use of PPMO in anti-MTb therapy (Muhammad et al. 2012). The studies conducted from 2010 to 2015 produced encouraging results but a compelling business model could not support the modest research expenses (our drug would be expensive compared to other antibacterial drugs).

The worldwide prevalence of TB is 1.7 billion people with 10–15 million in the USA and 340 million in India. A population four times the size of the USA with 30 times the burden from TB. Indeed, TB is the national disease in India accounting for more than one-fifth of the global TB population. Every year approximately two million people in India contract TB leading to 1000 deaths every day. In 2012, I decided to use more than one million frequent flyer miles to book a flight to India. I wanted to know the urgency in India for a new TB therapeutic. My wife, Laurie, and my friend, Tom Stewart, and his wife Sue and son Tommy accompanied me. Tom arranged a meeting with his UCLA classmate, Arvind Singhal, an advisor to the India medical establishment.

Mr. Singhal provided a primer for healthcare in India at his offices at Technopak located in a modern industrial park called Cyber City. We discussed challenges in providing healthcare in India, which included a need for 2–2.5 million hospital beds, an additional one million doctors, and 1.8–2.0 million nurses by the year 2020. The aging population of India is associated with an increasing burden of heart disease, diabetes, and cancer. Fortunately, household income is increasing, greater access to health insurance, and medical tourism help drive resources to meet the rising demand. Mr. Singhal arranged for a variety of visits to healthcare facilities around New Dehli including: (1) Medanta- the Medicity, (2) the LRS Institute, (3) the All India Institute of Medical Sciences, (4) the Rajan Babu Institute for Pulmonary Medicine and Tuberculosis, and (5) the Maharishi Valmiki Infectious Disease Hospital.

I met Dr. Himanshu Garg, a senior consultant for respiratory and sleep medicine at Medanta, the Medicity in Gurgaon. The facility was a large, clean, modern structure filled with human movement. Lines formed at the elevators with instructions for each line associated with a particular destination indicated on the floor, we were directed to the fifth floor. The receptionist was not obscured by a computer monitor and instead retrieved large format book stacked on a small table to locate the schedule of the doctor we came to visit. A few nonverbal gestures to an assistant and we were escorted to our meeting room. Dr. Garg was generous in taking time to meet given the obvious chaos of his oversubscribed day. He wasted no time in commenting, “TB is India’s National Disease” and proceeded to add color to our growing appreciation of how healthcare works in India. Government hospitals are locations for treating people at the bottom of the economic strata, the “have nots.” Private hospitals such as this Medicity are growing and provide treatment for those with resources and insurance. When you compare this to the US healthcare system, is it different or the same?

Dr. Garg pointed out that nearly every day, “some guy just shows up” who has travelled hundreds or thousands of miles, a trip that will have required multiple

days. This drop-in patient does not understand the concept of an appointment, only that they need to be seen by a physician. A Dr. Garg puts it, "What can I do, I must see these patients?" Add a couple more hours to an already long day. Devoted people willing to give of themselves for the greater good meet the chaos of the 1.2 billion population of India every day.

The next day, I met Dr. Arnand Jaiswal at the LRS (I don't know what this stands for), at the front lines of TB healthcare in India. Dr. Jaiswal spends every day attending to patients but took time for a short visit. He was conversant about the molecular biology of MTB as well as mycobacterium other than tuberculosis (MOT, eg. *M avium* and *M bovis*). The LRS has 40 beds devoted to pediatric TB cases and sadly it is children that represent the large share of new TB cases. As we left, a woman was being helped out of an ambulance. She wore a beautiful sari with vibrant reds, blues and yellow but she appeared as a skeleton. Obviously a lady of substance that is loved but she finds herself in an unfortunate circumstance.

A visit to the All India Institute of Medical Sciences (AIIMS) and Dr. S.K.Sharma, Chief of the Division of Pulmonary Care. No drugs have been developed to treat latent TB and so this remains an area of unmet medical need. One problem is the uncertainty in the accuracy of animal models of TB. This combined with the complexity in monitoring efficacy in latent infections makes drug development exceptionally challenging. Dr. Sharma related a recent visit to Mumbai where TDR-MTb had been recently observed. "The genesis of the drug resistant surge of TB in Mumbai is not a mystery." Many employers do not want people under treatment in the workplace so patients stop taking drugs so they can return to work. The stigma associated with TB means people do not want a formal diagnosis of TB and self-medicate by purchasing readily available ciprofloxacin at the local pharmacy- no questions asked. They discontinue treatment when they feel better which means they are not likely to eradicate MTb in their bodies. These understandable behaviors serve to select for drug resistant MTb.

We visited the Rajan Babu Institute for Pulmonary Medicine and Tubercuosis (PBIPMT) located in a region called the Kingsway Camp of Dehli. This is the largest TB hospital in India, probably the largest TB hospital in the world. Dr. Mittal, director of PBIPMT, shared a cup of tea with us and then arranged for us to meet with three of the top physicians at the institute: Drs. Chaudery, Anuaj, and Bhatnaggar. They provided perspectives on their treatment population. Diabetics represent 7–10 percent of the patients because they are more prone to develop severe disease because the infection interferes with glycemic control leaving these patients' immune compromised. Children under 8 years old represent 14 percent of their patients. About 5 percent of their patients are co-infected with HIV which limits therapeutic options for treating TB. Finally, 85 percent of their TB patient population are smokers. Smoking irritates the lungs and promotes TB disease progression.

Tuberculosis is a force of nature. A decline in disease cases in the United States preceded drug intervention with roughly 113 cases per 100,000 in 1920 to less than 40 cases per 100,000 by 1940 prior to the introduction of streptomycin and isoniazid. The spread of TB in a population of people is like the spread of a forest fire

through densely packed trees. Tremendous expense and human effort may not change the course of the forest fire and it seems this may be true for the spread of TB. Nations like the United States would benefit from daily healthcare lessons of India taught in the face of enormous challenge.

Horizontal Gene Transfer

Transfer of genes between closely related bacteria has been reported. Diphtheria bacilli were observed as "avirulent" and "virulent" in the early 1900's. Investigations by Frobisher (Frobisher et al. 1947) in 1947 described isolating nontoxigenic diphtheria strains that gained "subtle, unexplained, pathogenic power." Virulent, diphtheria toxin producing, *C. diphtheria* were created from avirulent strains by incubating them with lysates from virulent strains that carry a lysogenic bacteriophage (Freeman 1951). They observed horizontal gene transfer (HGT) of the diphtheria toxin gene by bacteriophage transduction. The transfer of pathogenic virulence by HGT is now observed for numerous bacteria.

HGT is responsible for the rapid emergence of antimicrobial drug resistance. Antimicrobial resistance genes can become mobile in transposable elements that are sequences of DNA within bacterial chromosomes or plasmids. *Acinetobacter baumannii* is a Gram-negative bacterium with impressive genome plasticity has emerged as an important human pathogen that has become resistant to carbapenems, "last line agents" recently approved antimicrobial drugs designed to prevent resistance. *A baumannii* are considered environmental bacteria but have recently emerged as human pathogens. Carbapenem resistance involves acquisition of beta-lactamase enzymes which are most often encoded on plasmid DNA suggesting conjugation as a key mechanism for horizontal gene transfer (Da Silva nad Doningues 2016) but phage mediated transduction appears to be the mechanism for transfer of the New Delhi Metalloproteinase (NDM). HGT of antibacterial drugs is a common observation in numerous bacterial pathogens, an emerging concern for the treatment of bacterial infections in man.

Transfer of DNA from bacteria to eukaryotic cells has also been observed. The most fundamental example of bacterial transfer to eukaryotes is the acquisition of mitochondria and chloroplasts from alpha-proteobacteria and cyanobacteria. When the genome sequence of the unicellular amoeba, *Dictyostelium discoideum*, was completed 18 genes were identified as bacterial genes. These acquired genes provided new functions and possible survival benefit to the amoeba (Lacroix and Citovsky 2016). Examples of HGT in multicellular organisms has been described for *Wolbachia* bacteria that are endosymbionts associated with arthropods and nematode worms. The *Wolbachia* grow intracellularly and HGT has been observed in aphids, mosquitoes, beetles, fruit flies, and parasitic wasps (Hotopp 2011). The *Aedes aegypti* mosquito is linked to transmission of chickungunya, dengue, and zika viruses. *Wolbachia*-carrying *Ae aegypti* mosquitos can restrict virus infection and transmission. The Eliminate Dengue Program has conducted field trials in Australia,

Indonesia, Vietnam, Brazil and Columbia using *Wolbachia* to reduce mosquito transmission of these viruses.

The initial analysis of the human genome sequence included 223 suspect bacterial genes (Lander et al. 2001) but skepticism led to a re-analysis of the human genome sequence which confirmed 40 bacterial genes in the human genome presumably through HGT (Salzberg et al. 2001). If these genes are found in cells from multiple organs and are vertically transmitted (from parent to offspring) then HGT has involved germ cells. The probability of HGT to germ cells is much lower than to somatic cells of the body suggesting transfer frequency may be very frequent in cells that do not lead to vertical transfer.

A Strange Case of Horizontal Gene Transfer

Human chorionic gonadotropin (hCG) is a member of the glycoprotein hormone family which includes luteinizing hormone, follicle stimulating hormone, thyroid stimulating hormone and hCG. These hormones are made of two subunits, they share an alpha subunit but differ in their beta subunits, in the case of βhCG the subunit is 145 amino acids of which the terminal 37 are unique to hCG. The best-known function of this hormone is growth stimulation, promoting blood vessel formation, and selective immune suppression protection the fetus from maternal responses during pregnancy. However, hCG is expressed by tumor cells of virtually every histologic type and in metastatic disease. hCG expressed by neoplasia has been used to monitor anti-cancer treatment because it is normally undetectable in patients not harboring tumors (Broder et al. 1977).

hCG expression provides advantages for the tumor which is why inhibiting hCG expression leads to inhibition of tumors. In addition to growth and angiogenesis stimulation, hCG binds to a G-protein coupled receptor (GPCR) which leads to stimulation of MAP kinase (MAPK) and subsequent induction of the oncogenes *c-myc* (Czerwiec et al. 1989) and *raf*. Human prostate cancer cell lines were injected into athymic mice (nude) which lack immune responses so the human tumors grow in the mice. We blocked the expression of hCG in the human prostate tumor bearing mice and observed reduction in the tumor growth from 450 mg in control mice to 252 mg after inhibition of hCG expression (Devi et al. 2002).

An hCG anticancer vaccine made from β-hCG amino acids 109–145, the C-terminal 37 amino acids (CTP37). The immune system is designed not to react to "self" so the CTP37 was conjugated to diphtheria toxin, a foreign protein, to induce an immune response. This vaccine strategy produced antibodies to hCG in humans as it was found to be an effective form of birth control. Investigators then used the CTP37 vaccine in animal models of cancer. The vaccine significantly reduced tumor mass in immune competent Fischer rats with mammary adenocarcinoma from 5.2 g in controls to 2.8 g in CTP37 vaccinated rats (Kellen et al. 1982) and in immune competent C57BL mice with lung carcinoma the vaccine reduced tumors from 7.0 g to 4.1 g in vaccinated mice (Acevedo et al. 1987). A disturbing paradox in these

observations is that only primates express chorionic gonadotropin, rodents do not. How could an immune response to a human protein that is not expressed in a rodent lead to an effective anti-tumor immune response?

The relatively small peptide vaccine is made of type epitopes leading to production of two antibodies, one to CTP16 (5C10; amino acids 130–145) and the other to CTP21 (15H7; amino acids 109–130). We developed human monoclonal antibodies to the two epitopes as an antibody approach to cancer therapy and as a tool to dissect the antitumor mechanism of action. While the two antibodies will not reduce tumor cell viability in cell culture, addition of complement will lead to tumor lysis. The tumor efficacy requires the correct IgG isotype, IgG1, and demands that both antibodies be present at the same time (Iversen et al. 2003). Historically, hCG expression during pregnancy was thought to suppress the maternal immune response against the conceptus. Maternal immune responses do not react to the male fetus expressing "foreign" male antigens. However, the immune system of pregnant women continues to respond to vaccines they received earlier in life to prevent infectious diseases. One function of hCG is to induce IgG heavy chain class switching from IgG-1 to IgG-2 so that the "neoantigens" presented by a male fetus to the mother result in an ineffective, IgG-2, immune response. Similarly, tumors expressing hCG induce their host to switch from the effective IgG-1 to ineffective IgG-2 antibodies thus crippling the immune surveillance.

The potential resolution to the vaccine paradox involves the cytotoxic T-cell (CTL) response. While antibodies created by B-cells in response to the CTP37 vaccine may bind to circulating hCG and effectively clear the hormone from circulation, antibodies are not likely to directly kill tumor cells. We observed the expression of hCG was localized to the tumor cell membrane but it has biochemical signals that should cause it to be secreted from cells where it is synthesized. We explored the mechanism of anti-tumor effect from the CTP37 by moving the hCG gene into a mouse tumor cell line, P815. The mice were vaccinated with CTP37 or ovalbumin prior to injecting the tumor cells. All of the mice produced antibodies expressing the effective IgG-1 heavy chain prior to tumor injection but considerable IgG-2a heavy chain antibodies were detected after tumor growth pointing to the class switching mechanism. Those mice with IgG-1 antibodies produced CTL that attacked the tumor while those with IgG-2a antibodies produced very few CTL capable of attacking the tumor cells (Mourich et al. 2004). The CTL response is dependent on Dendritic cells (DC) which are necessary for antigen presentation that signals the immune response. Further, the DC engulf antibody coated tumor cells and may cross-present antigens associated with the antibody binding. In the case of a tumor, the cross presentation of antigens are generally overexpressed by the tumor cells leading to immune responses to these tumor associated antigens. Hence, the CTP37 vaccine may lead to CTL attack of tumors based on several hCG associated tumor antigens. The anti-tumor CTL action may not require hCG expression by the tumor cell.

The origins of this CTP37 vaccine are rooted in observations from 1974. Chorionic gonadotropin (CG) was found to be produced by a bacterium (Livingston and Livingston 1974) and the observations were confirmed (Cohen and Strampp

1976). These reports were controversial in that a human protein was found in a bacterium and the studies were limited in that only protein fragments were identified and the purity of the CG was called into question. Later, a bacterium called *Progenitor cryptocides* was found to produce CG with biological activity equivalent to hCG and bound antibodies directed to the C-terminus of β-hCG (Maruo et al. 1979). The CG-like protein was then found in aerobic and anaerobic bacteria that were isolated from patients having a variety of malignant neoplasms. Further, the CG-like protein was not found in bacteria from noncancer, control, tissues pointing to bacteria acquiring the human gene from tumor cells (Acevedo et al. 1981). The bacteria may benefit from expression of CG in that immune responses capable of clearing the bacteria are blunted providing a survival advantage. The tumor associated bacteria expressing CG was confirmed by a second group that cultivated the *Staphylococcus epidermidis* from tumors of patients within 6 months (Backus and Affronti 1981). Assembly of information led to cultivating the bacteria expressing β-CG for the preparation of a vaccine for the treatment of cancer (Domingue et al. 1986).

The Environment and Antibiotics

The search for antibacterial drugs has been dominated by searching the environment for ecological clues to killing bacteria. In 1922, Alexander Fleming left petri dishes laying around for over a week and found most covered with large yellow bacterial colonies but clear areas were observed where his own nasal mucus had dripped onto the dish. He had discovered lysozyme, a component of the body's innate immune system that works to damage bacterial cell walls. In 1928, Flemming's similar study leading to the discovery that *Penicillium notatum* mold also prevented growth of bacteria on a petri dish left open near a window for weeks introduced an environmental concept. Mold produces substances that prevent bacteria from invading their space. The observation led to the first humans treated with penicillin in 1942. The 14-year journey inspired others to search for antibiotics in fungi and treatment of humans including Streptomycin in 1944 (Macrolide family), Tetracycline in 1948 (tetracycline family), Chloramphenichol in 1949 (Amphenicol family), Erythromycin in 1952 (Macrolide family), Vancomycin in 1955 (Glycopeptide family), Virginamycin in 1959 (Streptogramin family), Cephalotin in 1964 (Cephalosporin family), Rifampicin in 1967 (Ansamycin family), Clindamycin in 1968 (Lincosamide family), Ncardicin A in 1977 (Monobactam family), Imipenem in 1985 (Carbapenem family), and Linazolid in 2000 (Oxazolidone family) all of which are members of antibiotic classes derived from environmentally acquired fungi (Table 5.3). These families represent the bulk of currently available antibiotics and conjures two critical thoughts: (1) the spectrum of antibiotic discovery has been somewhat limited to fungi and (2) bacteria had billions of years to adapt to these environmental challenges so emerging resistance should not be surprising.

Table 5.3 Comprehensive antibiotic drug discovery

Year	Antibiotic class	Source	Prototype drug
1490	Heavy metals	Plausible chemistry	Mercury
1911	Asenicals	Tissue stains	Arsphenamine
1932	Sulfonamide	Analine dye chemistry	Sulfanilamide
1936	Anti-metabolite		Mandelic acid
1937	Sulfone	Analine dye chemistry	Diaminodiphenyl sulfone
1942	Penicillin	Penicillium chrysogenum	Benzylpenicillin
1942	Peptide antibiotic		Gramicidin S
1944	Aminoglycoside	Streptomyces griseus	Streptomycin
1946	Thiosemicarbazones	Thiosemicarbazide	
1948	Tetracycline	Streptomyces aureafaciens	Chlortetracycline
1949	Amphenicol	Streptomyces venezuelae	Chloramphenicol
1952	Macrolide	Sacharopolysora erythraea	Erythromycin
1955	Glycopeptide	Amycolatopsis orientalis	Vancomycin
1959	Streptogramin	Streptomycin virginiaca	Virginiamycn
1960	Nitroimidazole		Metronidazole
1961	DHFR inhibitor	Chemical synthesis	Trimethoprim
1962	Steroid		Fusidic acid
1964	Cephalosporin	Cephalosporium acremonium	Cefalotin
1967	Ansamycin	Amycolaptosis mediterranei	Rifampicin
1967	Quinolone		Nalidixic acid
1968	Lincosamide	Streptomyces lincolnensis	Clindamycin
1977	Monobactam	Chromobacterium violaceum	Nicardicin A
1985	Carbapenem	Streptomyces cattleya	Imipenen
1985	Fluoroquinolone	Derived from chloroquine	Ofloxacin
2000	Oxazolidinone	Based on S venezuelae	Linezolid
2001	Ketide		Telithromycin
2005	Glycylglycine		Tigecycline
2009	Lipoglycopeptide		Telavancin
2012	Diarylquinolone		Bedaquiline

Creativity and innovation are alive and well in the search for new antibiotics. New approaches to synthesis of complex chemical antibiotics structures continues to advance which permits innovative but rational design to evade current resistance mechanisms, improve metabolic stability, and enhance efficacy. New approaches to the use of killer bacteriophage as antibacterial agents are making progress to therapeutics with acceptable drug-like properties. New concepts are emerging like use of rival bacteria to alter the microbiome ecology squeezing pathogenic forms out. Antibacterial peptides called defensins expressed in plants and animals are exploited for their therapeutic potential. Advanced methods to identify active antibacterial agents from the environment that exploit technologies revealing previously unknown organisms that have never been cultivated in the laboratory have been developed. In addition, sophisticated molecules capable of attacking specific bacterial DNA

sequences are in development. All new agents will be the "first-in-class" that are accompanied by burden of proof of safety and efficacy and the financial risks to development. Unfortunately, the past success in retrieving active compounds from fungi has been relatively cheap, led to easy manufacture, and the regulatory demand for new drugs can be daunting. The antibiotic landscape is not likely to experience broad changes soon.

Microbiome Considerations

The diversity of microorganisms and their association with animals and plants now forces consideration of living entities as biomolecular networks composed of host and associated microbes, holobionts (Bordenstein and Theis 2015; Mindell 1992). The holobionts are encoded by the hologenome (collective nuclear genome, organelles and microbiome) which is adaptable in form, function and fitness to influence the origin of species. Evolution is then a composite of adaptations in the nuclear genome and the environmentally acquired microbiome.

The microbiome is neither static nor continuous. Our body does not carry bacteria while we are in the womb but we quickly acquire bacterial symbionts after birth. As we reach adulthood, our bodies have roughly 30 trillion eukaryotic cells and 100 trillion bacterial and fungal cells. Bacteriodetes and Firmicutes represent 99.9 percent of the bacteria phyla in our bodies but an additional 8 or so phyla also find home in our body. A different microbiome (collection of bacterial species) occupies each part of the body in that the area under the arm is different from between the toes, which is different from the inside of the nose. The mouth bacteria reside on teeth, on the tongue, the cheeks and the gingival crevice. The acidic environment of the stomach selects for *Helicobacter pylori* while the colon is populated with *Escherichia coli*. The metabolic activities of the microbiome generate nearly all of the chemicals in the bloodstream, the circulatory metabolome.

The gastrointestinal tract is colonized by hundreds of microbial species creating an ecology that contributes to maintenance of health and resistance to several intestinal pathogens. Treatment with antibiotics alters the microbiome that leads to varied susceptibility to pathogenic infection from *Clostridium difficile* (Buffie et al. 2015). For example, treatment with clindamycin leads to long-lasting susceptibility, ampicillin induced transient susceptibility, and enrofloxacin did not increase susceptibility. *C difficile* treatment with fecal microbiota transplant (FMT), an attempt to restore microbiota complexity, can resolve recurrent infections (Van Nood et al. 2013). Sequence data of the 16S ribosomal RNA of bacteria in the gut measures bacterial diversity or operational taxonomic units (OTS). The sequence information provided a hypothesis that *C. scindens* is associated with resistance associations. Transfer of *C. scindens* in patients ameliorated *C. difficile* infection and mortality. The influence of a 7α-hydroxysteroid dehydrogenase enzyme encoded by the *baiCD* gene found in *C. scindens* restored host secondary bile acid biosynthesis appears to be responsible. Indeed, administration of bile acids deoxycholate (DCA) and

lithocholate (LCA) inhibit *C. difficile*. A powerful message that one species in the microbiome synthesizes a precursor to secondary bile acid synthesis by the host resulting in resistance to *C. difficile*, an emerging pathogen.

A case for not using antibacterial drugs is emerging. The Yanomami people of Venezuela have been isolated for ~11,000 years have unprecedented levels of bacterial diversity, a strong microbiome ecology. The observation may facilitate "restoration" of the perturbed human microbiome in the developed world to the ancestral condition. Further, the Yanomami have never been exposed to antibiotics yet they harbor bacteria that carry functional antibiotic resistance genes which may explain the rapidity with which resistance has developed to antibiotics (Clemente et al. 2015). Antibiotic use is likely to have diminished the diversity of the human microbiome which has been linked to the rising incidence of asthma (Kozyrskyi et al. 2007; Chen and Blaser 2007), obesity (Cox et al. 2014; Trasande et al. 2013), type 1 diabetes, and Celiac disease (Blaser 2014).

Summary

Humans spread pathogenic bacteria rapidly as evidenced by the global spread of New Delhi Metallo-beta-lactamase 1 (NMD-1) gene. First identified in December 2009 in a Swedish man with *Klebsiella pneumonia* acquired in India (Yong et al. 2009). In May 2010, a case of NDM-1 in *E coli* was identified in the United Kingdom (Muir and Weinbren 2010). In June 2010, three cases of NDM-1 in *Enterobacteriaceae* were reported in the United States (MMWR 2010). The NDM-1 gene was in *Acinetobacter baumannii* in Chennai India in July 2010. On August 21, 2010, a case was reported in Ontario Canada (CTV News 2010) and cases were confirmed in British Columbia and Alberta with no relationship with India. On September 6, 2010, Japan reported its first case. Finally, on December 16, 2013, NDM-1 was found in two wastewater treatment plants in northern China (Science Daily 2013). Humans are only hours apart from exposure to emerging infections anywhere on the planet.

The centuries of suffering from bacterial infections drove science, economics, industry, and clinical practice. As each advancement matured, it became more complex, more expensive, and less likely to ever revert to a simple discovery industry, a collection of irreversible processes. It is not likely that doctors will reduce antibiotic prescriptions, that agriculture will stop using feed enhancement, that the economy will support dramatic changes in antibacterial drug discovery, or bacteria will stop adapting. We can expect "crisis management" in which a tipping point in the number of deaths from antibiotic resistant bacteria is unacceptable.

Bacteria dominate the planet, can adapt to severe environments, and are becoming resistant to antibacterial drugs. Human population is expanding exponentially, people do not adapt quickly, and are approaching critical mass for infections. We face a paradox, treat with antibiotics and save millions of lives while possibly predisposing our population to increased obesity, type I diabetes, asthma, Celiac

disease, and an endless list of unknown consequences. We continue to perturb an impossibly complex global ecosystem that may end in catastrophic human loss.

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Chapter 6
CURE 2000

Abstract Cancer cells differ from non-cancer cells and face elimination from the body. The cancer persists because these cells are remarkable resilient. The evade immune responses and proliferate faster than they die. Medical interventions employed are both highly selective and effective in killing tumor cells preferentially over normal cells. The tumor resilience extends to development of resistance to chemotherapies such that some tumors reappear over time. Circumstances where resilience poses a threat confirms our genomes capacity to support the survival of the cell, even a tumor cell.

Keywords Leukemia · Oncogenes · p53 · OL(1)p53 · bcr-abl · *c-myb*

Introduction

Imagine sitting in a ballroom with over 500 people that share a common cause and every last person is crying? This was not on the agenda but virtually everyone in attendance of the Leukemia Society of America Annual Leadership Conference in November of 1996 was pretty certain this would be one of the events. We assembled in a Hyatt Hotel in La Jolla California with a singular purpose of creating "A Climate for the Cures." We were the Leukemia Society volunteers, researchers, and staff with a mission to share a vision and accountability to accelerate finding cures for the various forms of leukemia. People become involved in the Leukemia Society because it is highly likely that someone close to each volunteer has died from the leukemia. There are inevitable events in such a gathering that remind every participant of personal loss. Further, there was no need to explain why these people all responded with this collective cry because we all knew their thoughts were with departed love ones. Indeed, this was not the first time for these people to cry over the careless ravage of leukemia.

The story of a "little old lady" from New York describing her creation of a novel fund raising mechanism called "Run for Life" that triggered uncontrollable sobs from the entire group simultaneously. This woman had lost her husband to chronic myelogenous leukemia (CML) a couple of years ago. He had suffered with the

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disease for over 5 years so it was not a surprise when he died. Her grief was great and she found it necessary to go out and walk then subsequently run to put sad thoughts out of her mind. As months passed she became a very competent runner for an individual in her late 60s. The relief afforded by running led to her to enter the New York City Marathon. She approached friends and relatives asking them to donate a couple of dollars for every mile she was able to complete to help leukemia patients and support leukemia research. Most donors considered this a modest gamble as they anticipated this apparently frail person would not run very far. The money would be donated to a good cause. These donors were both surprised and proud of our champion's completion of the 26-mile course. She raised thousands of dollars which inspired her to recruit more runners with the common goal of raising money to fight leukemia. By 1996 she had generated hundreds of thousands of dollars for the Leukemia Society. As she stood at the podium in the well-appointed ballroom many of us strained to see her as the lectern hid her small size from view. I was taken by the fact that one individual can be so powerful in making a significant difference. I was also impressed by the deep appreciation expressed by the entire group. The humor and sincerity of the moment penetrated to the heart of each person bringing them to tears.

I attended this conference because the research in my academic laboratory at the University of Nebraska Medical Center was focused on leukemia but I also lost my mother-in-law to CML. I was interested in learning about future funding opportunities for my research in discovering novel, gene-specific inhibitors that may be utilized to treat leukemia. I was part of a very small team of investigators that had recently exploited a discovery that inhibition of a gene called p53 appeared to cause acute myeloid leukemia (AML) cells to commit suicide (referred to as apoptosis). At the time, our approach appeared to be analogous to the use of weed killer to rid the lawn of dandelions. Inhibition of p53 expression encouraged the leukemic blast cells to grow themselves to death. The wild type p53 functions, in part, as a "checkpoint" in the cell division process so loss of p53 allowed cells with problems to proceed through the replication growth event. We had devoted the past year and a half to studies with leukemia cells in culture and prepared an investigator initiated investigational new drug application (IND) to the US Food and Drug Administration (FDA). The clinical stages of drug development were new to us, the gene was new to the scientific community, and our approach to specifically inhibit the p53 gene expression was new. In fact, our application was the first to propose use of this strategy for the treatment of leukemia.

A Brief Look at History

A pattern of identification, search for the cause, then development of a cure has extended back into the history of man for over 5000 years. The identification of cancer naturally evolved into the epidemiology of certain tumors. The most colorful yet innovative history is reflected in the exploration of the various causes of cancer.

The treatment history reveals an apparent rediscovery and refinement of surgical removal and physical destruction of the tumor.

Anthropologists have observed evidence of cancerous tumors in Egyptian mummies of people that lived 5000 years ago, 3000 BCE. Most animal species can get cancer leading to the conclusion that the genetic basis of this malady has been with us for a very long time. Historical investigations from these early times fail to provide information about how these cancers arose but the need to treat was recognized. Evidence of surgical removal of tumors and treatment of stomach cancers with boiled barley and dates can be tracked back to 1400 BCE. Cancer, a human problem seeking a solution for over 5000 years.

The term cancer was applied by the Greek father of medicine, Hippocrates, 300 BCE to describe the appearance of blood vessels of a tumor that spread out like the legs of a crab, karkinos. The body was believed to be composed of four humors: blood, phlegm, yellow bile and black bile. Imbalance of these humors is the cause of disease, and cancer was an excess of black bile. The Roman physician Galen considered cancer curable in early stages by removal of tumors by surgery and cauterized the wound in 168 BCE. He also noticed that sometimes the tumors grew again, early evidence of tumor resilience.

Our focus on cancer shifted to a deeper understanding of the disease. In 667 AD, Paul of Aegina recognized cancer of breast and uterus were the most common forms. Evidence of outcomes based research comes from studies that breast cancer removal by surgery was superior to cauterization. Further refinement came in 1190 AD as Moses Maimonides recognized removal of tumor and roots up to the point of healthy tissue.

In 1649–1652 AD, Zacutus Lusitani and Nicholas Tulp independently publish the contagion theory of cancer. This idea led to a frequent desire to avoid contact with cancer patients, deeply rooted fears of the patient. In 1779 AD, the first cancer hospital in Reims, France was created out of fear cancer was contagious and would spread. Occupation and behavior became associated with cancer as Bernardino Ramazzini observed absence of cervical cancer in nuns but high incidence of breast cancer in 1713 AD. In 1750 AD, John Hunter put forward the lymph theory of cancer. In 1761, Giovanni Morgagni localized cancers of nose and the danger of tobacco based on associations between use of snuff. In 1775, Percival Pott reported repeated exposure to soot in chimney sweeps was linked to scrotal cancer in chimney sweeps. Multiple probable causes and multiple different cancers.

Cancer cells come from “blastema,” the undifferentiated tissue from which the tumor cells arose leading Joseph Claude Anthelm Recamier to recognize cancer metastasis in 1829. In 1838, Johannes Muller established pathological history of cancer as a discipline. In 1889, Steven Paget proposed the “seed and soil” theory of cancer, tumor cell “seeds” have a specific affinity for specific organs, the “soil” expanding on the understanding of tumor metastasis.

Wilhelm Conrad Rontgen detected tumors with X-rays and within 4 years Tage Anton Ultimus Sjogren used X-rays to treat cancer. In 1946, Louis Goodman discovered nitrogen mustards could treat cancer. First chemotherapy for Hodgkin’s Disease, lymphosarcoma and leukemias.

The last century began with the discovery by Francis Peyton Rous in 1910 that a virus could cause cancer. The virus is known as the Rous Sarcoma Virus and definitive support of the contagion theory of cancer. Not to be forgotten, the Perceval Pott observations were supported by 1915 studies of Katsusaburo Yamagiwa and Koichi Ichikawa at Tokyo University that showed application of coal tar to skin of rabbits would induce cancer. An emerging understanding of hormones led Charles Brendon Huggins discovers hormones are necessary for growth of certain cancers, eg. Androgens and prostate cancer.

The modern era involves cancer cause involves the discovery of oncogenes and tumor suppressor genes. The link between viral infection and cancer first proposed by Rous was elaborated on by Howard Temin in 1960 where he proposed a DNA provirus hypothesis of cancer. He elaborated that certain RNA viruses can insert their genetic material into the DNA of host cells. Harold Varmus and J. Michael Bishop discovered the first cellular oncogene, src in 1976. The list of viral infections leading to cancer now includes chronic infection with HBV or HCV and cancer of the liver, the Epstein-Barr herpes virus and non-Hodgkin lymphoma and nasopharyngeal cancer, the human immunodeficiency virus (HIV) as Kaposi sarcoma and non-Hodgkin lymphoma, and human papilloma viruses (HPV) and cancer of the cervix, vulva, vagina, anus and penis.

Tumor suppressor genes generally provide signals within a cell to constrain proliferation. These genes are associated with tumor formation when mutations lead to loss of function. Prototypical tumor suppressor genes behave in a recessive manner so that mutation in a single allele is tolerated and tumors appear only after both alleles acquire mutations. Retinoblastoma, a tumor in the retina, arises in children that carry a single mutation in the Rb gene. These children acquire a “second hit” mutation to the remaining Rb allele in their youth and the retinoblastoma then appears. A collection of tumor suppressor genes are known including p53, p16, Adenomatous Polyposis Coli (APC), Neurofibromatosis Type 2 (NF-2), renal cell carcinoma linked to von Hippel-Lindau Disease (VHL), and Wilm’s Tumor (WT-1).

Leukemia

Leukemias are cancers that begin in the bone marrow, a hematopoietic tissue. Wilhelm Ebstein recognized rapidly progressive leukemia as acute leukemia in contrast to a buildup of mature blood cells and a more indolent leukemia he called chronic leukemia in 1889. Myeloid cells were named by Franz Ernest Christian Neumann in 1869 referring to white cells from bone marrow in contrast to lymphocytes found in the spleen. The differentiation of leukemia as myeloid and lymphocytic was coined by Otto Naegeli in 1900. Combining the terms permutations such as acute lymphocytic leukemia (ALL) and chronic myelocytic leukemia (CML) describe broad groupings of leukemia.

Certain chemicals and radiation can interfere with hematopoiesis resulting in leukemogenesis. Early observations linked exposure to benzene with development of AML (Hunter 1939). Japanese atomic bomb survivors show enhanced cumulative leukemia incidence linking radiation to development of leukemia. There is a 12 percent excess risk of childhood leukemia per millisievert of cumulative red-marrow dose from gamma radiation but exposure did not increase risk of other childhood cancers (Kendall et al. 2017). AML is the dominant leukemia associated with drug or chemical exposure followed by myelodysplastic syndrome (MDS). Drugs include alkylating agents used in cancer chemotherapy such as cyclophosphamide, melphalan, busulfan, chlorabucil, and nitrosoureas (carmustine and BCNU; Greene et al. 1986). Non-alkylating chemotherapy agents including azathioprine, procarbazine, doxorubicin, and bleomycin are also associated with AML/MDS risk (Carver et al. 1979). Some exposures remain leukemia suspect such as cigarette smoking, 1,3-butadiene, and nonionizing radiation from microwaves and electromagnetic sources. One conclusion that can be drawn from drug and chemical leukemogenesis that is the bone marrow is the most sensitive tissue to exposure.

Leukemia is observed more in the developed world with highest rates in Australia and the United States. The incidence is about 2.9 percent of all cancers with roughly 45,000 new cases a year in the USA. Acute myeloid leukemia is rare with a little over 10,000 cases a year in the United States. Treatment involves a two phase regimen: induction with cytarabine and daunorubicin (an anthracycline) to attain remission (no disease can be detected) followed by consolidation with allogeneic stem cell transplantation or interleukin-2 and histamine dihydrochloride. Induction therapy for the M3 subtype of AML, acute promyelocytic leukemia involves all-trans-retinoic acid (ATRA) and an anthracyclene. Patients that relapse, a common event, face fewer treatment options that includes a hematopoietic stem cell transplant of arsenic trioxide, an FDA approved agent. Cure rates range from 20 to 45 percent with higher rates associated with younger people and older people have a less optimistic outcome.

Guardian of the Genome

Simian virus 40 (SV40) was discovered by Maurice Hillman in 1960 as a contaminant in polio vaccine stocks. Polio virus was manufactured in African monkey kidney cells followed by inactivation to create the live attenuated vaccine. Implications of the SV40 contamination were brought into focus in 1961 when the virus was observed to cause tumors in newborn hamsters. Americans continued to be vaccinated into 1963 so that 10 to 30 million were injected with contaminated vaccine. SV40 is known to transform human cells in culture into malignant cells, a sentinel for human cancer. Follow up studies by the Centers for Disease Control (CDC) and the National Cancer Institute (NCI) failed to find the virus in tumors concluding there is no evidence of increased risk of cancer in people vaccinated with contaminated polio vaccine (NCI 2004).

Several researchers were looking for the mechanisms invoked by SV40 in transforming cells. The large T antigen (Tag) was considered the transforming viral protein so purification of this protein led to the search for the host protein bound by Tag. David Lane found p53 bound to Tag in his studies at the Imperial Cancer Research Foundation (ICRF) in London reporting his observation in 1979. Tag binds p53 preventing function, a mechanism raising concern for uncontrolled cellular proliferation and cancer.

I was a biology research technician in the laboratory of Dr. Martin Rechsteiner at the University of Utah. The laboratory atmosphere and the cellular and molecular biology environment in the Biology Department included Drs. Gordon Lark (plant DNA expert), David Wolstenholm (mitochondrial DNA expert), Mario Capecci (Nobel Prize winner and gene transfer authority), and Baldimore Olivera (coneshell toxin expert). Marty's lab developed a red blood cell preparation to deliver macromolecules including tRNA into cultured eukaryotic cells. My project involved investigation of SV40 virus transformation in non-permissive cells in contrast to lytic infection in monkey cells. The hypothesis was that mouse 3 T3 cells were transformed with SV40 virus (SV3T3 cells) due to failure of the virus to synthesize "late" proteins due to lack of specific iso-accepting tRNAs. I isolated tRNA from lytic TC7 African Green Monkey cells then loading the tRNA into red blood cells that were then fused to SV3T3 cells delivering tRNAs. We expected the SV3T3 cells to release SV40 virus into the medium but our experiments failed to support the hypothesis (Schlegel et al. 1978).

Tumor protein p53 gene (TP53) encodes the tumor suppressor protein p53. The p53 gne is located on chromosome 17p and encodes a 393 amino acid protein.

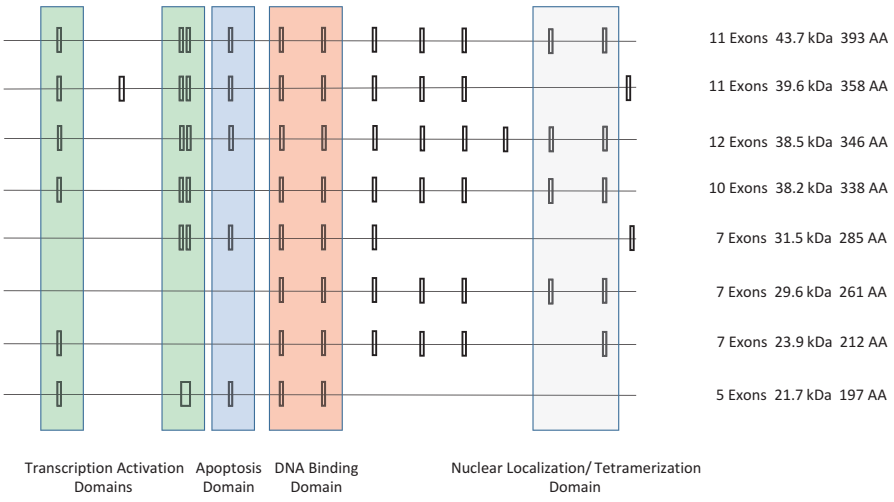

Fig. 6.1 Exemplary p53 Splice Variants. Each line represents a different splice variant with exons depicted as open squares. The summary number of exons, size of the protein, and number of amino acids for the variant are indicated at the right. The colored boxes indicate domains of protein function. If no exons are in the colored box the protein lacks that function

Inactivation of p53 signaling pathways can allow cell proliferation and replication of damaged genomes leading to tumor formation. The name is based on the apparent molecular mass of 53 kilodaltons (kDa) but the predicted mass is 43.7 kDa. There are many protein isoforms of p53 ranging in size from 3.5 to 43.7 kDa. There are four sites used for initiation of translation; at the first AUG, the AUG at codon 40, the AUG at codon 133, and the AUG at codon 160 (p53α, p53β, p53γ, Δ40p53α, Δ40p53β, Δ40p53γ, Δ133p53α, Δ133p53β, Δ133p53γ, Δ160p53α, Δ160p53β, and Δ160p53γ). A selection of splice variant forms of p53 are shown in Fig. 6.1. Exclusion of some exons leads to loss of specific protein functions such as nuclear localization, tetramerization, DNA binding, apoptosis signaling, and transcriptional activation. Mutation or deletion of TP53 is observed in over 50 percent of human tumors (Hollstein et al. 1991). Substitution of arginine for proline at codon 72 (P72R) have been linked to increased incidence of cancer. However, meta-analyses failed to show a link to cervical cancer (Klug et al. 2009) in 2009 and no association with colorectal cancer (Wang et al. 2011) and endometrial cancer (Jiang et al. 2011) in 2011. However, the proline mutation at codon 72 is linked to pancreatic cancer in males (Sonoyama et al. 2011), lung cancer (Piao et al. 2011), and proline homozygosity is associated with decreased risk for breast cancer in Arab women (Alawadi et al. 2011).

The way p53 guards the genome and suppresses tumors involves expression coupled to DNA damage. p53 binds DNA through a DNA binding domain (DBD) and enhances transcription of a variety of genes including WAF1 encoding p21. The p21 protein binds cyclin dependent kinase 2 (CDK2), a complex that creates the G1/S checkpoint in the cell cycle (NCBI 2018). This checkpoint means cells cannot divide preventing proliferation. DNA damage leads to enhanced p53 expression and creation of the G1/S checkpoint means the damaged DNA will not be duplicated in the synthesis phase (S) of the cell cycle. Preventing copies from a damaged DNA genome means the cell avoids creation of potentially deleterious mutations. If p53 expression is inhibited or if mutations prevent successful DNA binding, p21 will not be synthesized with the result that cells will progress through cell division successfully proliferating. Loss of the p53-p21 complex following DNA damage can lead to mutations, genome instability, and mitotic catastrophy resulting in cell death.

Human embryonic stem cells (hESC) have low expression of p53 that maintains "stemness." Elevation of p53 in hESC leads to elevated p21 and loss of S-phase as well as prolonged G1 phase of the cell cycle. The delay in cell division promotes cell differentiation.

Cells maintain low levels of p53 through rapid synthesis coupled to degradation. Functional levels of p53 lead to expression of MDM2 that binds p53 and promotes degradation of p53 creating a negative feedback loop. MDM2 binding sequesters p53 protein in a non-functional state, transports p53 from the nucleus to the cytoplasm of the cell, and marks p53 for proteolytic degradation by ubiquitin ligase. Polymorphisms in MDM2, SNP309 an A2164G, are associated with enhanced cancer susceptibility and in combination with TP53 codon 72 mutations lead to earlier appearance of some tumors in women (Yu et al. 2011). The concept that a small molecule may be designed to break the interaction between p53 and MDM2

resulting in greater cell levels of p53 inspired anticancer drug discovery leading to Nutlin among others.

A viral vector designed to infect tumor cells and express wild type p53 was first taken into clinical trials by Jack Roth in 1996. The therapeutic strategy was to provide a functional p53 in cells lacking p53 or with a non-functional p53. The initial studies were promising in that the p53 gene was transferred to the tumor cells, protein was expressed, and there were no significant adverse events. The key limitation was the immune system detected the retrovirus vector clearing it from the system and ending expression of p53. The delivery was changed from a retroviral vector to an adenoviral vector resulting in two products in development, Advexin and Gendicine. Gendicine was approved for use in China in 2003 and is used in combination with radiation in treatment of patients with head and neck cancer. Supporting data for 5 year survival rates were 14 of 27 (52%) patients in the control group compared to 17 of 26 (65%) in the treated group. The US FDA declined approval of Advexin, an adenovirus type 5 virus in which the E1 region is replaced with the cDNA of the p53 gene where the cytomegalovirus promoter drives expression. The 2008 decision was based on insufficient evidence of efficacy. The controversy of a product approval in China and disapproval in the US will only be resolved with larger numbers and time.

A replication-conditional adenovirus, ONYX-015, was constructed in which a modified early regulation protein E1B binds and inactivates p53. Cells with intact p53 inhibit replication of the E1B deficient virus but cells lacking p53 allow efficient viral replication leading to cell death- an oncolytic virus. ONYX-015 clinical trials began in 1996 revealing the treatment was well tolerated even after multiple daily injections. A phase II study in patients with head and neck cancers produced partial or complete responses in 10 to 14 percent of the treated patients. Viral replication correlated with p53 status as 58 percent of tumors with mutant p53 showed viral replication while none of the tumors with wild-type p53 showed viral replication. Subsequent phase II studies combined intratumoral injection of ONYX-015 with cisplatin and 5-fluorouracil in patients with recurrent squamous cell carcinoma of the head and neck. Endpoint measures included 11 of 30 (36%) of patients had a partial regression and 8 of 30 (27%) had a complete regression of tumor (Vattemi and Claudio 2009). ONYX-015 was sold to Shenzhen Si Bono Gene Technologies in 2003. A variant version of ONYX-015, Oncorine, was developed by Shanghai Sunway Biotech was approved by the Chinese regulatory authorities in 2005.

Leukemia and lymphoma cells almost always have wild-type p53, a clear break from solid tumors. The difference provides the basis for consideration of p53 addiction in these blood tumors and the genesis of our effort to block expression of p53 to treat leukemia.

OL(1)p53

Ernest Armstrong McCulloch joined the Ontario Cancer Institute in 1957 following his education at the Upper Canada College and obtained an MD in 1948 at the University of Toronto in Canada. He pursued research interests at the Lister Institute in London, England. McCulloch collaborated with Dr. James E. Till to provide scientific evidence for the existence of stem cells. They irradiated mice to deplete blood formation but injecting bone marrow cells to test the hypothesis that bone marrow held the cells responsible for creating blood. Nodules observed in the spleens of mice occurred in direct proportion to the number of bone marrow cells injected leading them to speculate that each nodule arose from a single bone marrow cell (McCulloch and Till 1960). McCulloch and Till confirmed their hypothesis that each nodule arose from a single cell that was capable of self-renewal, a defining feature of stem cells (Becker et al. 1963). They then developed methods to quantify stem cells based on growth of spleen colony-forming cells in culture (Till et al. 1964), a critical analytical tool at the heart of stem cell biology.

McCulloch focused attention on the growth of malignant blast stem cells obtained from patients with Acute Myeloblastic Leukemia (AML). A model of AML was developed in which malignant blast cells are organized in a manner similar to normal myelopoietic cells (McCulloch and Till 1981) but despite their uniform appearance, these cells are heterogenous (McCulloch et al. 1988). A graduate student in McCulloch's lab, Larry Smith, investigated the molecular basis of AML finding the recently discovered oncogene p53 is overexpressed in AML blast cells (Smith et al. 1986). Larry joined a bone marrow transplantation group at MD Anderson Cancer Center and developed the aberrant programming model of cancer.

The aberrant programming model is based on the combination of transcription factors that are unusual in malignant conditions. Examples of the transcription factors include c-myc, CREB, p53, Rb, and Rel but many more candidates are known. Altering the pattern of transcription factors will upset the aberrant program resulting in therapeutic benefit. Larry moved to the University of Nebraska Medical Center in 1989 eager to test the aberrant program model of AML by blocking the expression of p53 with antisense oligonucleotides. We were introduced by Dr. Jerry Zon at Applied Biosystems and immediately began to collaborate with Dr. Eliel Bayever, a pediatric oncologist, on the discovery of OL(1)p53.

Kathleen Haines, a hematologist in Eliel's lab recovered leukemic blast cells from AML patients from peripheral blood and bone marrow. These enriched malignant blast cells were cultivated in the presence of at least six different phosphorothioate oligodeoxynucleotide (PSO) sequences, four targeting p53 and two unrelated sequence controls. The PSO targeting exon 10 of p53 reduced the viability of the blast cells, an anti-leukemic effect, from every AML patient by 60 percent within 7 days in culture compared to unrelated PSO sequences or media only controls. The antisense p53 PSO also blocked self-renewal of the leukemic blast stem cells by over 90 percent. Critical to the stem cell model, the p53 PSO did not have any effect on the in vitro growth of cells recovered from normal bone marrow (Bayever et al.

1993a). None of the PSO had any inhibitory effect on the growth and viability of the AML cell line HL60 which is known to have lost the p53 gene. These studies were based on the most credible scientific foundation with lineage back to the genesis of the existence of stem cells.

Questions of how a PSO might enter mononuclear cells needed to be addressed. We injected PSO with a fluorescent tag into mice then recovered spleen, lymph node, bone marrow, thymus and peripheral blood to examine the association of the PSO with mononuclear cells. PSO binding to mononuclear cells was not homogenous with B-cells binding more than T-cells. However, when T-cells were stimulated to proliferate the binding of PSO was enhanced (Iversen et al. 1992a). Transition from binding to a cell to entry into the cell was investigated using both fluorescent and radioactive tags on PSO. We found the PSO distributed primarily to the cell membrane and cytosol but were able to recover tagged molecules from the cell nucleus and the mitochondria (Iversen et al. 1992b). The feasibility of delivery of an oligonucleotide to proliferating mononuclear cells was demonstrated while mechanisms leading to cell uptake remained poorly understood.

Our p53 team began to focus on transition from the research bench to the patient's bedside. Studies to investigate pharmacokinetics and potential toxicity in healthy animals were initiated. We injected mice, rats, rabbits, and monkeys with increasing dose regimens of our lead candidate OL(1)p53 and observed minimal events of concern (Iversen 1993). However, concern was raised when bolus intravenous injections led to severe but transient hypotension in nonhuman primates. The OL(1)p53 was well tolerated with no cardiovascular effects when administered as a continuous intravenous infusion (Cornish et al. 1993). The cardiovascular effects of bolus injections of PSO were observed by other groups shortly after our report. These other groups attributed the hypotension to activation of complement, essentially a form of anaphylaxis. Our observations ruled complement activation out since we did not observe leaky vasculature but rather linked the hypotension to loss of sympathetic vascular tone due to inhibition of the alpha-1 adrenergic receptor (Iversen et al. 1999). These mechanistic studies are largely ignored and the FDA continues to request studies related to complement activation for synthetic oligonucleotides in drug development.

Two years of carefully orchestrated studies led to submission of an IND to the FDA for systemic treatment of AML patients. Our group worked intently to prepare the IND document but we had minimal understanding of this process. Fortunately, Jerry Zon had previously been employed by the FDA and he and his group at Lynx Therapeutics provided necessary guidance and support to complete the document. The FDA allowed us to proceed with our clinical trial, the first systemic use of an antisense synthetic oligonucleotide.

These activities were relatively rare in the university setting back in 1992 and we had generated interest in university leaders as well as interest among the oncology group at the medical center. The activity was new to me as I had expected my career to be one of writing grants and research papers. I inherited a pivotal role in interacting with Lynx Therapeutics as they were the only source for the manufacture of our therapeutic compound. Lynx provided critical experience in working with the FDA

as well. The progress of this project led to a change in the focus of attention from research laboratory work to clinical activities led by oncologists. Once the FDA allowed us to proceed with human trials, a change in leadership was natural (Bayever et al. 1992a). The oncologists generously allowed the research team to participate in monitoring each patient's progress.

We announced the enrollment of our first patient in a guest editorial in 1992 (Bayever et al. 1992a). The preclinical work was presented at annual hematology and cancer research (Bayever et al. 1992b) meetings and were swamped by interested scientists. Meeting organizers visited our posters and invited us to a conference on gene therapy for cancer (Spinolo et al. 1992), antisense technology in France (Bayever et al. 1992c), and an antisense meeting in Japan (Iversen et al. 1992c). The university arranged for a well-attended press conference. Four of us sat at a table in front of a collection of television cameras and a grouping of 7 or eight microphones all bound together. A group of approximately one hundred reporters sat taking notes. My most vivid memory was of the new CNN International cameras. Our greatest challenge was to explain our achievement in language that non-scientist might understand. The session was relatively short and the questions did not probe into details. One interesting outcome of the CNN broadcast of the event was a phone call from an oncologist calling from the bedside of the King Husain of Jordan after he had been diagnosed with lymphoma. They invited us to travel to Jordan to treat their king. This and other head swelling events followed and included a plaque from the Nebraska Chapter of the Leukemia Society with the slogan "CURE 2000" on the front. The intention was to focus attention to scientific breakthroughs that might lead to a cure for leukemia by the year 2000. As I look at this plaque in early 2018 my thoughts are whiskey tango foxtrot (WTF) happened.

The life as an academic scientist often involves building a laboratory with equipment and people that operates like a small business. Activities in the lab focus on the subject focus of the lab often creating a silo of interest. The leukemia project was a team effort that involved medical oncologists with research interests and basic researchers from the Departments of Anatomy, Physiology, Pathology, and Pharmacology. Our team also included a few people from a small biotechnology company, Lynx Therapeutics. My lab served as a hub for the assembly of the IND and the point of contact with Lynx. Submission of the IND required signatures from the chancellor of the medical center, an administrative official so far up the food chain that I had only seen her on the stage at campus-wide faculty meetings and on television when notable events on campus become newsworthy. Department heads and the Dean all expressed their desire that our effort not embarrass the medical center, a justified concern because most of our team had little experience, as we were young.

Our first patient was a 19-year-old man from New Jersey with AML that had relapsed and refractory to all current treatments. A physician's assistant, Mark, came to my lab asking if I would like to meet the patient. I felt this was an unusual privilege while at the same time experienced fear. I wanted to respect the patient's privacy and was concerned he might think of himself as an elaborate guinea pig. However, he deserved to have someone to ask any questions regarding what we

knew about the drug. Mark tutored me on procedures necessary before we entered the patient's room, a gown, mask, and shoe covers. The light level was low in the small room occupied by two people, Chris, the patient, and his mother. They had never been to Nebraska but immediately recognized my unease.

The lesson began, as Chris put my mind at ease. He knew he only had 6 weeks to live and there was no time for non-essential emotions or pleasant chatter. He was obviously ill and the trip included his first time ride on a plane where he spent most of the time with his head in an airsick bag. Astonished by our human-to-human interaction, our meeting was brief. Chris wanted me to know that he understood his role in this trial was to help future patients. He would prefer that we offered a cure but had reached an acceptance that he did not have a future. I left the bedside forever changed by the lesson that as death nears we become the raw essence of humanity and it is beautiful.

The 10-day infusion of OL(1)p53 at 0.05 mg/kg/hr. in the first five patients provided insights into future directions. This low dose patients confirmed that most of our PSO was eliminated in the urine but between 9 and 18 percent remained intact and distributed throughout the body. The primary objective is safety and this dose of OL(1)p53 was not toxic and did not lead to enhanced growth of malignant cells. Bone marrow samples recovered after the infusion showed lower cumulative blast cell production, an encouraging observation in support of diminished stem cell self-renewal (Bayever et al. 1993a, b). Escalating the dose of OL(1)p53 from 0.05 to 0.25 mg/kg/hr. in 16 patients confirmed the minimal toxicity with the exception of injection site reactions, some elevation of hepatic enzymes, and flu-like symptoms. While significant anti-leukemic effect was not observed in treated patients, we did observe a dose dependent reduction in production of leukemic cells *in vitro* (Bishop et al. 1995). The emerging role of p53 as a tumor suppressor posed a contradiction that inhibition of p53 would decrease cell proliferation.

Reflection on our studies revealed oxygen tension in air at 20 percent as a driver of the diminished cell proliferation in culture. The 4 percent oxygen tension in bone marrow failed to lead to OL(1)p53 mediated apoptosis (Copple et al. 1994a, b; Bayever et al. 1995). We were able to recapitulate the failure of OL(1)p53 effects in cultures with low levels of oxygen. Our despair led to discovery as we found adding anthracyclines to low oxygen cultures would restore the OL(1)p53 apoptosis in leukemic blast cultures (Copple et al. 1994a, b). The hypothesis that chemotherapeutics like mitoxantrone would damage DNA but inhibition of p53 removes the checkpoint for cells moving into the cell cycle. These cells will replicate the damaged genome leading to mitotic catastrophy and cell death.

We were able to test our revised strategy in two patients, combining OL(1)p53 infusion with administration of Ara-C and mitoxantrone. Significant reduction in leukemic blasts cells in circulating blood was observed but reduction of these malignant cells in bone marrow was nearly eradicated. The encouraging observations could not overcome the growing sentiment that inhibiting a tumor suppression gene considered the guardian of the genome was contraindicated. Our commercial partner Lynx backed out of future drug development and evaluation. Our program was closed and I left the university to join AVI BioPharma in Corvallis, Oregon in 1997.

The OL(1)p53 project was taken over by Eleos, a small biotechnology company formed in Omaha, Nebraska. The interactions with the FDA were resumed and manufacturing of GMP material was arranged with a CMO. A generic drug name, Cenersen, was created and a phase II study in AML conducted. The study enrolled fifty-three first-salvage AML patients treated with combinations of cenersen and idearubicin alone or with cytarabine. Ten of 53 patients (19%) responded to the treatment with 8 complete remissions (CR) and 2 CR with incomplete platelet recovery (Cortes et al. 2012). Earlier studies revealed the PSO chemistry would scavenge reactive metabolites of acetaminophen possibly influencing antisense efficacy (Copple et al. 1995). Some patients in the phase 2 trial (17 of 53) received acetaminophen and or antioxidants during treatment and none of those patients responded to treatment. These are encouraging results for refractory and relapsed AML.

Beyond p53

Chronic myelogenous leukemia (CML) has been linked to a chromosome 9 translocation with chromosome 22 that results in a fusion of ABL with BCR to form BCR-ABL. The translocation is referred to as the Philadelphia chromosome and the BCR-ABL fusion gene contributes to the pathogenesis of CML. Dr. Bruno Calabretta reasoned that an antisense PSO designed to bind to the unique fusion site in BCR-ABL would selectively degrade this mRNA and suppress leukemic growth. We treated a CML mouse model, a SCID mouse transplanted with a BV173 CML cell line, with a PSO targeting BCR-ABL and observed suppression of the BCR-ABL mRNA and protein. The CML mice that were untreated, treated with a sense sequence control, or a mismatched PSO survived 9.7 weeks (± 0.9 weeks) while the BCR-ABL antisense PSO treated CML mice survived 8 to 13 weeks, median survival of 19.4 weeks (± 1.4 weeks; Skorski et al. 1994).

The potential to remove CML cells from bone marrow recovered from a CML patient might enable a tumor purging strategy. This would allow for autologous transplantation for consolidation treatment of CML. The studies involved mixing normal peripheral blood mononuclear cells with CML cell lines and monitoring the presence of BCR-ABL as a marker of residual tumor cells. The cultures were treated with 80 μg/mL BCR-ABL PSO for 36 h at 37 °C. The treatment eradicated the CML cells from the cultures providing encouraging support for bone marrow purging (Wu et al. 1995).

These preclinical data justified toxicology studies and preparation of an IND to the FDA. However, financial constraints on Lynx Therapeutics prevented progression into human trials.

Dr. Alan Gewirtz was an oncologist pioneering an antisense PSO treatment for leukemia. He had observed *c-myb* as a cell cycle checkpoint in human hematopoietic cells (Gewirtz et al. 1989). Inhibiting the expression of the oncogene c-myb will inhibit the growth of both acute and chronic myelogenous leukemias (Ratajczak

et al. 1992). Alan was part of our Lynx group and I participated in preclinical toxicology studies supporting the IND to the FDA for treating leukemias with PSOs targeting c-myb. Our group at UNMC extended the *c-myb* utility to include Burkitt lymphomas (Joshi et al. 1996). Alan was able to enroll 20 patients into a leukemia trial but the study was stopped prematurely because the drug supply was discontinued due to financial constraints on Lynx Therapetuics.

The Modern Era

Children with Down syndrome, constitutional trisomy 21 or an extra copy of chromosome 21, have a higher risk of developing leukemia but a lower risk of developing solid tumors (Hitzler and Zipursky 2005). The risk of developing acute megakaryoblastic leukemia (AMKL), a rare form of AML, is 500 times greater than in children without trisomy 21 and the risk of acute lymphoblastic leukemia (ALL) is 20 times higher. A transient leukemia or transient myeloproliferative disorder (TMD) occurs in at least 5 to 10 percent of newborns with Down syndrome. The majority, 85 to 90 percent, of TMD resolve spontaneously within the first 3 months of life (Mateos et al. 2015), significant evidence of resilience. The resolution of leukemia in children with Down syndrome provides insights into all leukemia and an encouraging outlook for a cure.

It was clear in the mid-1990s that even if we had an exceptional treatment that it could not complete studies leading to approval by the year 2000. Indeed, the millennial milestone provided a round number to express our enthusiasm that technology would soon find a cure. We have witnessed incremental progress in the treatment of leukemia and enthusiasm remains for reliable cures. The immune therapies such as CAR T-cells are likely to bring about cures and cumulative approvals are taking place. The goal of CURE 2000 is on the way it will just take longer than anticipated.

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Chapter 7
Chemicals in the Environment

Abstract There are no anthropic chemically naïve humans on earth anymore. The past century was the first in human history in which humans harnessed the power of chemistry to enhance survival and quality of life on a global scale. The age of the pharmaceutical industry, the rise of the agricultural industry, and the petroleum era coincide over the past 100 years. The excitement and profits of the chemistry century lead to accumulation of concern. Dilution is not really a solution to pollution but degradation helps, the levels of chemicals in our environment is the net result of chemicals released minus chemicals degraded. Life on earth is demonstrating just how resilient it can be to new potent chemicals.

Keywords Pharmaceutically active compounds · Endocrine disrupting chemicals · Carcinogen · Volatile organic compounds · Pesticides

Introduction

Our global population is exposed daily to chemicals, some of which were not found on the earth 150 years ago. The better living through chemistry slogan parallels the unprecedented growth in human population. The expansion of the chemical boundaries is the basis of emerging philosophical belief that humans are the center of the living world. A single-minded effort to defeat threats to human life resulted in longer life expectancy. Only now does the question, "For how long?" appear.

The good news was that scientific discovery expanded our boundaries of knowledge while improving the human condition. A simultaneous creation of a business model hungry for discovery emerged. Solving problems involving threats from infection, aging, food production, and energy production. The pharmaceutical industry provided discoveries and implemented availability to patients. Pesticides improved food security and quality of life. Retrieval of fossil fuels permitted efficient energy and transportation that are a part of civilized life.

The chemical exposures can lead to adverse human health effects. The fact that we survive is the foundation for resilience and a somewhat optimistic view of the future. Where does the capacity for resilience reside?

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The Pharmaceutical Industry

The pharmaceutical industry arose in past century beginning with isolation of drugs from plants provided by apothecary shops. A notable example is the isolation of morphine from opium in the mid 1800s distributed by Merck, Hoffman-La Roche, Burroughs-Welcome, Abbott Laboratories, Eli Lilly, and Upjohn apothecaries. The industry was transformed with the emergence of organic chemistry based on coal tars and the manufacture of dyes. Methods of organic synthesis and purification of organic molecules offered access to unlimited therapeutic opportunities.

Moving beyond plant sources new drugs began to appear early in the twentieth century. Epinephrine was identified in 1897 by John Abel at John Hopkins University leading to the inhaled, Primatine Mist, for the treatment of asthma in 1929 sold by Parke Davis. The sleep aid, Veronal, containing phenobarbital was marketed by Bayer pharmaceuticals in 1904.

The search for anti-infectives was the major driver for drug discovery and refinement beginning with arsphenamine (Slavarsan) in 1907 championed by Paul Erlich. German chemists exploited their inventory of dye compounds to discover sulfonamide antibacterial agents typified by Prontosil in 1932. Gerhard Domagk was awarded the 1939 Nobel Prize in Medicine for the discovery of the early sulfonamides. Alexander Flemming discovered penicillin in 1928 but it took over a decade and significant contributions of Flory and Chain to bring this antibiotic to patients in 1942. They were awarded the 1945 Nobel Prize in Medicine for its discovery and isolation.

Success in sales of morphine, antibacterial agents, and dietary supplements supported growth of the pharmaceutical industry. However, contaminated formulations and unsubstantiated therapeutics accompanied quality therapeutics in the marketplace. In response, the Federal Food, Drug, and Cosmetic Act was passed in 1938 to require pre-market drug safety to be demonstrated before a drug could be sold. The ethical drug industry proved resilient and flourished as consumer confidence supported rapid growth.

Antibacterial drug development brought forth cephalosporins developed at Eli Lilly, the aminoglycosides beginning with streptomycin discovered by Merck, tetracyclines beginning with chlortetracycline developed at Lederle Laboratories, the macrolides beginning with erythromycin discovered at Eli Lilly, chloramphenicol, and thiosemicarbazones. These antibacterial agents were associated with a 42 percent drop in incidence of the incidence of bacterial disease in the period from 1946 to 1955 (Government Printing Office 1958). Mortality from syphilis, dysentery, scarlet fever, whooping cough, meningococcal infections and pneumonia was reduced by 56 percent and a 75 percent reduction in deaths from tuberculosis. Effective management of bacterial infections provided by antibacterial drugs extended life expectancy post the World War II era.

Treatments for high blood pressure including hydrazaline in 1952 developed by Ciba and chlorothiazide in the mid-1950s by Merck and Co. Antihypertensive drugs have been shown to reduce the risk of death, stroke, coronary heart disease, and

heart attack. Within a decade, combination therapy began with the co-administration of furosemide sold by Hoechst Pharmaceutics as Lasix in 1963, beta blockers introduced by ICI Pharmaceuticals in 1964, and angiotensin converting enzyme inhibitors (ACE). The combination therapy further reduced risk of death as well as delay in the onset of kidney disease in people with hypertension.

The first oral contraceptive, Envolid, was marketed by Searle & Co. was approved by the FDA in 1960 based on synthetic methods developed at Syntex. The "pill" was an immediate commercial success associated with cultural controversy. Convenient birth control led to changes in lifestyle, delay in marriage, and pre-marital cohabitation. The social changes were not accompanied by concern for the environmental impact of these compounds in wastewater.

The advance of big pharmaceutical projects led to development of the cholesterol lowering drugs, statins. Statins inhibit conversion of mevalonate to cholesterol by inhibiting the enzyme HMG CoA reductase. Lead compounds were isolated from a variety of molds with the first statin, Mevacor, marketed by Merck in 1987. Zocor, a newer statin, reduced cholesterol levels by 35 percent associated with a 43 percent reduction in the risk of dying from a heart attack. Akira Endo, a Japanese biochemist, was awarded the 2008 Lasker-DeBakey Clinical Medical Research Award for his pioneering research into the statin class of drugs.

Marketing success and consumer confidence created powerful "Big Pharma" through blockbuster drugs, those that sell over $1 billion a year. Conflicts of interest muddy the waters and obscure the light of medicine for pharmaceutical companies. Conflicts are germinated from the seeds of greed and financial gain, an obligate part of doing business as a public company. Other conflicts are based in arrogance and delusion created by zealous individuals seeking to stand atop the executive teams of these public companies. In the beginning, the pharmaceutical train leaves the tracks in pursuit of money. The perceived objective of a pharmaceutical company is to create and distribute medicine, literally the mediator between heaven and earth. The historic background established an accepted baseline philosophy that the company, "will do well by doing good." A company would not be in business long if they did not bring in more money than they spend. Translate this to the pharmaceutical executive with a singular objective in maximizing profit and share price creating a growing separation from the customer's singular objective seeking to maximize improvement in health.

Pharmaceutical fraud is now monitored seeking evidence for violations in Good Manufacturing Practice (GMP), Off Label Marketing, Best Price Fraud, and Medicaid Price Reporting. Off-label promotion violations led to staggering financial settlements such as $3 billion to GlaxoSmithKline for Paxil, $2.3 billion to Pfizer for Lyrica, $1.5 billion to Abbott Laboratories for Depakote, and $1.4 billion to Eli Lilly for Zyprexa all under the False Claims Act. At this point settlements in the hundreds of million dollars barely make the newspaper. The massive billion dollar settlements have not crippled the industry as in some cases these amounts represent a couple months revenue.

Over a century of drugs that extend life expectancy and reduce suffering, establish pharmaceutical companies as essential to modern life. There will always be a

few bad actors in these companies when they employ tens of thousands. Megatons of active pharmaceutical materials pass though treated patients before entering the environment. Scientists can monitor and estimate the risk of single potent molecules excreted into the environment but prolonged exposures to low levels of hundreds of these molecules exceeds the capacity of modern science. Understanding will require greater financial support of sophisticated studies backed by political will. The threat is now recognized but the remedies fall behind in a cloud of ignorance and indifference.

Drugs in the Environment

Over 10,000 prescription drugs and over 300 over-the-counter drugs are currently in use and produced. These pharmaceutically active compounds (PhACs) are detected in the aquatic environment worldwide at sub-therapeutic concentrations. The compounds are designed to target specific biological pathways and can disrupt sensitive processes in humans and non-targeted organisms. The PhACs enter the environment following excretion of drugs and metabolites in urine and fecies. Removal from sewage by wastewater treatment plants is highly variable due in part to the chemical diversity of PhACs. However, properties desired in PhACs such as hydrophobic character and stability leads to their persistence and accumulation in aquatic ecosystems (Dong et al. 2013). Little or no data are available regarding the fate and transport of PhACs in the environment, their toxicity to organisms and humans at environmentally relevant doses, and the net effect of environmentally available combinations.

One great challenge in prescribing drugs is patient compliance. It is frustrating when a patient drug supply is depleted and delays occur in resupply. The opposite problem, the one where a patient doesn't use the drug and discards it, is more important to the environment. The problem is incorrect disposal has several societal consequences as well. If we consider the mass of prescription drugs that are utilized by people which are then excreted un-metabolized and are not removed by waste water treatment plants we have the environmental mass load (ML in kg/yr). The ML for metformin HCL, a drug used to treat people in the process of becoming type 2 diabetics, exceeds a ML of one million kg/yr. (1000 metric tons). The United States is experiencing an unprecedented growth in type 2 diabetes so this environmental contaminant will only grow in a non-linear manner in the future.

Local input of pharmaceuticals into the environment can be high in areas adjacent to pharmaceutical manufacturing sites and hospitals. Concerns over these regional release sites led to emergence of ecopharmacovigelence, environmental pharmacology, and ecopharmacology all with a goal of establishing environmental risk assessment (ERA) of pharmaceuticals. Forward thinking pharmaceutical companies have implemented environmental risk management plans (ERMP) to show good stewardship of their products and a learning process to mitigate environmental impact of future products.

Antibacterials

Nearly 3.3 million kg of antibiotics were sold for human use and 13.6 million kg sold for animal use in the United States in 2011. In 2013, China consumed nearly 162 million kg of antibiotics. While the European Union has forbidden antibiotics to promote animal growth, eight million kg of antibiotics were used in animals in 2012. Projections of antibiotic use in Brazil, Russia, India, China and South Africa will increase by 99% (doubled) from 2010 to 2030 (Wang Xu et al. 2015). Up to 95% of the administered dose of human and veterinary drugs can be excreted unmetabolized into wastewater (Table 7.1) (Milic et al. 2013).

Antibiotics of the families tetracyclines, aminoglycosides, amphenicols, lincosamides, macrolides, oxazolidinones, and streptogramins block mitochondrial peptide synthesis. Tetracyclines bind the A-site of bacterial 30S ribosomal subunit preventing the aminoacyl-tRNA access leading to blockade of protein synthesis. Tetracyclines also inhibit mitochondrial protein synthesis with $IC_{50} = 2.1$ μM. Doxycycline altered mitochondrial function in mammalian cell lines, worms, fruit flies, mice, and in plants. Aminoglycosides induce misreading and premature termination of mRNA translation by binding to the 30S ribosomal subunit. Aminoglycoside side effects include kidney injury, ototoxicity, and vestibular toxicity which are hallmarks of mitochondrial toxicity. Antibacterial drugs activity extends into the environment where activity equals toxicity.

Mitochondria are subcellular organelles that originated from endosymbiotic α-proteobacteria and similarly chloroplasts are subcellular organelles unique to plants that originated from endosymbiotic cyanobacteria-like prokaryotes. Both mitochondria and chloroplasts carry their own circular DNA genomes (mtDNA and cpDNA) that encode a few polypeptides, tRNAs and rRNAs. They are also bacterial-type ribosomes distinct from the 80S ribosomes in the cells cytoplasm; chloroplasts carry 70S ribosomes while mitochondria have 55-60S ribosomes. Mitochondria are essential in their role in oxidative phosphorylation, the conversion of nutrients to the energy currency molecule of life, adenosine triphosphate (ATP). Antibacterial drugs intended to kill bacteria can impair mitochondria inside eukaryotic cells.

Summary. Antibacterial drugs in the environment present bacteria an opportunity for emerging resistance. The antibiotic resistance phenomenon is entering crisis phase and a time when few new antibacterial drugs are being approved. The amount

Table 7.1 Environmental mass loading of selected antibiotics (Data from 2009)

Antibacterial	Antibacterial class	ML (kg/yr)
Amoxicillin HCl	Beta-lactam	8.24×10^5
Cephalexin (Keflex)	Cephalosporin	7.48×10^5
Trimethoprim (Proloprim, Monotrim, Triprim)	Dihydrofolate reductase inhibitor	4.39×10^5
Ciprofloxacin HCl	Quinolone	1.46×10^5
Levofloxin	Quinolone	1.00×10^5
Sulfamethoxazole	Sulfonamide	9.57×10^4

of discarded antibacterial drugs is not known but is significant given the propensity of patients to not complete their course of therapy.

Antivirals

Sofosbuvir was discovered in 2007, approved by the FDA in 2013, and now is sold as Sovaldi for the treatment of hepatitis C virus with cure rates of 30 to 97 percent (Gounder 2013). The chemical structure is a fluorinated uridine nucleic acid analog. The recommended treatment is 400 mg/day for 12 weeks or 33.6 grams per patient (84 days * 0.4 grams/day). The majority is excreted in the urine and feces as a fluorouridine metabolite so that each patient will pass approximately 30- grams in the sewage system. If 130 to 170 million chronically infected people take Sovaldi, then the environmental burden will be at least 3.9 million kilograms. The fluorinated structure of Sovaldi was designed to be stable and the environmental impact is not known.

Environmental risk studies have been conducted on antiviral compounds concluding that they do not pose a threat. Studies conducted on oseltamivir (Tamiflu), used as a prophylaxis and post-exposure treatment for influenza, conclude the drug is unlikely to impact the environment (Hutchinson et al. 2009). The no concern status for environmental impact of antivirals should be accompanied by skeptical vigilance.

Analgesics

Analgesia is the reduction in pain without the loss of consciousness. As the name implies the non-steroidal anti-inflammatory drugs (NSAID) are analgesics lacking a steroid chemical structure and generally do not inhibit the NFkB inflammation pathway. NSAIDs inhibit arachidonic acid cyclooxygenase (COX-1 and COX-2), prostacyclin synthase, or thromboxane synthetase. Ibuprofen is a NSAID that is the third most highly-consumed pharmaceutical in the world detected in the parts per thousand (ppt) range in the environment. Risk assessment studies conclude ibuprofen is a probable environmental risk (Bouissou-Schurtz et al. 2014). Endocrine disruption effects were observed in Australian blue mussel, *Mytilius galloprovincialis,* at 250 ng/L which represents environmentally feasible concentrations. Membrane damage in the digestive glands of mussels was also observed after 7 days of exposure. Concentrations as low as 11.5 μg/L cause changes in skeletal development, respiration and immune function.

Hormones

Birth control pills contain ethinyl estradiol (EE_2), a potent hormone that effectively prevents pregnancy, that is excreted into waterways where harm comes to wildlife (Parry 2012). The global use of birth control pills represents an important problem. Proposed efforts to remove EE_2 from wastewater reveal a significant challenge accompanied by high cost. Upgrading the 1360 wastewater treatment plants across England will cost between $41 and $47 billion. EE_2 influences male fish and frogs interfering with their ability to reproduce. Such males have been observed to produce eggs in their testes. Some believe EE2 is diluted to inconsequential levels in the environment but others point to the fact that fish have more estrogen receptors than humans making them more sensitive to low levels of the hormone (Lund University Press 2016). Credible studies by the US Geological Survey confirm EE2 in drinking water and point to the influence of endocrine disruptors on fish for multiple generations (McGovern 2015).

Antidepressants

Selective serotonin uptake inhibitors (SSRIs) are used to treat depression, generalized anxiety disorder, obsessive compulsive disorder, and eating disorders. The indolamine neurotransmitter serotonin plays a key role in behavior of both vertebrates and invertebrates. SSRI agents include citalopram (Celexa), escitalopram (Lexpro), fluoxetine (Prozac), paroxetine (Paxil), and sertraline (Zoloft). Fluoxetine has been detected in the acquatic environment apparently responsible for subtle effects on predatory behavior (Martin et al. 2016), reproduction (Mennigen et al. 2010) and foraging behavior (Schultz et al. 2011). However, a recent review concludes environmental concentrations to affect wildlife behavior are not achieved (Sumpter et al. 2013).

Prozac emerged as a therapeutic while I was an assistant professor of pharmaoclogy in 1988. The potential for environmental contamination came to my attention when I observed Prozac drug wrappers along the path as I walked to class. The notion that such a specific drug wrapper repeatedly found along the sidewalk was confirmation of widespread use. While Prozac is the third most prescribed SSRI (behind Zoloft and Citalopram), over 24.4 million prescriptions were filled in the United States. Indeed surface water concentrations globally range from 0.012 to 1.4 μg/L (Christensen et al. 2009) and between 13 and 540 ng/L in North American rivers (Brooks et al. 2003). Human effective plasma concentrations range from 50 to 500 μg/L. While Prozac is known to accumulate in brain, liver, and muscle, the impact resulting from chronic environmental low-level exposure remains unknown.

Beta-Blockers

Propranolol (Inderal) is known as a generic beta-blocker referring to the interference with the beta-adrenergic receptor system. James Black was awarded a Nobel Prize in Medicine in 1988 for the discovery of propranolol. The –olol nomenclature is reserved for beta-blocker medications. Listed on the World Health Organizaton's (WHO) essential medicines, it is used to treat high blood pressure and essential tremors. Atenolol is a selective β_1 receptor antagonist that is the most widely used beta-blocker in the United Kingdom and over 33.8 million prescriptions in the United States.

Several beta-blockers are found in wastewater treatment plant effluence including: atenolol 550–980 ng/L, metoprolol 41–69 ng/L, and propranolol 93–388 ng/L (Petrie et al. 2015). A therapeutically effective concentration of propranolol is in the range of 120 μg/L which is on the order of 500 times that of environmental levels.

Hormone–Mimetics

Bicalutamide is a nonsteroidal antiandrogen (NSAA) was discovered based on the antibacterial agent flutamide in the 1980s. The drug is designed to block the androgen receptor, the biological target of testosterone and dihydrotestosterone. Roughly 400,000 prescriptions a year are for treatment of benign prostatic hyperplasia (BPH) and prostate cancer.

Endocrine Disrupting Chemicals (EDC)

Endocrine disrupting chemicals are foreign compounds, xenobiotics, that alter the normal actions of the endocrine system. They interfere with the production, release, transport, metabolism, binding action, or elimination of natural hormones in the body. DES PAHs are EDCs interfering with binding to estrogen receptors, eg. DMBA, 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) found in charred foods. Bisphenol A (BPA) is found in plastics used to make drink containers, epoxy resins that seal metal food cans, dental sealants and paper products. BPA has a 5.3-hour half-life in adults but plasma levels remain steady due to continual exposure. BPA mimics estrogen binding to ERα and ERβ thus exhibits EDC character. TCDD is a product of combustion and found in manufactured chemicals and is an EDC binding to the AhR. Phthalate esters are used as plasticizers added to polymers including polyvinyl chloride (PVC) to increase pliability. Perfluorooctanoic acid (PFOA) is a synthetic surfactant used in production of non-stick and stain-resistant products, flame resistant foams, dental products, and the lining of popcorn bags. PFOA are so abundant they are detected in every American sampled and has

a body half-life of 3.8 years. PFOA are EDCs because they disrupt cholesterol, immune and thyroid function. Phytoestrogens are naturally occurring estrogens produced by plants. Genistein is a phytoestrogen is found in soy products with estrogen-like properties. Resveratrol, a polyphenol, is a phytoestrogen found in grapes, red berries and red wine (1.4 mg/glass). Zearalenone (ZEA) is a mycoestrogen/mycotoxin produced by the mold fungus *Fusarium*. ZEA is found in oat, barley, corn, wheat, and rice producing an estrogen-like response.

Summary. The exposure to biologically active manufactured chemicals in the environment is relentless. The exposure repertoire is vast encompassing molecules capable of disrupting multiple organ systems of the body. Even if you could know details of your personal exposure, the complex actions of near infinite combinations of environmental exposures would be impossible to precisely estimate your personal risk.

A speculative analysis to estimate the consequence of exposure favors molecules that are lipophilic and relatively stable. Exposure leads to distribution to sites in the body containing fat. Body fat is likely to act as a chemical reservoir capable to retaining these compounds for weeks or even years. The brain is an organ that can accumulate lipophilic molecules. Hence, speculation that low-level exposure to thousands of lipophilic environmental molecules will lead to behavioral anomalies.

Perhaps we should consider these exposures as the causative basis for the emerging frequency of autism. What would happen if the outbreak of school shootings is rooted in a contaminated environment? The political impasse in which blame is placed on availability of guns versus those who claim people are to blame can be supplanted by greater emphasis on a clean environment. While most people have developed impressive resilience to chemical exposure, other may have not. This line of reasoning points to finding markers of poor human resilience.

Chemicals in Food

Numerous chemicals are added to our food to preserve from spoiling, improve visual appeal, and to support texture and consistency. We are exposed to these chemicals nearly every day so even mildly toxic agents may be of concern. Indeed food additives are the subject of routine news stories intended to evoke concern for possible adverse influences on health (Wilson and Christensen 2014).

Antimicrobial preservatives prevent bacterial degradation including sorbic acid, sodium sorbate, benzoic acid, hydroxybenzoate, nitrite, lactic acids, sulfites, and propionic acid. Sodium nitrite ($NaNO_2$), is used as a meat preservative. The World Health Agency includes sodium nitrite on the list of essential medicines. The International Agency for Research on Cancer (IARC) report ingested nitrate and nitrite can lead to nitrosation and is probably carcinogenic to humans.

Antioxidants prevent fats from truning rancid when exposed to oxygen including ascorbic acid, butylated hydroxytoluene, butylated hydroxyanisole, gallic acid, and

tocopherols. Propyl gallate (3,4,5-trihydroxybenzoate) is used as an antioxidant in foods containing oils and fats to prevent spoilage. Earlier studies found little or no evidence for carcinogenesis but more recent studies indicate propyl gallate is an estrogen antagonist (Amadasi et al. 2009). Butylated hydroxyanisole (BHA, 2-tert-butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole) is used as an antioxidant in food and some drug formulations. Animals administered high doses of BHA in their diet can develop papillomas and squamous cell carcinomas of the forestomach. The U.S. National Institutes of Health reports BHA as an anticipated human carcinogen. However, analysis of human population statistics show no significant association with an increased risk of cancer. Tert-Butylhydroquione (TBHQ) is used as a preservative for unsaturated vegetable oils. A review of scientific literature determined TBHQ has a wide margin of safety between levels consumed in foods and doses that produce adverse effects in animal studies (Vanesch 1986).

Tartrazine or Yellow dye number 5 (trisodium 1-(4-sulfonatophenylazo)-5-pyrazolone-3-carboxylate) is a common food additive. Tartrazine is associated with food allergy and intolerance reactions in approximately 360,000 people in the U.S (Elhkim et al. 2007). often seen in asthmatics and those with aspirin intolerance. The Center for Science in the Public Interest requested the FDA ban Yellow 5 in 2010 but after a review of data, the FDA did not ban the dye.

Silicon dioxide (SiO_2), silica and calcium silicate, an additive to prevent powders from aggregating. If large amounts are inhaled it can cause irreversible lung damage. Silica crystals in the lung are taken up by macrophages and dendritic cells where it causes production of pro-inflammatory cytokines. Triacetin (1,2,3-triacetylpropanone) is used as a food additive in chewing gum and gummy candy.

Air Pollution

October 27, 1948 Donora, PA experienced smog so dense that high school quarterbacks could not see teammates to complete a pass (Science News 2017). 50 people died following this episode of smog. The Clean Air Act of 1970 has reduced six common air pollutants by 70 percent. However, a 2013 study from MIT estimates 200,000 premature deaths occur each year in the US because of air pollution. The Global Burden of Disease study reports worldwide air pollution impacts up to 3.1 million deaths in the year 2010 (Lim et al. 2012). Air pollution is lumped into: (1) gasses like carbon monoxide, sulfur dioxide, and nitrogen oxides, (2) particulate matter, and (3) ozone (Table 7.2). These pollutants trigger physiological coping mechanisms throughout the body. Long-term air pollution is repeatedly linked to atherosclerosis and most coronary events (Newby et al. 2015). An increase of 10 micrograms of fine particulates per cubic meter of air increases the risk of cardiovascular health by 24 percent and the risk of dying from heart attack or stroke by 76 percent (Samet et al. 2000). Fine particulates are likely to induce inflammation, heart rate variability, and blood vessel damage (Mills et al. 2009). Modest

Table 7.2 Highlights in air pollution

Where	When	Impact	Pollution
Meuse Valley, Belgium	1930	60 people dead	Fluorine
St; Louis, Missouri	Nov. 28, 1939	Unknown	Smoke $PM_{2.5}$; PM_{10}
Los Angeles, California	July 26, 1943	Unknown	Volatile organics
Donora, Pennsylvania	Oct. 27, 1948	50 people dead	Smoke $PM_{2.5}$; PM_{10}
London, England	Dec. 5–9, 1952	12,000 dead	Smoke $PM_{2.5}$; PM_{10} 3000
New York City, New York	Nov. 28, 1953	168 dead	Volatile organics
Bhopal, India	Dec. 2–3, 1984	3787–16,000 dead	Methyl isocyannate
Eastern China	Dec. 2013	Unknown	Smoke $PM_{2.5}$ 500; PM_{10}
Dehli, India	Nov. 12, 2017	Unknown	Smoke $PM_{2.5}$; PM_{10} 1000

particulate concentrations can increase risk of stroke by 34 percent within a day of the exposure (Wellenius et al. 2012).

The Meuse Valley fog of 1930 in Belgium killed 60 people. The older people were vulnerable to the lethal effects of the severe air pollution (Nemery et al. 2001). Fluorine gas from a nearby factory is attributed as the killer (Roholm 1937).

Tuesday, November 28, 1939 is referred to as "the day the sun didn't shine" in St. Louis, Missouri. A temperature inversion trapped emissions from coal burning for 9 days. The problem was recognized as early as 1893 when the City Council passed an ordinance prohibiting thick smoke emissions within St. Louis corporate limits. In 1933 a citizen smoke committee was created to facilitate enforcement to improve air quality.

A smog event on July 26, 1943 in Los Angeles, California was initially thought to be a Japanese chemical warfare attack. Los Angeles had become synonymous with smog during much of the twentieth century. Strict regulations on car emissions has resulted in a decline in volatile organic compounds by a factor of 50 between 1962 and 2012.

London smog of 1952 in December 5 to 9 created by stagnant air, fog, and burning of coal. Estimates were 4000 people died as a direct result of the fog and 100,000 more were made ill. Retrospective studies put deaths at near 12,000 (Bell et al. 2004) and reveal mortality remained elevated for a couple of months after the smog event. The London basin was home to over a million coal stoves. In addition, regional coal powered factories added to the sulfurous smoky fog, smog, that brought the city to a standstill. The PM10 probably reached 3000 μg/m^3 that December. Concerns for air pollution date back to 1306 when Edward I banned coal fires in London.

Air pollution in New York City reached alarming levels in 1953, 1963 and 1966. The event on Thanksgiving holiday of 1966 resulted in 10 percent of the city's population suffering adverse health events and 168 deaths.

The world's worst industrial disaster occurred as a result of a gas leak at the Union Carbide India Limited (UCIL) pesticide plant in Bhopal, India on the night of December 2, 1984. The toxic gas was methyl isocyanate and other chemicals. There were confirmed 3787 immediate deaths, up to 8000 deaths within 2 weeks,

and 8000 deaths since the accident. In addition, 558,125 injuries to people in the area. Union Carbide paid $470 million to settle litigation in 1989. In June of 2010, seven UCIL employees were convicted of negligence leading to death and sentenced to 2 years in prison and a fine of approximately $2000, the maximum punishment allowed by Indian law. Numerous complaints of chemical leaks at the plant were recorded beginning in 1976.

The Eastern China smog in December of 2013 exposed 600 million people to PM2.5 levels of 300–500 μg/m^3. Microorganisms in the PM2.5 included 86.1% bacteria, 13% eukaryotes, 0.8% archaea, and 0.1% viruses (Cao et al. 2014).

Koustav Das 12 Nov 2017 "Delhi's air quality turns deadly, but is it the worst in the world?" The air quality index (AQI) exceed the hazardous PM10 level of 300 to over 900, touching 1000 in some areas. The PM2.5 levels are 30 times greater than the World Health prescribed safe limits. By comparison, two cities in Mexico, Monclova and Piedras Negras reached 869 and 814, respectively. However, the WHO indicates Zabol, Iran as the most polluted city on the planet with PM2.5 of 217 indicating the greater threat from smaller particles.

Dublin Ireland banned all burning of bituminous coal resulting in a 70 percent reduction in black smoke. 10 years later deaths from respiratory and cardiovascular causes were reduced by 15.5 and 10.3 percent, respectively (Clancy et al. 2002). Now evidence reveals air quality contributes to excess body weight- obesogens. Conversely, prenatal traffic-related air pollution is associated with low birth weight (Lakshmanan et al. 2015). The observation now extends to diabetes and air pollution [2010]. Finally, air pollution appears to accelerate brain aging (Babadjouni et al. 2017).

The actual mortality from air pollution is not precise ranging from 6.5 to ten million a year:

1. WHO 25 March 2014 "7 million premature deaths annually linked to air pollution"
2. New York Times 27 June 2016 "Study links 6.5 million deaths each year to air pollution"
3. Lancet recently estimated nine million premature deaths with 2.5 million Indians die prematurely each year due to air pollution and 1.8 million in China (Landrigan et al. 2017).
4. Recent report of ten million deaths this year from air pollution.

Volatile Organic Compounds (VOC)

Volatile organic compounds (VOC) are carbon-containing, organic, molecules that evaporate from their liquid or solid form at ambient temperatures into the surrounding air, a trait called volatility. Human activities produce 142 tetragrams (1.42 x 10^{11} g) of carbon per year as VOCs. This is the amount of matter converted into energy by the Sun in a second or near the mass of the Great Pyramid of Giza. The

VOCs associated with human activities include chlorofluorocarbons and chlorocarbons often used in refrigerants, solvents such as ethyl acetate and acetone used in paints and coatings, fossil fuels often gasoline and the products of gasoline combustion, methylene chloride used in adhesives, and formaldehyde used in building materials.

People inhabiting newly constructed buildings reported a collection of nonspecific symptoms that the World Health Organization called "Sick Building Syndrome" in 1984. The symptoms included respiratory, allergic and immune effects that are often challenging to find the triggering agent. The causation was linked to VOCs released from building materials into the indoor air. The American Society of Heating, Refrigeration and Air Conditioning Engineers (ASHRAE) established ventilation rates for indoor air based on levels of carbon dioxide in 1989.

Biological sources emit 1150 tetragrams (1.15×10^{12} gr) of VOCs, most of which are isoprenes created by plants. Isoprenes are building blocks of terpenes and mycrene of interest as precursors of pheromones and other plant hormones. Naturally occurring isoprenoid compounds include vitamin A, vitamin E, phytol, and carotene.

Summary. Mortality from air pollution is a significant part of modern life. Air pollution has been associated with larger cities for centuries. Crisis events continue in winter months in India and China.

Pesticides in the Environment

Humans have developed an adversarial relationship with the environment exemplified by Rachel Carson's Silent Spring (Carson 1962). Carson pleaded for unity, in what seems to be a "can't we just get along" plea. The premise that we place man as a part of the environment implores that all that needs to be done is to find our natural place in the environment. Indeed, Carson's book served as an anchor to an emerging environmental movement. The Native American Indians exemplified this philosophy in showing respect for the environment and living as a sustainable culture with the environment. Jarad Diamond observed the breakdown in this philosophy in his book, "Collapse" describing deforestation practices prior to the collapse of ancient civilizations. Are humans destined to destroy their own environment?

The industrial revolution introduced air pollution and chemical discharges creating water pollution near the end of the nineteenth century. Britain created the Alkali Acts in 1863 to regulate release of muriatic acid (hydrochloric acid) into the air. The growing demand for agricultural products from India led to the Madras Board of Revenue in 1842 responsible for forest management. "Silent Spring" chronicled indiscriminate use of pesticides, which emboldened the environmental movement to ban DDT in 1972. Hundreds of well-funded foundations and institutes have arisen to set policy to limit organic chemical polluting practices.

The response to "Silent Spring" led to the formation of the Environmental Defense Fund in 1967, which focused on DDT. The Nixon administration created

the Environmental Protection Agency (EPA) in 1970 to relieve the conflict of interest within the USDA, which regulated both pesticides and interests of the agricultural community. The EPA enforced the 1972 Federal Insecticide, Fungicide, and Rodenticide Act in alignment with "Silent Spring." Alternatively, the Competitive Enterprise Institute posed a counter response stating, "Millions of people around the world suffer the painful and often deadly effect of malaria because one person sounded a false alarm." A 2012 Nature review (Dunn 2012) cited 60 to 80 million deaths resulted from a misguided fear based on poorly understood evidence. The conflict is not likely to be resolved in our lifetime.

The world used 2.4 megatonnes ($5.3*10^9$ lb) of pesticides in 2006 and 2007 (herbicides = 50%, insecticides = 17%, and fungicides =10%). There are 1055 active ingredients registered as pesticides used in over 20,000 pesticide products marketed in the United States. While the US used 1 kg per hectare of arable land, Japan used 5.9 kg, China used 4.7 kg, Italy used 2.5 kg, and the United Kingdom used 1.3 kg per hectare. The United States reduced use by nearly half since 1980 as organophosphates are nearly phased out and corn growers have switched over to transgenic Bt corn. The economic benefit of $10 billion in cost of pesticides adds approximately $40 billion in increased agricultural production. An additional offset is the $9.6 billion in health and environmental costs (Pimentel 2005). Based on current practices, the "juice is worth the squeeze" for use of pesticides.

Is the human population a natural adversary of the environment? The global population exceeds 7 billion, the demand for food production and energy is a force that will not change. The response to the environmental movement is that we now have 20,000 pesticide products, many sold in every store with a garden center in the United States. Life expectancy in 1962 was 70.12 years in the United States and during the pesticide onslaught has risen to 78.74 years. Increases in life expectancy are observed in India and Japan rising from 42.42 to 68.35 years and from 68.6 to 83.84 years, respectively, over the same 1962 to 2015 interval. Does this mean humans are winning their battle with the environment?

Consider the poor efficiency of pesticide delivery. 98 percent of sprayed insecticides and 95 percent of herbicides actually reach their target species (Miller 2004). The off-target effects include air, water and soil contamination. Finally, pesticide resistance is emerging in insect and weed species. As expense, eroding efficacy, and risk threaten benefit, the current pesticide practices can be displaced by alternatives including biological pest controls, genetically engineered plants, and genetic modifications of insects. Integrated pest management (IPM) strategies such as "push-pull" techniques are gaining broad acceptance.

Pesticides represent diverse chemical structures with diverse mechanisms of action; each poses a hazard to human health (Table 7.3). Oral, dermal and inhalation routes expose humans. The risk resulting from exposure is not precisely determined as LD_{50} (lethal dose to 50 percent of the population) calculations are an estimate associated with a range of confidence limits. Further, is LD_{50} the appropriate or best measure of human health risk? The spread in confidence limit range is likely a measure of differences in human resilience.

Table 7.3 Brief overview of pesticides

Pesticide class	Use [MOA]	Prototype agent	Year
Ancient	Insecticide	Sulfur	2500 BCE
Ancient	Insecticide	Poisonous plants	2000 BCE (Rig Veda)
Pyrethrum	Insecticide [Na channel in/out]	Chrysanthemum extract	1000 BCE
Pyrethroid esters		Allerthrin	
Metals	Insecticide	Arsenic	~1400
Nicotine sulfate	Insecticide [nAChR agonist]	Tobacco leaf extract	1763
Neonicitinoid		Clothiandin	2003
Rotenone	Rodenticide	Tropical roots	1800
Rotenoids	Insecticides	Crocetonone	
Organophosphate	Insecticide [AChE Inh]	Parathion	1940
Organochlorine	Insecticide [Na Channel]	DDT (Neocid)	1945
		Dieldrin, Aldrin, Kepone	
Carbamate	Insecticide [ChE inh.]	Sevin	1958
Avermectins	Parasites [GABA R ag]	Ivermectin	1975
Nitromethylenes	Insecticide [nAChR ag]	Fipronil	1979
Chlorophenoxy	Herbicides	2,4-D	1947
Bipyridyl-Violagen	Herbicides	Paraquat	1959
Chloroacetanilides	Herbicides	Alachlor (Lasso)	1965
Various	Fungicides	Captan	1940
Various	Fumigant	CS_2, CCl_4, PH_3	
Fluoroacetate	Rodenticides	Compound 1080	

Perhaps the most shocking pesticide development has been the exploitation of nerve agents, the organophosphates, originally developed for warfare are used as pesticides. In retrospect, the use of these agents is capable of flattening the forehead of even an ardent industrialist. How were these agents ever approved for use in the environment? The human health hazard of these agents is recognized and their use is now limited.

Summary. Pesticide exposure is now inevitable and represents a threat to human health. The fulcrum in the risk to benefit use of pesticides moves with demand for food and comfort. Estimating risk from exposure to a single agent is challenging but we are exposed to multiple agents in common use. At present, resources are limited to address the human health risk from exposure to combinations of pesticides. We just don't know- you are on your own.

Chemical Carcinogenesis

The process leading to neoplasia parallels evolution. A neoplasm is a **variant cell** capable of surviving host defenses similar to **natural selection**. The neoplastic cell **reproduces** often with sufficient frequency to produce a tumor. Each daughter neoplastic cell carries sufficient **heritable** information that daughter cells are also neoplastic. Finally, the neoplastic cell is relatively autonomous, with abnormal regulation of gene expression defining their adaption to a course of neoplasia. Given the plausible parallels between carcinogenesis and evolution, the case for chemical carcinogenesis provides the foundation for chemical evolution.

The rich history of chemical carcinogenesis is worth a brief review. Perhaps the earliest report linking chemical exposure to cancer was the 1775 report by Percival Pott. Pott recognized the occurrence of scrotal cancer among English chimney sweeps. He went on to conclude the exposure to the dark soot from the inside of the chimneys accumulates inside the apparel along the groin of the chimney sweeps causes this skin cancer. The soot-cancer concept resurfaced in observations over 100 years later as European chimney sweeps experienced lower incidence of scrotal cancers relative to British "climbing boys" which was associated with British low standards of hygiene. Definitive evidence of the soot/coal tar-skin cancer relationship came in an experimental animal model reported by Yamagawa and Ichikawa in 1915 (Yamagawa 1915). They painted the skin of animals with crude mixtures of coal tar and observed the appearance of skin tumors establishing a cause and effect relationship that was highly reproducible.

The growing sophistication in chemical synthesis and isolation techniques in the 1930s enabled investigators to identify polycyclic aromatic hydrocarbons from coal tar mixtures. The first, dibenz-(*a,h*)anthracene, was isolated and experimental models confirmed the purified chemical was a potent carcinogen. Numerous polycyclic aromatic hydrocarbons cause skin tumors when topically applied including but not limited to benzo (*a*)pyrene, chrysene, perylene, benzo (*e*) pyrene, dibenz (*a,c*) anthracene, and 3-methylcholanthrene. The concept of chemical carcinogenesis expanded in 1935 (Sasaki and Yoshidda 1935) to include consumption of an azo dye, 3-dimethyl-4-aminoazobenzene, and leading to liver tumors. The dietary route produced tumors that were not the first site of chemical contact. Once again, an expanding list of azo dyes produced tumors upon consumption and the organ site of tumors expanded. The compound 2-acetylaminoflluorene produced neoplasia in the mammary gland, ear duct and liver of rats (Miller et al. 1949) and bladder of mice (Miller et al. 1964). Chemical carcinogenesis is an established area of investigation expanding a matrix of chemical structures, routes of exposure; multiple species influenced, and dose dependent exposure.

Industry relies on a growing repertoire of chemicals ushering in the age of occupational exposure. Ethyl carbamate became popular as a cosolvent in manufacturing procedures from 1950 to 1975 at which time it was found to be carcinogenic in many tissues of the mouse. The nitrosamine compounds such as dimethylnitrosamine and NNK produced from nicotine in tobacco smoke are extremely potent

carcinogens. Surveillance of compounds in the workplace is essential with the Manufacturers Safety Data Sheet (MSDS) functioning as the foundation information at the worksite.

Environmental exposure to chemical carcinogens brings us back to the putative role of chemicals in evolution. Aflatoxin B_1 (AFB_1) produced by the mold *Aspergillus flavis* is a potent hepatocarcinogen. AFB_1 is found in numerous farm products such as grain and peanuts that have been stored in warm humid locations favorable to mold growth. AFB_1 and related compounds cause toxic hepatitis and neoplasia in parts of Africa and Asia.

Example benzo[*a*]pyrene:

Hypothesis: *Exposure to chemicals that have been in the environment as long as fire can cause human cancer so the same chemicals can facilitate evolution.*

My intent has been to avoid chemical structures in this book but Fig. 7.1 is included in order to sow the spectrum of structures derived from metabolism of benzo [a]yrene. Benzo [a] pyrene (BaP; $C_{20}H_{12}$) is a ubiquitous environmental pollutant that is produced by combustion of organinc compounds (e.g., cigarette smoke, diesel and automobile exhaust, urban dust, coal tar and atmospheric depositions). BaP has recently been classified as a class 1 carcinogen by the International Agency for Research on Cancer (IARC). BaP is a yellow powder that is relatively insoluble in water but soluble in ethanol.

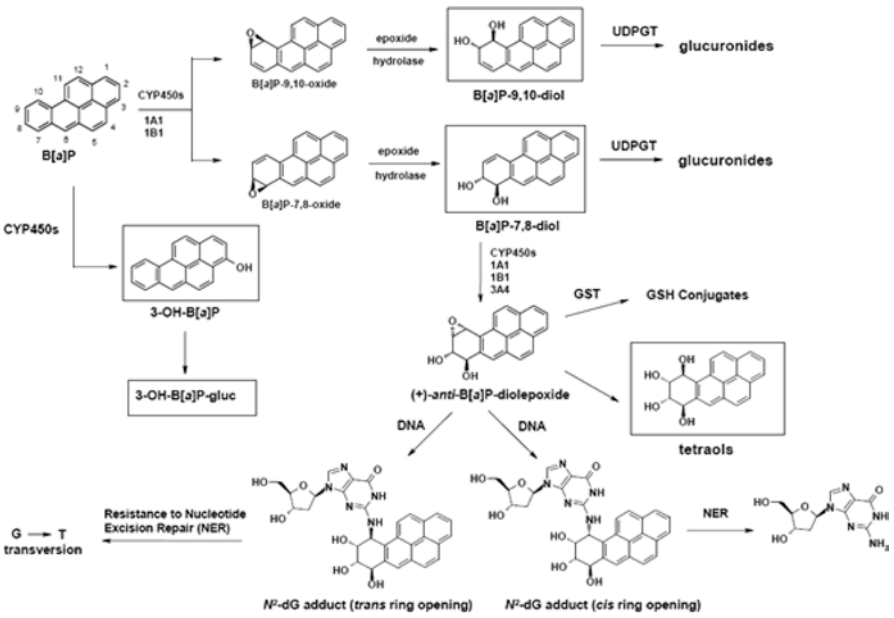

Fig. 7.1 Metabolic activation of benzo[a]pyrene

Humans exposed to PAHs, including BaP from a number of sources, the highest being occupational and smoking. Further, the examination of multiple routes of exposure to BaP in the general population has shown the great majority of exposure is through diet (9.5–43.5 inhalation; 1 water; 160–1600 diet; ng/day). The FDA action level is 35 ng/g for finfish and Health Canada action levels for olive oil is 3 ng/g.

BaP is one of the most extensively studies PAH environmental contaminants. BaP is a skin carcinogen in the rodent 2-stage model involving dermal application and promotion by TPA. In addition to dermal exposures, BaP has been documented as an animal carcinogen following oral or inhalation exposures. Target tissues include liver, forestomach, esophagus, auditory canal and oral cavity. Occupational exposures in humans are associated with increased incidences of cancers of the lung, skin and bladder.

Animal studies in mouse and rat confirm the carcinogenic potential of BaP when administered by the oral route, a mimic of human exposure. Dose dependent appearance of tumors can be observed in the forestomach, exophagus, tongue and larynx in a somewhat reliable manner.

The most well accepted mechanism for BaP carcinogenesis involves metabolic activation. Bioactivation to the ultimate carcinogen is initiated by epoxygenation at the 7,8-position. The epoxide is hydrolyzed by the action of the enzyme epoxide hydrolase and a second epoxygenation produces the ultimate mutagenic and carcinogenic metabolite, the BaP-7,8-dihydrodiol-9,10-epoxide. The (+)-anti-BaP-7,8-dihydrodiol-9,10-epoxide metabolite is the most mutagenic and carcinogenic form of BaP. The metabolite is formed by oxidative metabolism by CYP enzymes. The epoxide is chemically unstable and has been shown to react with the O^6 position in guanine in DNA, which can lead to mutations in genome sequence.

Cytochromes P450 (CYPs), predominantly CYP1A1 and CYP1B1, epoxygenate BaP at multiple sites. Bioactivation to the ultimate carcinogen is initiated by epoxygenation at the 7,8-position. The epoxide is hydrolyzed by the action of the enzyme epoxide hydrolase and a second epoxygenation produces the ultimate mutagenic and carcinogenic metabolite, the BaP-7,8-dihydrodiol-9,10-epoxide. CYPs can produce two stereoisomers when forming an epoxide and these produce either (R,S) or (S,S) 7,8-dihydrodiols (epoxide hydrolase always produces trans-dihydrodiols from epoxides. Upon epoxygenation a second time at the 9,10-position, again two possible epoxides are formed from each of the two dihydrodiols. Thus, a mixture of four enantiomers of the ultimate carcinogenic metabolite are produced. Studies have shown that the (+)-anti-BaP-7,8-dihydrodiol-9,10-epoxide is the most mutagenic and carcinogenic.

Other CYP-dependent BaP metabolites include phenols other than at the 3-position (1-OH, 6-OH, 7-OH and 9-OH). The BaP phenols and dihydrodiols can be conjugated with glucuronic acid by UDP-glucuronosyl transferases (UGTs) or with sulfate by sulfotransferases (SULTs) and the epoxides (with the exception of the 7,8-dihydrodiol-9,10-epoxide) with glutathione by glutathione-S-transferases (GSTs). These reactions represent detoxication. A number of peroxidases, including COX-2 can catalyze 1-electron co-oxidation of BaP to produce the 1,6; 3,6 or

6,12-quinones. If not detoxicated by a 2-electron reduction by NAD(P)H-quinone oxido-reductase (NQO), these quinones will redox cycle in the presence of oxygen generating reactive oxygen species (ROS, superoxide anion radical, hydrogen peroxide and hydroxyl radical). This pathway is associated with the oxidative stress or damage associated with BaP toxicity in some tissues. Finally, a third possible metabolic route is catalyzed by aldo-keto reductase (AKR) which converts dihydrodiols to catechols. BaP-dione is electrophilic and can directly bind to DNA. The catechol (or ortho quinone) can also redox cycle generating ROS and contributing to BaP-dependent oxidative damage.

Example Aflatoxins:

Hypothesis: *Exposure can cause cancer in humans so the same mutagenic process can facilitate the evolution of an emerging single-stranded RNA virus.*

Aflatoxins are a group of approximately 20 naturally occurring fungal metabolites produced by *Aspergillis* molds. Earliest appreciation of these compounds is linked to "Turkey X disease," an outbreak in 1960 in which liver necrosis led to the deaths of 100,000 turkeys in England and the USA. Moldy peanut meal used to feed the turkeys were the toxins identified and isolated. Environmental abundance varies with geographic location and agricultural practices. Growth of molds on food is the greatest concern to humans and is associated with pre-harvest, transportation, storage, and processing. Aflatoxin contaminated foods are most often associated with tropical and subtropical regions where warm temperatures and humidity support optimal mold growth. Highest concentrations of aflatoxins are found on cereal grains and nuts in Africa and rice in China and Southeast Asia.

The *Aspegillus flavus* and *Aspergillus parasiticus* are primarily responsible for the difurocoumarocyclopentens, AFB_1, AFB_2, AFM_1, AFM_2, AFM_{2A}, and AFL, while *Aspergillus arachidocola* makes the difurocoumarolactones, AFG_1, AFG_2, $AFGM_2$, and AFB_3. The designation of "B" and "G" in the name refers to the blue and green fluorescent colors produced under UV light for the respective compounds. The "M" refers to a metabolic product, initially found in milk from lactating animals fed moldy grains. AFB_1 is the most toxic aflatoxin classified as a class 1 carcinogen by the World Health Organization (WHO) due to the epidemiological associations with liver cancer and hepatobiliary carcinoma. AFB_1 causes death in children associated with malnutrition, kwashiorkor, and marasmus.

AFB_1 is metabolized by cytochrome P450 enzymes to the reactive metabolite, aflatoxin-8,9-epoxide (AFBO), that then forms covalent bonds with DNA and albumin. The epoxide is a reactive oxygen species forming DNA adducts similar to the 9,10 epoxide of benzo[a]yrene-7,8-diol, *eg.* N^7-guanine adducts. CYP3A4 and CYP1A1 are human enzymes converting AFB_1 to *exo*-8,9-epoxides. CYP3A5 is particularly active in African populations and creates both *exo*-eoxide and AFQ1 metabolites. AFBO is detoxified in the body by either microsomal epoxide hydrolase (mEH) catalyzing hydrolysis to the dihydrodiol and conjugation to glutathione by glutathione S-transferase (GST).

AFB1 is a genotoxic hepatocarcinogen arising from DNA adduct formation leading to DNA strand breaks, DNA base damage, and oxidative damage. The alterations in the DNA structure result in errors during replication in which an incorrect base is inserted in newly synthesized DNA. The epoxides form N^7, N^2 and N^6 guanine adducts that cause transversions, G to A mutations, including those at codon 249 in *p53* that result in substitution of arginine with serine associated with 50 percent of all hepatocellular carcinomas (HCC). The combined actions of detoxication and DNA repair mitigate the mutagenesis from AFB1 exposure but those DNA adducts that persist enhance the risk of HCC.

The exposure to AFB1 in individuals infected with Hepatitis C Virus (HCV; Chen et al. 2007) or Hepatitis B Virus (HBV; Yu et al. 1997) enhance the incidence of HCC greater than the additive effect of AFB1 plus HCV or AFB1 plus HBV. The infectious and AFB1 co-carcinogenesis synergy has been reproduced in animal models (Jeannot et al. 2012). Studies have focused on the role of inflammation, lipid peroxidation, and unfolded protein response (UPR) as the mechanism for the synergy. No studies have investigated the potential for AFB1 induced mutations in the HBV DNA or HCV RNA genomes in infected individuals.

Emerging Role of Ribonucleic Acid Damage

Exposure to reactive chemicals can result in adducts to DNA, RNA and protein, which poses a significant health threat to humans and all living creatures in our environment. While significant effort has been invested into understanding the biological impact of DNA and protein adducts, a more sophisticated understanding of RNA damage remains essential. Although DNA damage may produce nearly permanent, deleterious consequences for an organism, multiple DNA repair systems exist to effectively reduce the damage burden. In contrast, no analogous repair systems are known to exist for RNA, which is more abundant than DNA and more susceptible to damage due to a broader subcellular distribution. Observations linking RNA damage to inflammation, cancer, neurodegenerative disorders and certain autoimmune diseases confirm the significance of studies investigating the molecular consequences of RNA damage.

Polynuclear aromatic hydrocarbons (PAH) are environmental contaminants that cause human cancers, cancer in laboratory animals, and death, mutation and transformation in cultured mammalian cells. Generation of PAH induced damage depends on metabolic activation to reactive intermediates (RI) that form covalent adducts with critical cellular macromolecules. Covalent adducts of metabolites from the PAH benzo[a]pyrene reveal B[a]P-9,10-diol shows a near absolute preference for cellular proteins but derviatives of B[a]P-7,8-diol bind nucleic acids, both DNA and RNA (MacLeod et al. 1980). The optical enantiomers of 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE), are not equally carcinogenic (Buening et al. 1978). The (+)-anti-BPDE is 64 times more carcinogenic than (−)-anti-BPDE in CD-1 mice and 66 times more carcinogenic in sencar mice (Slaga

et al. 1979). However, the skin of Sencar mice exposed to (+) and (−) anti-BPDE resulted in only 3 times more DNA bound from the (+)-anti-BPDE than (−)-anti-BPDE, but no differences were observed in RNA bound BPDE (Pelling et al. 1984). In cultured cells exposed to BPDE the RNA adducts were more abundant than DNA adducts, RNA = 259 pmol/mg and DNA = 225 pmol/mg[5]. Further, the clearance of BPDE adducts on DNA and RNA suggests half-lives of approximately 48 h in contrast to a shorter half-life of 24 h for protein adducts. While data evaluating the environmental toxicity of RNA adducts remains minimal, it is reasonable to conclude that these adducts are formed and that they persist for several days post-exposure, making their unknown biological importance, increasingly relevant.

While only 1.4 percent of the human genome is transcribed for protein synthesis, as much as 80 percent of the genome is transcribed into RNA, focusing new attention to several emergent classes of non-coding RNA. Non-coding RNA can be divided into long non-coding RNA, lncRNA, and small non-coding RNA. The small ncRNA include: (1) miRNAs that are 18–21 nt in length and are involved in regulation of gene expression, (2) siRNA that are 21 nt segments regulate gene expression and transposon activity, (3) rasiRNA are 24–27 nt that help form centromeres and orient heterochromatin, (4) piRNA or piwi-interacting RNA are 26–30 nt molecules that regulate transposon activity and chromatin, (5) snoRNA are 60–300 nt in length that create 2'-O-methyl and pseudo uridylation modifications of other RNAs, and (6) snRNAs that are 100–300 nt which are involved in assembly of the spliceosome and nonsense mediated decay (NMD) of pre-mRNA. lncRNA are a novel class of functional RNAs that impinge on gene regulation involving recruitment of epigenetic modifier proteins, control of mRNA decay and DNA sequestration of transcription factors. The human genome appears to produce over 60,000 lncRNA, the majority expressed at low levels. Early examples of trans-acting lncRNAs are inhibitory polycomb repressive complex (PRC2) and the activating Trithorax/MLL chromatin modifiers HOTAIR and HOTTIP. The lncRNome contains cis-acting enhancer elements (eRNAs) which control the expression of neighboring protein coding genes. A lncRNA mediating alternative splicing via assembly of serine/arginine splicing factors within subnuclear components called speckles, MALAT1, is retained in the nucleus. A lncRNA called TINCR binds to the 3'-UTR and elicits Staufen-mediated decay (SMD). The human genome appears to produce over 60,000 lncRNAs, all of which are subject to altered function via radical oxygen damage or adduct formation.

The residue-by-residue transfer of information from genes encoded in DNA to RNA, which is translated into the amino acid sequences of a protein, is the embodiment of the now outdated "Central Dogma of Molecular Biology." Translation of mRNA can be initiated at alternative start sites leading to protein diversity by creating several different proteins (of different length) from a single transcript. Proteins translated from alternate start-sites tend to vary the subcellular localization as suggested by Blobel in his 1999 Nobel Prize lecture. For example, basic fibroblast growth factor (FGF) is translated from 4-CUG and 1-AUG initiation codons encoding 34, 24, 22.5, 22, and 18 kDa proteins. The larger CUG initiated, FGF proteins contain nuclear localization signals but the AUG-initiated 18 kDa protein, does not,

and is efficiently secreted from the cell in response to autocrine or paracrine signaling of the transmembrane receptor. Therefore, alternative splicing paradigms which place gene transcripts under the control of alternate promoters, or downstream from alternative open reading frames (i.e. uORF) can generate both quantitative and qualitative changes in gene expression that could not be predicted by simply sequencing an individual gene and its flanking promoter region.

The expanding roles for cellular RNA in epigenetic regulation of transcription and cellular regulation of translation make RNA damaging agents an attractive target of environmental toxins including the PAHs. A relatively common RNA adduct is 8-hydroxyguanine (8-OHG) and RNA is more vulnerable to oxidative damage than DNA. RNA oxidation has been linked to: (i) Alzheimer's disease due in part to accumulation of iron in the brain, (ii) elevated concentrations of 8-hydroxyguanine are observed in the cerebrospinal fluid of patients with Parkinson's disease, and (iii) RNA oxidation is observed in atherosclerotic plaques. We conclude that at least some RNA adducts can be associated with clinical disease.

Telomeres may represent a highly sensitive target for RNA adduct formation in the cell. These repetitive DNA sequences cap the end of chromosomes; they do not encode proteins, and are considered transcriptionally silent. However, transcription of G-rich, long non-coding, telomeric repeat-containing RNA (TERRA; i.e. 5′-$(UUAGGG)_n$-3′) has now been observed. TERRA forms G-quadruplexes (G4) that interact with DNA/RNA polymerases to influence replication and transcription, and G4-induced suppression signature (G4SS) genes are considered potential tumor markers. G4-RNA are found throughout the transcriptome and their functions include: (1) switching translational start-sites (Bonnal et al. 2003) and repression of translation (Beaudoin and Perreault 2010), (2) splice enhancers as seen in Pax9 intron 1 (Ribeiro et al. 2015), TP53 intron 3 (Marcel et al. 2011), alpha/beta tropomycin (Sirand-Pugnet et al. 1995), and thyroid hormone receptor Munroe et al. 2015) as well as splicing silencers as in proinsulin intron 1 (Kralovicova et al. 2014), (3) alternate polyadenylation and mRNA shortening (Beaudoin and Perreault 2013), and (4) mRNA localization. We previously developed a dominating position for the therapeutic telomere mimic oligomer based on a phosphorothioate oligodeoxyribonucleotide, 5′-d(TTAGGG)-3', that inhibits Burkitt's lymphoma cells and tumor growth in a mouse model (Mata et al. 1997). The 5'fluorrescein conjugate of this oligomer became the best-selling product of CalBiochem. Therefore, we speculate that RNA adduct damage to TERRA can potentially alter global gene expression patterns in highly-unpredictable ways.

The G4 RNA sequence, 5′-$(UUAGGG)_4$–3′, is a potent antagonist of TLR9 and can also inhibit NFkB transport to the nucleus. We wish to test the hypothesis that RNA damage from environmental factors can disrupt the G4 structure by forming adducts on guanine resulting in linear RNA structures that activate NFkB and promote cellular replication (Fig. 7.2).

Pathogen-associated molecular patterns (PAMPs) identify pathogen molecules distinctly from host molecules. Among the PAMPS, 13 membrane-bound, Toll-Like Receptors (TLR) have been identified in humans. TLRs generally form homodimers but can form heterodimers (eg. TLR2:TLR1 or TLR2:TLR6) as a result of ligand

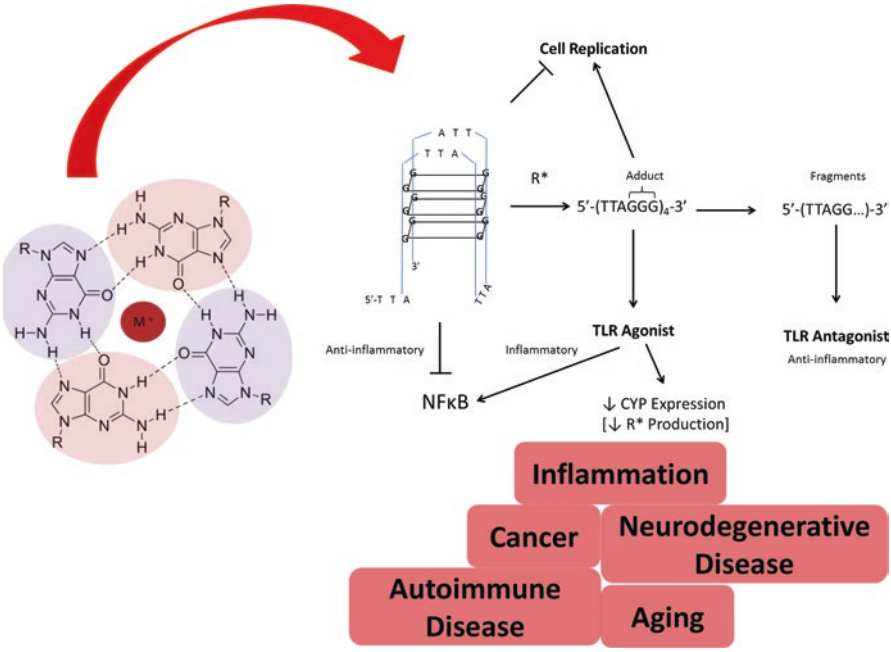

Fig. 7.2 Telomeric RNA forms G-quartets that are recognized by cells

binding. Once a TLR dimer forms, MyD88, an adaptor protein that ultimately promotes NFkB to enter the nucleus. MyD88 recruits IRAK4, IRAK1, and IRAK2 which phosphorylate TRAF6. TRAF6 ubiquinates TAK1 and itself which promotes binding to IKK-β. Once bound TAK1 phosphorylates IKK-β which then phosphorylates Ikβ causing its degradation and allowing its biding partner, NFkB, to diffuse from the cytoplasm to the nucleus. NFkB initiates transcription of a collection of inflammatory cytokines. In addition, TRAF6 activates the mitogen-activated protein kinases (MAP 3Ks).

In addition, the RIG-I (retinoic acid-inducible gene 1; also DDX58)-like receptor family represents a group of soluble PAMPS. The RIG-I (retinoic acid-inducible gene 1; also DDX58)-like receptor family is a DEAD box helicase in superfamily 2 and is conserved from DNA and RNA viruses to E coli to humans. Three members of the RIG-I family include RIG-I, melanoma differentiation-associated gene 5 (MDA5), and Laboratory of Genetics and Physiology 2 (LGP2). RIG-I and MDA5 have CARD domains but LGP2 does not. Unlike TLRs, these RLRs are cytoplasmic proteins detecting RNA in the cytosol. PolyI:C was the prototype ligand but viruses with negative sense genomes do not make dsRNA leading to the discovery that the 5'ppp and 10–18 nt of a panhandle can activate RIG-I. Recognition is short (<4000 nt) 5′ triphosphate uncapped dsRNA or ssRNA. RIG-I (DDX58) and MDA5 activate MAVS and trigger an innate immune response and IFNs. dAdT is a template for RNA pol III generating 5'pppAU-polymers which activate RIG-I. RIG-I is

essential for IFN induction by paramyxoviruses, influenza virus and Japanese encephalitis but MDA5 is essential for picornavirus detection. Viral dsRNAs differentially activate RIG-I and MDA5 based on size, <4 kb for RIG-I and > 7 kb for MDA5. RIG-I binding of 5'ppp-RNA to the C-terminal domain uncovers the CARD domain in the presence of ATP. TRIM25, an E3 ubiquitin ligase, conjugates the lysine 172 in the CARD domain with a lysine 63-linked polyubiquitin chain. This allows the CARD domain of RIG-I to bind the CARD domain of MAVS on the mitochondrion leading to signaling to TRAF6 and TBK 1 and transcriptional activation of IFNs (IRF3/7) and cytokines (NFkB). MAVS can also localize on peroxisomes where interferon-independent action provides short term antiviral activity. Disruption of RIG-I leads to development of progressive myeloproliferative disorders in mice.

Cytosolic DNA induces human cells to synthesize c-GMP-AMP using the cGAS enzyme. This appears to be related to cyclic-di-GMP signaling molecules in bacteria. cGAS is similar in structure to oligoadenylate synthase (OAS1) but links the 2'-OH of GMP to the 5′-phosphate of AMP and the 3'-OH of AMP to the 5′-phosphate of GMP, a 2'3'cGAMP. 2′3'-cGAMP binds STING leading to interferon signaling.

Conclusion

Every breath you take, every move you make, you are exposed to chemicals. The famous quote, "Dose makes the difference between a toxin and a therapeutic" predates the extensive chemical combinations found in our daily environment. Those of us seeking to understand mechanisms of toxicity for chemicals in the exposome are challenged by how little we know about complex exposures to hundreds of different chemicals.

Pharmaceuticals created to act on living systems are particularly potent chemical pollutants. The dialog surrounding lower drug prices and a greater reach for healthcare does not entertain what happens after patients are treated. These drugs face transformation in the environment through exposure to ultraviolet light, oxidation, and metabolism by the Earth's most abundant creatures. Degradation is not necessarily the termination of biological activity, in many cases just reduced potency or a shift in the site of action. However, we have been exposed for multiple generations and our society has stronger athletes, taller children, and we live longer. This should focus our attention to how we become resilient to these chemical exposures.

Gene expression defines our phenotype. While earlier research interest centered on chemically induced damage to our DNA, the interactions with RNA are likely to reveal our resilience. A single oxygen at the 2′-position of RNA defines the chemical difference between DNA and RNA. This means chemicals capable of interacting with DNA are capable of interacting with RNA. In fact, the 2'-OH of RNA adds to sites of chemical interaction. However, the lack of RNA repair mechanisms means RNA retains a "memory" of the chemical assault. This is an excellent characteristic

of RNA in that renewal of RNA ranges from minutes to months. Chemically modified RNA can then signal through recognition receptors to signal exposure information to the transcription apparatus leading to responses in gene expression. Coordination of immune and metabolic responses are among the gene expression events signaled by chemically modified RNA. These adaptive responses represent an early recognition that RNA mediates resilience.

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Chapter 8
Immune Defense

Abstract The world is full of life forms that threaten human health but they meet the most sophisticated immune defense system on Earth. The immune system continues to evolve shaped by changing pathogens. Pathogens counter with their own evolution probing for weak immune defenses. However, the ongoing emergence of new and re-emerging viral threats and drug resistant bacteria, fungi, and parasites mean immune defenses can rapidly adapt. This resilience is reflected in RNA transcripts that are part of the complex immune defense system.

Keywords Restriction endonucleases · Innate immunity · Natural T-killer · Pattern recognition receptors · Adaptive immunity · Autoimmunity · Immunosuppression

Introduction

"The immune system has used a remarkably extensive variety of solutions to meet fundamentally similar requirements for host protection" (Litman et al. 2005). A mechanically created barrier may have driven evolution of the innate immune diversification. A variety of cell surface molecules mediate key defensive metabolic processes. Pathogens have developed multiple ways to change their DNA during invasion of the host including DNA recombination, gene conversion, gene hypermutation, and complex epigenetic events to evade host detection. To counter this, the host has developed a mirror set of genomic changes in a continuous exchange of selective pressures.

All mammals, birds, reptiles, and amphibians show antibody synthesis and delayed allergic reactions evident of lymphoid cells and adaptive response. In contrast, immune responses in lobsters, crabs, shrimp, earthworms and horseshoe crabs do not have an adaptive response. Early responses were agglutins, bactericidins, and suppuration but not one with specificity or memory. Agnathans, elasmobranchii, chondrosteans, holosteans, and teleosts reveal early evidence of lymphoid systems but no gamma globulins. Hagfish possess cellular expansion in response to antigens and macrophage like cells in the pronephros, lamina propria, and the gills. Sea lampreys develop a prothymus in the second to sixth pharyngeal pouch complete with

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a foci of lymphoid cells. They have granulopoiesis, erythropoiesis (in the notochord) and thrombocytopoiesis in a site near the spiral valve. Lampreys have delayed allergic responses, exhibit immunologic memory, and immunoglobulin proteins (Good and Finstad 1968).

Our immune system comes from the same stem cells as blood. Immune cells circulate in blood creating an extensive defensive network within our bodies. If our arteries contained a simple aqueous solution, one liter would carry 3 mL of O_2 and the heart would need to pump 1000 liters per minute to support tissues of the body. Erythrocytes are about one quarter volume of hemoglobin with 163 μm^2 surface area (70% greater than a sphere). One liter of blood contains 150 g of hemoglobin that binds 8.7 nmols of O_2 increasing capacity over 30 times a simple solution. Erythropoiesis is nearly independent of external iron supply because of efficient iron recycling which involves macrophage scavenging of red cells. In this way, iron recycling in the spleen is at the frontier between oxygen transport and an immune system. Macrophages capable of discriminating between old and young RBCs represents primitive expression of self-recognition (De Sousa 1992). The stage is set to explore the immune defense system.

Prokaryotic Defenses: A Primitive Immune System

The Sun's radiation exposes the inhabitants of Earth to damage to the molecules of life. Primitive defense includes DNA repair of ultraviolet (UV) radiation damage, which involves pyrimidine dimers. Clear evidence is provided by bacterial host promotion of survival of infecting UV-irradiated bacteriophages in a process called host cell reactivation (Hanawalt et al. 1979). The reactivation observed was closely associated with loss of dimers from DNA in a process of nucleotide excision repair. Five enzymes *uvrA*, *uvrB, uvrC*, *uvrD*, and *polA* mediate the repair.

DNA damage from UV-light, ionizing radiation, mitomycin, nitrogen mustards, hydrogen/organic peroxides, nucleic acid base analogs, and starvation arrest DNA replication. Recognition of DNA damage is an internal distress signal, "danger", which initiates expression of *recA* and *lexA* genes that suppress the expression of a number of bacterial genes. This response called SOS repair involves a response to inhibition of DNA replication. Conversely, SOS responses can de-repress genes required or bacteriophage production leading to phage induction and cell lysis. A curious version of defense is expression of genes that lead to cell death.

Bacteriophage λ that grow well in one strain of bacteria but not others means the resistant bacteria are restricted hosts. The restricted hosts degrade the bacteriophage DNA after insertion into the bacterial host cell. Such recognition of DNA posits a problem, how can the bacteria discriminate between the infecting foreign DNA and their own DNA? The defense mechanism was found to involve a collection of restriction enzymes that cleave DNA based on the recognition of a specific DNA sequence. More than 3000 restriction enzymes are known so far with over 600 available commercially for use in genetic engineering. The enzymes are named for their

bacterial origin, for example, *EcoR1* comes from *Escherichia coli*. The enzymes often cleave palindromic DNA sequences (read the same sequence from the reverse strand), for example, EcoR1 cuts 5'-GAATTC-3′ leaving a "sticky end" by cutting after the G leaving a single stranded 3′-TTAAG-5′ sequence at the cleaved fragment end. The host bacteria can protect their own genomic sequences from restriction enzyme cleavage by modifying their own DNA through DNA methyltransferases (Arber and Linn 1969). The paired elements of nonself-recognition and development of self-recognition through DNA modification is a potent lesson for host defenses in more complex organisms.

Recent excitement in molecular biology has been exploiting another bacterial host defense mechanism, clustered regulatory interspersed palindromic repeats (CRISPR). This prokaryotic immune system provides a version of acquired/adaptive immunity to bacteria. When a virus infects a microbe, the DNA is captured and inserted into a CRISPR locus as a spacer DNA segment. Hence, DNA spacers follow CRISPR palindromic sequences from previous exposures to foreign DNA from viruses and plasmids. The clusters of palindromic repeats called the CRISPR-associated system (cas) direct a nuclease to regions of spacers that guide the nuclease to degrade corresponding sequences of the invading foreign DNA. A Cas9/CRISPR system now dominates strategies for genome editing and recent RNA versions for RNA editing.

Prokaryotic host defenses include a mirror set of genomic changes in a continuous exchange linked to selective pressures of environmental threats. Even this primitive defense system utilizes recognition of non-self, genome modifications to define self, and both innate and adaptive components. Further, these defense strategies are integrated linking metabolic changes to recognition and the ultimate fight of flight response involving programmed cell death.

Innate Immune System

Multicellular organisms view microorganisms as a threat. "The immune system evolved under selective pressure imposed by infectious microorganisms." (Medzhitov and Janeway 1997) The innate immune system is phylogenetically ancient and is characterized by germline-encoded receptors that recognize pathogens. In contrast, the adaptive immune system is based on receptors that are generated during ontogeny of each organism. Innate recognition involves pathogen associated molecular patterns (PAMPS) but not particular structures. The germline encoded receptors responsible for binding PAMPS are pattern recognition receptors (PRRs), a small number of genes capable of binding a wide variety of molecular structures associated with pathogens. The ancient response pathway responds linking Toll receptor to activation of NFκB which initiates transcription of antimicrobial peptides and in mammals' transcription of B7, IL-1, IL-6, and IL-8. Activation of T lymphocytes requires a second costimulatory signal, B7, while B cells require help from activated CD4 T cells.

All multicellular organisms express host defense genes but only vertebrates have developed the adaptive immune system. Invertebrates developed innate, non-clonal, immune responses but not adaptive, clonal, responses, which is associated with r-selection. This type of evolutionary selection is associated with relatively short lifespan (<1 yr), very large number of offspring, rapid development, small body size, and a single reproduction cycle. There is little need for memory in an organism that lives a single season. The innate response is competent and requires less energy, which is well suited for rapid development in a small body size. In contrast, the longer life span and repeated reproductions of vertebrates benefit from long-lasting immunological memory and efficient allocation of clonal expansion for a given environment.

The human natural killer T-cells (NK) of the innate immune system play an analogous function to cytotoxic T cells of the adaptive system. NK cells express HLA-I killer cell immunoglobin-like receptors (KIR). Antigen presenting cells (APC) present a foreign antigen simultaneously with MHC class I molecules to engage cytotoxic T-cells (Tc). Pathogens evolve to evade adaptive immune responses by interfering with MHC-I expression. CMV and EBV encode genes that interfere with antigen processing and blockade of HLA class I expression. Tumorigenesis often down regulates MHC class I expression. Escape from adaptive immunity can occur but NK cells counteract these evasive maneuvers. KIR diversity defines the NK cell repertoire (Uhrberg 2005). During pregnancy cells of the trophoblast induce KIRDL4, an NK inhibitory receptor, protects the embryo from NK cell-mediated death. NK cells release small granules from their cytoplasm including peforin and granzymes in the proximity of target cells resulting in apoptotic cell death. NK cells also release antimicrobial peptides, α-defensins that directly kill bacteria by disrupting their cell walls in a manner similar to that of neutrophils (a parallel adaptive immune response).

Hemorrhagic Fever Viruses Exploit NK Cells Viral infections often elevate NK cell numbers mediated by release of IFNα and IFNβ by macrophages as they engage and phagocytize the virus. The NK cells attack virally infected cells based on their missing-self surface antigens in the absence of MHC class 1 expression suppressed by viral infection. The activated NK cells release IFNγ inducing inflammation at the site of infection leading to infiltration of the site by immune cells. The activated NK cells also release cytoplasmic granules containing perforin, a cytolytic protein causing formation of transmembrane channels through which calcium enters, serene proteases, and granzymes, agents that induce target cell apoptosis and self-degradation.

Early studies evaluating lethal challenge of cynomolgus macaques with Ebola kikwit (EBOV-kik) revealed a general reduction of CD4+ and CD8+ cells with a 75 percent decrease in NK cells in circulating blood. The NK cell decrease in blood is associated with increased NK cells in the spleen and probably infected organs. This decrease in CD4+, CD8+, and NK cells was also observed in Balb/c mice infected with mouse adapted EBOV. Synthetic peptides containing MARV glycoprotein (GP) immunosuppressive motif (L_{585}INRHAIDFLIARWGGTC) inhibited IL-1 and

decreased NK cell activities. A similar NK suppressing motif is also found in EBOV VP40. Finally, virus like particle (VLP) vaccines significantly protect mice and guinea pigs from lethal challenge from EBOV. The protected mice had high IFN, TNFα, and NK cells. The VLP vaccines were not effective in mice deficient or depleted of NK cells. Leading Jens Kuhn to conclude, "The mechanism of innate protection against ZEBOV was shown to be independent of IFNγ but dependent on perforin." (Kuhn 2008).

CD160 receptor is selectively expressed on the fraction of NK cells with the highest cytotoxic functions (Maiza et al. 1993). CD160 is a glycosylphosphatidylinositol-(GPI) anchored protein with homology to killer-cell immunoglobin receptors. CD160 binds to the herpesvirus entry mediator (HVEM), a TNF member binding MHC class I causing suppressed T cell responses Cai et al. 2008). HVEM inhibits T cell effector responses and the innate response through interactions with the B and T lymphocyte attenuator (BTLA). CD160 regulates cytokine production by NK cells (Tu et al. 2015). Further, CD160 expressed with PD-1 defines a subset of HIV-specific CD8+ T cells with advanced dysfunction associated with down regulation of NF-κB (Peretz et al. 2012a). Six splice variants are known including variants lacking exon 4 and exons 3 and 4. CMV and HIV CD8+ T cell proliferation and cytokine secretion were rescued after blocking the engagement of CD160 with HLA-C receptor (Peretz et al. 2012b). Three CD160 alternatively spliced transcript variants have been identified encoding a CD160 that lacks the Ig domain, CD160ΔIg-GPI, and a variant expressing the transmembrane and intracellular domains but not Ig domain, CD160ΔIg-TM (Giustiniani et al. 2009). These observations suggest PMO targeting splicing will simply shift the relative frequencies of naturally occurring CD160 splice variants.

Premise NK cells attack infected cells and release immune signaling molecules but also can suppress adaptive immune responses. The Jekyll and Hyde character could be due to expression of various splice variants of CD160 induced by the expression of filovirus proteins. A PMO designed to shift the spice variant profile in CD160 associated with NK cells may provide a survival benefit to mice from lethal challenge of EBOV or MARV.

Methods Phosphorodiamidate morpholino oliogmers (PMO) were designed to manipulate the expression of transcripts in mice challenged by lethal exposures to mouse adapted Ebola virus (EBOV). The experimental design involved injection of 50ug/mouse by the intraperitioneal route (ip) route 4 h prior to infection. The mice were challenged with 1000 pfu of mouse adapted Ebola Zaire. Each treatment group involved 10 mice (of mixed gender) and the endpoint was survival to day 14 post infection. The protocol was an established model in which the PMO and PPMO targeting the VP35 viral gene was evaluated (Warfield et al. 2006; Enterlein et al. 2006). All studies were conducted in BSL4 containment at USAMRIID laboratories in Fredrick MD.

Negative controls in which no PMO was administered or untreated controls for these studies involved 22 experiments with 220 mice (10 mice per experiment) with

a total of 40 survivors for an average survival of 18 ± 16 percent. Negative controls also involved PMO targeting unrelated targets including influenza A (19 mice in 2 experiments with 2 survivors for 10.5 percent), arenavirus (10 mice with 3 survivors for 30 percent), human globin (10 mice with 1 survivor for 10 percent), and a scramble sequence (10 mice with 1 survivor for 10 percent). Together the unrelated sequence controls involved 5 experiments with 49 mice with a total of 6 survivors for an average survival of 15 ± 10 percent which is not different from the untreated controls. If we consider 18 percent as a base and two standard deviations (32 percent) will be 50 percent survival as a significant survival threshold to identify a likely benefit from a test sequence.

Positive controls involved administering a PMO targeting VP35 at the same dose and by the same route based on prior studies confirming this sequence is effective. Two experiments with 20 mice led to 17 survivors for 85 percent survival. This would represent a significant survival benefit by targeting a viral target.

Freshly isolated splenic cells were magnetically sorted using the nouse NK Cel Isolation Kit (Miltenyi Biotec). The NK cells were identified with CD8a+, CD4+ and CD49b markers by flow cytometry. Non-sorted splenic cells had 3.24 percent NK cells while enrichment with magnetic beads had 32.5 percent NK cells. These enriched NK cell preparations were seeded into 24-well plates at 5 x 10^5 cells/well and incubated with up to 5 μM PMO for 48 h. Analysis of the NK cells included CD160 mRNA exon skipping using RT-PCR and CD160 protein expression using CD160 antibodies.

Results Mouse survival data are presented in Fig. 8.1. Targeting interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), Granzyme A, and granzyme B at three different sites in each transcript had no significanct influence on survival. This was expected since these proteins have either beneficial or no effect on innate immune protection from EBOV. CD160 transcript was targeted at four sites in the transcript with two sites having survival (SD4 and SA3). These splice switching oligomers create a novel CD160 with poor ligand binding and diminished signal transduction. CD160 signals the B- and T-lymphocyte attenuator (BTLA). The translation inhibitor targeting BTLA also provided significant survival benefit confirming the significance of the CD160/BTLA pathway in filovirus pathogenesis.

The most effective CD160 knockdown in splenic NK cells was from the SD4 PMO with 82 percent reduction following the 5.0 μM PMO incubation. The SA4 and SA3 PMOs reduced CD160 by 32 and 23 percent, respectively. Thee expression data correlate well with mouse survival data as SD4 had 82 percent knockdown is associated with 90 percent survival, SA4 had 32 percent knockdown associated with 50 percent survival, and SA3 had 23 percent knockdown and 60 percent survival.

The mechanism of CD160 knockdown is qualitatively different in skipping exon 3 versus exon 4. An exon 4-skipped transcript was observed with SD4 and SA4 determined by RT PCR of CD160 mRNA. No exon 3-skipped transcript was observed suggesting mRNA degradation by nonsense mediated decay. Hence, targeting of exon 3 is expected to produce loss of CD160 if effective while targeting

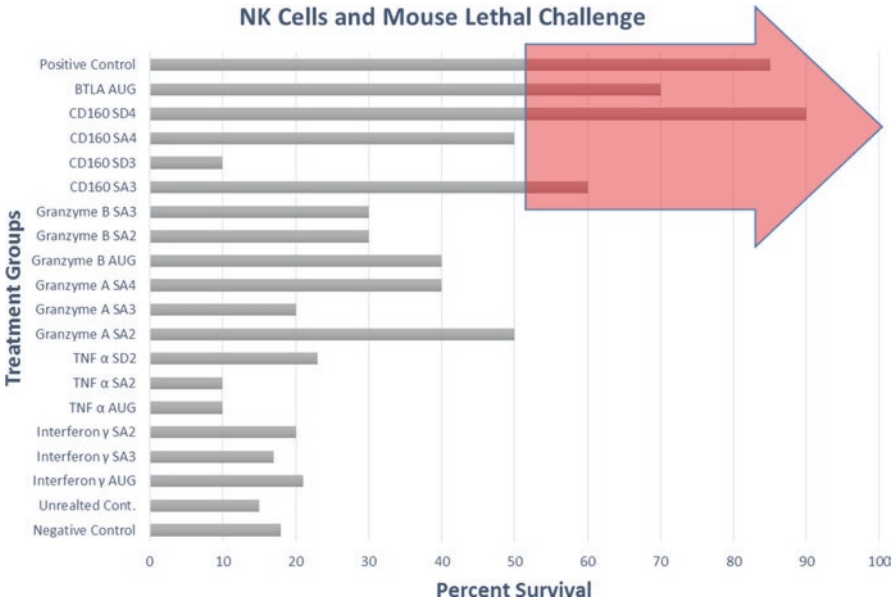

Fig. 8.1 Targeting NK cell functions. Targets covered by the large red arrow indicates highly significant benefit provided by selected NK cell genes

exon 4 produces a truncated CD160 protein that may have either altered or no function.

Conclusions PMO targeting variant expression of CD160 provided a survival benefit in mice challenged with lethal EBOV and MARV infections. BTLA interacts with TNFRSF14 and CD160 negatively regulating T-cell immune responses and inhibiting BTLA expression also provided significant survival benefit. Hemorrhagic fever virus infections induce NK cell trafficking out of blood into the spleen and infected tissues. NK cell activation is expected to release perforin and granzyme so that infected target cell lysis is accompanied by apoptosis. Speculation that the immunosuppressive activities of GP and VP40 may preferentially influence release of granzyme over perforin. The resulting target cell lysis that is not accompanied by apoptosis could benefit the viral infection by facilitating release of virus from infected cells.

The strategy employed in these studies is unique. Drug discovery practices generally seek validation from gene expression or protein interacting studies. This practice would not likely lead to NK cell targets since these cells represent a small fraction of immune cells and the gene expression signal falls below the attention of the unbiased search. The strategy in these studies relied on literature reports indicating the potential role of NK cells and a global immunologic perspective to identify the CD160 gene target. We employed the PMO as a probe to find the NK cells in the body during infection to interrogate function.

Pattern Recognition Receptors (PRRs)

Pathogen-associated molecular patterns (PAMPs) identify pathogen molecules distinctly from host molecules. The Toll Like Receptors (TLR) and interleukin-1 receptors form the IL1 superfamily (Table 8.1). So far 13 TLRs have been identified in humans. TLRs generally form homodimers but can form heterodimers (eg. TLR2:TLR1 or TLR2:TLR6) as a result of ligand binding. Once a TLR dimer forms, MyD88, an adaptor protein that ultimately promotes NFkB to enter the nucleus. MyD88 recruits IRAK4, IRAK1, and IRAK2 which phosphorylate TRAF6. TRAF6 ubiquinates TAK1 and itself, which promotes binding to IKK-β. Once bound TAK1 phosphorylates IKK-β which then phosphorylates Ikβ causing its degradation and allowing its biding partner, NFkB, to diffuse from the cytoplasm to the nucleus. NFkB initiates transcription of a collection of inflammatory cytokines. In addition, TRAF6 activates the mitogen-activated protein kinases (MAP 3Ks).

Nucleic acid-based drug development must be viewed through a lens, which recognizes that humans are well equipped to recognize both foreign and endogenous DNA/RNA in the circulation. This protective recognition arises either as part of an innate, microbial DNA immune response or in response to cellular danger signaled by DNA damage-associated molecular patterns (DAMPs) (Akira et al. 2006; Tang et al. 2012). Now that synthetic analogues of DNA and RNA are being developed as therapeutics for both acute and chronic treatment regimens, it is critical that we

Table 8.1 Toll-like receptors

Receptor	Ligands	Ligand source	Adaptor	Location	Cell Type
TRL1	Triacyl lipopept	Bacterial LPS	MyD88/MAL	Cell surface	Monocytes/ Macs
TRL2	HSP70; Lipoprot	Bacteria/host	MyD88/MAL	Cell surface	Monocytes/ Macs
TRL3	dsRNA, poly IC	Viruses	TRIF	Endosome	DC, B cells
TRL4	LPS, nickel, hsp	Gr(−) bacteria	MyD88/MAL/ TRIF	Cell surface	Mono; Macs
TRL5	Bac flagellin	Bacteria	MyD88	Cell surface	Mono; Macs
TRL6	Diacyl lipopept	Mycoplasma	MyD88/MAL	Cell surface	Mono; Macs
TRL7	ssRNA; imidazoquinone	RNA viruses	MyD88	Endosome	DC, B cells
TRL8	ssRNA	RNA viruses	MyD88	Endosome	DC, B cells
TRL9	CpG DNA	Bact; DNA vir	MyD88	Endosome	DC, B cells
TRL10	Unk				
TRL11	Profilin	Toxoplasma	MyD88	Cell compart	DC, B cells
TRL12	Profilin	Toxoplasma	MyD88	Cell compart	DC, B cells
TRL13	rRNA CGGAAAGACC	Viruses; bacteria	MyD88/TAK-1	Cell compart	DC, B cells

clarify the extent to which these emerging therapeutics result in chronic or inappropriate activation of these nucleic-acid sensors.

Double-stranded RNA polymers designed to activate TLR3 began with the discovery that poly (I:C) induced interferon titers in rabbits (Field et al. 1967). Ampligen incorporated an improved poly(I):poly($C_{12}U$) motif to induce interferon was evaluated in healthy volunteers at up to 600 mg by the intravenous route did not induce measureable levels of IFNα or IFNγ (Hendrix et al. 1993). Treatment of HIV infected individuals with repeated 300 mg doses administered twice weekly with Ampligen did not produce an observable clinical effect (Strayer et al. 1991) ending development for AIDS. Ampligen (Rintatolimod) development shifted to treatment of severe cases of chronic fatigue syndrome. A double-blind, placebo-controlled phase III trial produced objective improvement in exercise tolerance and reduction in related concomitant medicines (Strayer et al. 2012). However, Rintatolimod failed to gain approval by the USFDA.

Early studies in my laboratory focused on oligodeoxyribonucleotides that were designed to inhibit the newly emerging human immunodeficiency virus. Initially, we used nuclease sensitive phosphodiester chemistry conjugated to a lysine polypeptide to enhance cellular uptake (Stevenson and Iversen 1989). A more sophisticated approach used phosphorothioate nuclease resistant oligodeoxyribonucleotides (PSO) targeting HIV-rev (Matsukura et al. 1989). My research efforts shifted toward collaboration and evaluation of the feasibility of this PSO for human therapy (Iversen 1993). Injecting the PSO into mice once a day for 2 weeks led to a surprising ten-fold enlargement of the spleen. Closer inspection of these mice revealed proliferation of blood forming cells, hematopoiesis, in the liver and spleen. The observation was connected to the sequence, 5'-T**CG**T**CG**GTCTCTC**CG**CTTCTTCTTGCC-3′, in activation of the innate immune system through TLR recognition of CpG motifs (bold and underlined).

Phosphorothioate oligomers can be ligands for several toll-like receptors (TLRs) including TLR 3, 7, 8, and 9 (Kandimalla and Agrawal 2012). Paradoxically, synthetic oligonucleotides containing poly-dG sequences may inhibit TLR9 (Ashman et al. 2011). Dozens of human trials have been conducted with immune modulator oligomers (Krieg 2012). These compounds are utilized as vaccine adjuvants and immune therapies for allergy, cancer and infectious diseases including CpG7909, ISS1018, GNKG168, and IMO-2055. In addition, TLR7, 8 and 9 antagonists have progressed to clinical trials which incorporate 7-deaza-dG and the 2'-O-methyl phosphorothioate (Kandimalla et al. 2013).

An off-target effect of synthetic phosphorothioate oligodeoxynucleotides is elicited by the CpG motif (Klinman et al. 1999), which induces rapid and coordinated secretion of interleukin 6 (IL-6), IL-12, interferon γ, by eliciting B, T, and natural killer cells to secrete cytokine. The optimal "alert" sequence motif is GACGTT in rodents and GTCGTT in humans (Hartmann et al. 2000). Further refinement of this paradigm revealed multiple distinct classes of CpG including: (1) CpG-A class with palindromic 5'-RRCGRYCGYY-3′ sequences (where R is purine and Y is pyrimidine) that stimulate plasmacytoid dendritic cells, (2) CpG-B class containing 5'-GTCGTT-3′ sequences that stimulate B-cells, (3) CpG-C class with a

5'-GTCGTT-3′ motif in the 5′-region and a palindromic GC rich region near the 3′-end, and (4) finally the CpG-S class that efficiently block TLR9 dependent activities with 5'-GCGGG-3′ sequence motifs (Vollmer and Krieg 2008). The complexity and variety of sequences capable of eliciting both broad and relatively narrow spectrum activities, with both stimulatory and inhibitory actions, indicate that most, if not all, phosphorothioate oligomers will have some degree of immunomodulatory effect in mammals.

Mipomersen (Kynamro) is 20-base phosphorohtioate is composed of a 5–10-5 motif in which the terminal five linkages are 2'-O-methoxyethyl modified sugars. Mipomersen targets apolipoprotein B (ApoB) mRNA and was developed to treat homozygous familial hypercholesterolemia (HoFH), a lethal genetic condition (FDA Briefing Document, 2012). A subcutaneous dose of 200 mg/week for 12 to 24 months led to a change from baseline in LDL-C of +86.1 to −89.5% in phase 3 trials. The USFDA approved Mipomersen but approval was not granted in Europe (EMEA) because of the high discontinuation rate, 73/261 (28%) discontinued mipomersen vs. 9/130 (6.9%) with placebo. Mipomersen was found to be immunogenic in humans. The sponsor reported 102/142 (71%) of patients were positive for anti-DNA antibodies (ADA) in an open label study. The appearance of antibodies increased from 4% at 13 weeks to 33% by week 50 and ultimately reached 71%. (FDA Briefing Document).

Soluble PRRs The RIG-I (retinoic acid-inducible gene 1; also DDX58)-like receptor family is a DEAD box helicase in superfamily 2 and is conserved from DNA and RNA viruses to E coli to humans. Three members of the RIG-I family (Table 8.2) include RIG-I, melanoma differentiation-associated gene 5 (MDA5), and Laboratory of Genetics and Physiology 2 (LGP2). RIG-I and MDA5 have CARD domains but LGP2 does not. Unlike TLRs, these RLRs are cytoplasmic proteins detecting RNA in the cytosol. PolyI:C was the prototype ligand but viruses with negative sense genomes do not make dsRNA leading to the discovery that the 5'ppp and 10–18 nt of a panhandle can activate RIG-I. Recognition is short (<4000 nt) 5′ triphosphate uncapped dsRNA or ssRNA. RIG-I (DDX58) and MDA5 activate MAVS and trigger an innate immune response and IFNs. dAdT is a template for RNA pol III generating 5'pppAU-polymers which activate RIG-I. RIG-I is essential for IFN induction by paramyxoviruses, influenza virus and Japanese encephalitis but MDA5 is essential for picornavirus detection. Viral dsRNAs differentially activate RIG-I and MDA5 based on size, <4 kb for RIG-I and > 7 kb for MDA5. RIG-I binding of

Table 8.2 Soluble pattern recognition receptors

Receptor	Ligands	Ligand	Adaptor	Location	Cell type
RIG-I	5'pppRNA/DNA <4000 nt	RNA viruses	MAVS-CARD	Cytosol	All cell types
MDA5	5'pppRNA/DNA >7000 nt	RNA viruses	MAVS-CARD	Cytosol	All cell types
LGP2	5'pppRNA/DNA	RNA viruses	No CARD domain	Cytosol	All cell types

5'ppp-RNA to the C-terminal domain uncovers the CARD domain in the presence of ATP. TRIM25, an E3 ubiquitin ligase, conjugates the lysine 172 in the CARD domain with a lysine 63-linked polyubiquitin chain. This allows the CARD domain of RIG-I to bind the CARD domain of MAVS on the mitochondrion leading to signaling to TRAF6 and TBK 1 and transcriptional activation of IFNs (IRF3/7) and cytokines (NFkB). MAVS can also localize on peroxisomes where interferon-independent action provides short term antiviral activity. Disruption of RIG-I leads to development of progressive myeloproliferative disorders in mice.

PRR and Hemorrhagic Fever Viruses I conducted studies to explore the role of pattern recognition receptors in hemorrhagic fever virus pathogenicity.

The retinoic acid-inducible gene I (RIG-I) is an innate PRR capable of detecting foreign RNA sensing viruses such as influenza A, Sendai virus, and flaviviruses. Ebola VP35 binds blunt ended dsRNA as well as the phosphate backbone competing with RIG-I and prevents RIG-I and MDA-5 responses. VP35 also disrupts the RIG-I pathway by blocking IRF-3 phosphorylation. RIG-I also binds the PKR Activator (PACT) and PACT binds VP35 (Fabozzi et al. 2011) in a way that inhibits interactions with L reducing the efficiency of viral RNA synthesis and viral genome replication (Luthra et al. 2013). While VP35 antagonism of the RIG-I pathway is important, reduced expression of RIG-I may shift PACT binding to VP35 leading to antiviral responses.

The lack of antiviral activity from the designed inhibitors of TLR3, TLR4, and TLR9 is expected so the observations from mouse lethal challenge studies are not surprising (Fig. 8.2). Targeting the TLR signal transduction adaptor, MyD88 is also inactive. The design of splice altering PMOs such as those evaluated targeting RIG-I are active approximately 50 percent of the time so lack of antiviral activity may be due to PMOs that fail to induce exon skipping. The RIG-I translation inhibitor, RIG-I AUG, provided significant benefit with all ten of the challenged mice surviving. This is surprising given the clear role RIG-I plays in mediating innate immune responses to infection. That is why we do the experiments- to find observations like the RIG-I effect. At present, the most likely explanation of the potent antiviral effect is the transient reduction in RIG-I shifts PACT binding to VP35 and inhibition of RNA and viral genome synthesis.

The use of nucleic acid based drugs to probe immune gene functions can lead to confusing results due to PRR recognition of the oligonucleotide chemistry. A structure versus off target recognition study reveals the PMO chemistry is well suited to explore immune functions (Iversen 2016). Nucleic acids can unexpectedly distribute to new subcellular compartments leading to the induction of immune responses. For example, cytosolic DNA induces human cells to synthesize c-GMP-AMP using the cGAS enzyme. This appears to be related to cyclic-di-GMP signaling molecules in bacteria. cGAS is similar in structure to oligoadenylate synthase (OAS1) but links the 2'-OH of GMP to the 5′-phosphate of AMP and the 3'-OH of AMP to the 5′-phosphate of GMP, a 2'3'cGAMP. 2′3'-cGAMP binds STING leading to interferon signaling.

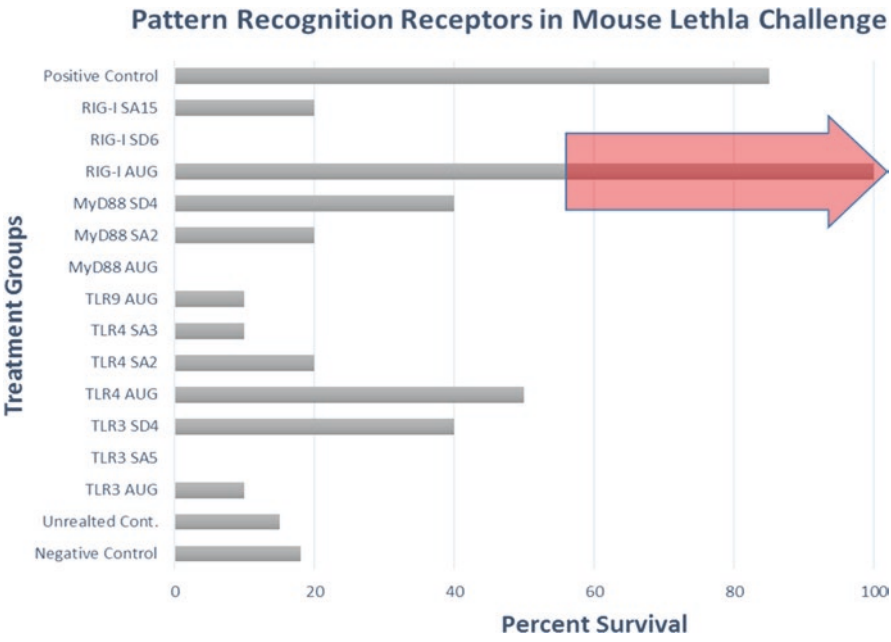

Fig. 8.2 Targeting pattern recognition receptors. Blocking RIG-I provides highly significant survival benefit in mouse lethal challenge

Adaptive Immune System

The repertoire of vertebrate immune system originated from one transposon insertion event, a precursor of RAG-1 and RAG-2 that occurred 450 million years ago (Agrawal et al. 1998). The vertebrate innate and adaptive immunities are functionally intermingled: (1); mannose receptor in antigen processing, (2) Toll receptors link innate and adaptive immunity, (3) regulation of IgM repertoire and formation of B-cell memory by complement, (4) role of stress proteins in both innate and adaptive immunity, (5) self-tolerance, and (6) immune responses in absence of costimulation (Rinkevich 1999).

DNA ligase IV is exclusively nuclear involved in non-homologous end joining (NHEJ). NHEJ is the main repair of DNA double strand breaks caused by ionizing radiation and some chemical mutagens. DNA ligase IV is required for V(D)J recombination in immunoglobin and T-cell receptor rearrangement (Martin and NacNeill 2002). Recombination signal sequences (RSS: 5'-**CAC**AGTG[spacer 12-23 bp] ACAA**AA**ACC-3′) are recognized by recombination activating genes (RAG-1 and RAG-2) that cut and repaired to create diversity in B- and T-cell receptors. The BCR combine heavy (H) and light (L) chains while the TCR combines α and β chains. Diversity is created by variable (V), diversity (D), and joining (J) gene segments in a process called V(D)J recombination (Table 8.3). The BCR H and TCR β chains

consist of V, D, and J segments while BCR L and TCR α chains are made with only V and J segments (Market and Papavasiliou 2003).

RAG−/− mice have neither mature B not T cells and are SCID phenotype. A similar type of immunodeficiency in man is Omenn syndrome. SCID is also seen in DNA-PKcs loss resulting in loss of constant region to J region (CJ) ligation. Other recombination proteins involved in V(D)J joining are Ku70, Ku80, Artemis, XRCC4, and DNA ligase IV. RAG has remnant transposon domains and RSS resemble ends of transposable elements. If SJ DNA is not ligated into a minicircle they can invade the genome and cause chromosomal translocations observed in B- and T-cell cancers.

TCRs and MHC molecules evolved together early in vertebrate lineage which would associate allorecognition and antigen presentation as co-evolutionary events. Rearrangement of subgenic elements composed of two VJ or three VDJ arrays with a constant C domain generates receptor repertoires. Hence, a mammalian immune system requires antigen receptors (immunoglobin and TCR), antigen presentation (MHC), and gene rearranging proteins (RAG-1 and RAG-2). Cartilagenous fish are the most primitive ancestor with these three elements. Further, the shark has three types of MHC loci (I, IIA, and IIB). Which of these molecules evolved first? MHC may have served as peptide transporters in non-TCR receptors- NK cells have MHC class I but no TCR. TCRs may have recognized antigen in the absence of MHC. The challenge is the emergence of RAG genes which are intronless. Hypothesis: RAG genes were involved in retrotransposons and were horizontally transferred from yeast or bacterium (Barti et al. 1994).

The adaptive immune response is antigen-specific relying on recognition of "non-self" antigens to bring a response to action while ignoring "self" antigens. Specialized cells, lymphocytes, are responsible for action as well as inaction. Cells involved in body defense come from pluiripotent stem cells that divide into a myeloid stem cell and a lymphoid stem cell. The myeloid stem cells make neutrophils, monocytes, eosinophils, erythrocytes or red blood cells, megakaryocytes, and mast cells. The lymphoid stem cells make B-cells and T-cells. The B-cells make antibodies and represent the "humoral" immune response often referred to as the

Table 8.3 Diversity of BCR and TCR

	Immunoglobin		TCR	
Element	H	K + λ	β	α
V segments	65	70	52	70
D segments	27	–	2	–
J segments	6	5K 4λ	13	61
V region comb	3.4×10^6	3.4×10^6	5.8×10^6	5.8×10^6
Jct diversity	3×10^7	3×10^7	2×10^{11}	2×10^{11}
Total diversity	10^{14}	10^{14}	10^{18}	10^{18}

Th2 response. Mature B-cells have rearranged immunoglobin heavy and light chains so that they produce antigen specific antibodies.

The T-cell is a multipotent stem cell capable of making CD4+, CD8+, and γδT cells depending on the local environment of the stem cell. Exposure of the undifferentiated T-cell to IL-6 and TGFβ will lead to a Treg17 cell while exposure to IL-21 and IL-23 will lead to a Th17 cell. An antigen presenting cell (APC) will present antigen to undifferentiated T-cells leading to development of CD4+ cells and CD8+ T-cells. The CD4+ cells can then facilitate B-cell maturation or participate in the Th1 of cellular immune response along with the CD8+ cells. Together the collection of different cells can attack foreign antigens with antibodies, cytotoxic cells, and regulators of these responses.

Shifting Th1/Th2 Balance Survivors of EBOV-Zaire (ZEBOV) infection transiently produced increased levels of IL-1β, TNF-α, IL-6, IFN-γ, and MIP-1 α and β, followed by increased plasma levels of IL-6R, TNF-R, and IL-1RA (Baize et al. 2002). However, non-survivors generated high levels of IL-10, TNF-R, and IL-1RA, and moderate amounts of IL-6 and TNF-α prior to succumbing to the disease (Villinger et al. 1999). Inhibition of IL-10 can diminish pathogenesis from bacterial, nematode, fungal, and chronic viral infections (Couper et al. 2008; Ejrnaes et al. 2006). IL-10 can suppress Th1 responses limiting the cytotoxic T cells from attacking infected cells. Conversely, IL-10 shifts the immune response balance toward Th2, humoral immunity, creating a new Th1/Th2 balance. Creation of antiviral antibodies to filoviruses requires nearly 2 weeks (Warren et al. 2016) in a futile effort to provide benefit in an acute viral challenge that is lethal within 2 weeks. Viral infections may have selected induction of IL-10 and suppression of IFNγ to delay immune effector response, which provides sufficient time for viral egress.

Phosphorodiamidate morpholino oligomers (PMO) were designed to manipulate the expression of transcripts in mice challenged by lethal exposures to mouse adapted Ebola virus (EBOV). The experimental design involved injection of 50ug/mouse by the intraperitioneal route (ip) route 4 h prior to infection. The mice were challenged with 1000 pfu of mouse adapted Ebola Zaire. Each treatment group involved 10 mice (of mixed gender) and the endpoint was survival to day 14 post infection. The protocol was an established model in which the PMO and PPMO targeting the VP35 viral gene was evaluated. All studies were conducted in BSL4 containment at USAMRIID laboratories in Fredrick MD.

Examination of IL-10 SA4 reveals exclusion of exon 4 in pre-mRNA processing and reduced IL-10 protein expression in a dose-dependent manner (Fig. 8.3). Further, inhibition of IL-10 enhances cytotoxic T-lymphocytes (CTL) resulting in effective viral clearance. Our studies confirm the function of IL-10 in shifting the balance from cellular immunity to humoral immunity. Viral infections developed the capacity to induce IL-10 to their advantage. Inhibition of IL-10 expression then represents an approach to spare humans from pathologic reaction and improve human survival from infection. The inhibitor of IL-10 can also be used as a vaccine adjuvant to bolster the vaccine CTL response. The resources of the body do not

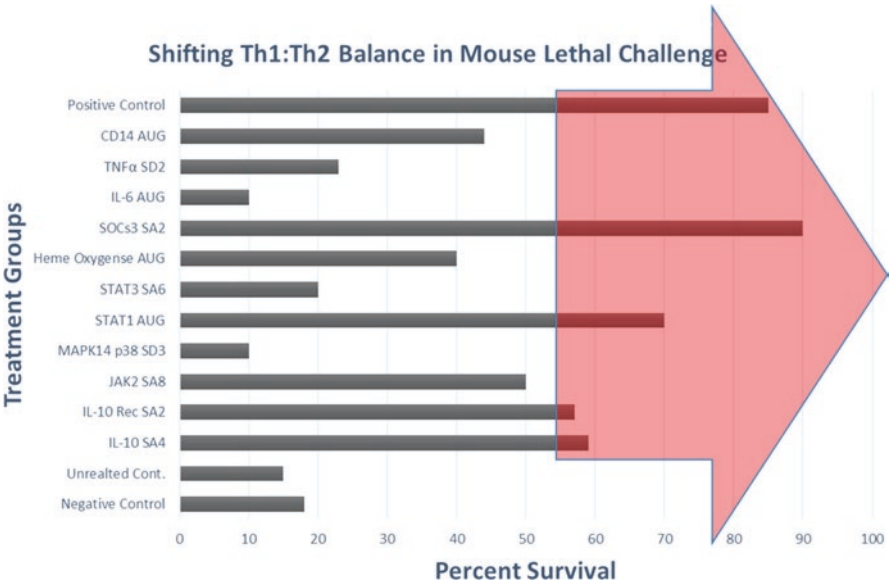

Fig. 8.3 Shifting the Th1:Th2 balance. Targeting disruption of the IL-10 signal transduction pathway reveals multiple effective gene targets

permit unlimited production of defenses so therapeutics that shift allocation of resources support improved defenses.

Manipulating the T-cell Repertoire T cells can produce inappropriate responses as chronically activated T-cells (resulting in autoimmunity) or naive T-cells responding to alloantigens (that is transplantation), or chemical modification of self-antigens (hapten-induced contact sensitivity). A protein is produced by T-cells to prevent or delay programmed cell death, apoptotsis, called the Caspace 8 FADD-like apoptosis regulator (CFLAR). CFLAR is produced when T-cells become activated in response to antigen and binds to caspace 3 preventing the formation of caspace-3 homodimers that initiate the apoptosis process. This activation-induced cell death (AICD) is a natural process that regulates the resolution of T-cell responses limiting the probability of autoimmunity, transplantation rejection, and hapten-induced contact sensitivity.

We developed an inhibitor of CFLAR synthesis (Mourich et al. 2009). In one study, we transplanted male DO.11 splenocytes (cells recovered from the spleen) into female BALB/c mice recipient mice. The male H-Y antigen is recognized as a foreign or alloantigen by the female mouse recipient T-cells resulting in rejection (Beaulieu et al. 1998). The experiment involved transplantation followed by no treatment, inactive inhibitor, or active CFLAR inhibitor then comparing the recipient mice for the number of transplanted T-cells 2 weeks after transplant. Female recipient mice with no treatment or an inactive treatment rejected the transplanted T-cells while those mice treated with CFLAR inhibitor did not reject the trans-

planted T-cells 2 weeks after the transplant. This is evidence that T-cells that were activated by the male H-Y antigen were eliminated in the female recipient mice.

A second study involved sensitizing mice by painting 2 percent solution of oxazolone (4-ethoxymethyene-2-phenyl-2-oxazolin-5) on the abdominal surface of the mouse. The oxazolone forms a chemical bond with proteins in the skin that are presented as antigens to the immune response. This initiates an immune memory response to these modified proteins. Six days later, one ear of the mice was painted with a 1 percent solution of oxazolone resulting in an inflammatory response that provides a model for contact dermatitis. The treated ear swells as T-cells infiltrate as part of the inflammatory response. One day later we measured the thickness of the ear and control mice showed an increase in ear thickness by 0.15 mm while those mice treated with the CFLAR inhibitor one day before the second antigen challenge showed only 0.05 mm increased ear thickness, a significant decrease in the inflammatory response. Re-challenge of the treated mouse ears two and 3 weeks later revealed significantly less inflammation, evidence that the oxazolone antigens have been tolerized. Treatment is long-lived because the immune system is unable to replenish highly avid antigen-specific T-cell clones once the precursor population has been removed from the T-cell repertoire. Once a T-cell is removed from the repertoire that T-cell response is lost. The specific elimination of T-cells producing inappropriate responses, such as responses to self-antigens, transplanted tissues, or allergens, would be a beneficial treatment option for numerous human diseases.

Autoimmunity

Immune responses to self-antigens represents a breakdown in the self-recognition process and often leads to autoimmune disease. Autoimmune diseases can be severe, are difficult to diagnose, and treatment options are limited providing some symptomatic relief. These diseases can be found in about 24 million people in the US or about 7 sercent affecting women more frequently than men. Examles of these diseases include celiac disease, type 1 diabetes (T1D), multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus (SLE). While some autoimmune disease is associated with genetic preference, some infections can precipitate autoimmune disease by molecular mimicry (the infecting agent makes an antigen that looks like a host antigen).

Type 1 Diabetes (T1D). T1D and a variety of other autoimmune diseases are associated with genetic risk. Mapping autoimmune disease to human chromosome 2q33 is associated with three T-cell regulatory genes, CD28, ICOS, and CTLA-4. These same three genes on mouse chromosome 1 are associated with the susceptibility of spontaneous type 1 diabetes in the NOD mouse (Ueda et al. 2003; Araki et al. 2009; Vijayakrishnan et al. 2004). CTLA-4 and its homologue CD28 are expressed by activated CD4+ and CD8+ T cells, both are triggered by B7 molecules

on antigen presenting cells (APCs). CD28 provides a positive co-stimulatory signal while CTLA-4 imparts a negative signal inhibiting cell cycle progression and IL-2 production (Krummel and Allison 1995; Krummel and Allison 1996) through an interaction with STAT5 (Srahna et al. 2006) so that functional activity depends on which signal prevails.

Knockout mice lacking CTLA4 rapidly develop lymphoproliferative disease and autoimmune destruction of tissues. Synthesis of CTLA-4 in which exon 3 has been omitted creates a soluble form of CTLA-4 (sCTLA-4) prevents CTLA-4 co-receptor signaling and is associated with autoimmune disease including T1D (Liu et al. 2003; Simone and Saverino 2009). A different splice variant of CTLA-4 in which exon 2 is excluded creates a ligand independent for of CTLA-4 (liCTLA-4) that signals as a T-cell co-receptor suppressing immune responses including responses to autoimmune antigens.

We created PMO designed to suppress expression of CTLA-4 (AUG), one that would induce skipping of exon 3 to create sCTLA-4 (SA3), and one that would induce skipping of exon 2 to create liCTLA-4 (SA2) (Mourich et al. 2014). Administration of CTLA-4 SA2 led to expression of liCTLA-4 in NOD mice resulting in a significant reduction in the incidence of T1D. Administration of CTLA-4 SA3 led to expression of sCTLA-2 in NOD mice resulted in a significant increase in T1D. The failure to develop T1D in these NOD mice is expected to be a life-long tolerance to autoantigens responsible for precipitation of disease. However, timing of the liCTLA-4 must coincide with the early presence of the autoantigen, once T1D has developed; the liCTLA-4 is not able to cure T1D.

Systemic Lupis Erythemotosis (SLE). Antigen-antibody complexes that are not effectively cleared can be deposited in tissues with small capillaries such as those in the renal glomeruli. These immune complexes can activate inflammatory cells capable of amplification of tissue injury. The deposition of immune complexes in walls of blood vessels leads to complement binding and the genesis of diseases like systemic lupus erythematosus (SLE), an autoimmune disease. SLE is a chronic, remitting and relapsing, multisystem disease that often confounds diagnosis. The incidence is 1 on 700 in women between 20 and 60 years in age but more rare in men with incidence of 1 in 7000 (about 10:1 women to men). While many different autoantibodies are found but the most frequent are antinuclear or anti-DNA/anti-RNA antibodies and autoantibodies to ribonuclear proteins. These antibodies and their antigen-antibody complexes lead to glomerulonephritis, arthritis, and vasculitis, all of which present symptoms shared by multiple diseases.

SLE is linked to ANA but ANA are also seen in the general population. High ANA Aby titers are linked to severe disease. The studies report mice that overexpress TLR7 do not develop spontaneous autoimmunity but when crossed with SLE susceptible mice (Sle1), the offspring develop severe disease. Mice with TLR7 in B cells develop antibodies to RNA/protein complexes and exacerbate SLE disease (Hwang et al. 2012). TLR7 is intracellular signaling following binding ssRNA and plays a pivotal role in autoantibody production. B-cells in the red pulp of the spleen

can produce class-switched IgG and are an important source of anti-RNA autoantibodies (Giltiay et al. 2013). Antibodies to single and double stranded RNA are seen in patients with SLE. An autoantibody to a 59 base sequence of 28S rRNA between nucleotides 1944–2002 was identified. Antibodies to tRNA and a 40 base segment of U1 snRNA are also reported (Chu et al. 1991).

The nucleic acid sensor/recognition system is a complex component of the human immune defense system (Table 8.4). An oligomer, A151, contains the telomere sequence $(TTAGGG)_4$ that is a potent antagonist of TRL9 (Grusel et al. 2003). This is due to novel stacking of G-quartets acting as an aptamer, which may block phosphorylation of STAT1 and STAT4. Self-versus nonself tRNA is determined by TLR7 inspection. This involves a 2'-OMe associated with G at position 18 in the tRNA as self (snoRNA mediated 2′-methylation). The fixed orientation of tRNA interaction with TLR7 suggests inspection (Kaiser et al. 2014). Cytosolic receptors RIG-I, the DExD/H-box helicase superfamily, inflammasome activators AIM2 and NLRP3, cyclic GMP-AMP synthase (cGAS) and STING. The exploration is a work in progress and full integration of recognition to response is not fully appreciated.

A cautionary note. Anti-drug antibodies (ADA) are reported in 71 percent of patients treated with 2′-methoxyethyl phosphorothioate oligonucleotide therapy from the FDA approved Kynamro (Mipomersen), and 29 percent of patients treated with 2'-O-methyl phosphorothioate RNA, Drisapersen that was not approved by the US FDA. Emerging therapeutics that employ CRISR/cas9 appear to evoke adaptive immune responses. Taken as a single drug these adverse events are concerning but in the greater context of antibodies directed at nucleic acids, these adverse events should be considered severe and life threatening.

Table 8.4 Nucleic acid sensors

Sensor	Ligand
TLR3	dsRNA, poly(I:C)
TLR7	ssRNA, R848, imiquimod
TLR8	ssRNA, R848
TLR9	Hypomethylated CpG
RIG-I	dsRNA, ssRNA, poly(I;C)
MDA-5	dsRNA, long poly(I:C)
LGP2	dsRNA, ssRNA
AIM-2	dsDNA
RNA-Pol III	dsDNA (AT-rich)
HIN-200	dsDNA
DAI/ZBP1	dsDNA

Immunosuppression

Reduction in the functioning of the immune system is a breakdown in a host defense as well as a public health threat (Table 8.5). Malnutrition, aging, chronic infection (such as HIV), certain cancers and some genetic disorders (including ataxia

Table 8.5 Agents known to cause immune suppression

Agent	Mechanisms of action	Occurrence
Corticosteroids	↓IL-1, IL-6, and IL-2 production	Stress, Anti-Inflammatory
Estrogens	↓CMI, DTH	DES, birth control
Azathiopurine	Inhibit purine biosynthesis, ↓proliferation	Tx-Histiocytic lymphomas
Methotrexate	Inhibit purine biosynthesis, ↓proliferation	Autoimmune disease, cancer
AIDS Therapeutics	Myelotoxicity (variable)	AZT, ddC, d4T, ddI
Cyclophosphamide	Cross-link DNA ↓proliferation ↑cell death	Autoimmune disease
Cyclosporin	Inhibit calcinurin; ↓IL-2, IFN-γ	Transplant rejection, GVHD autoimmune disease
Tacrolimus	Calcinurin inhibitor reducing NF-AT activity; ↓IL-2, IFN-γ	Transplant rejection
Rapamycin		
Polychlorinated Biphenyls (PCBs)	Observe ↓Humoral IgM/IgG and ↓cell mediated immunity (CMI)	Arachlor 1254
Polybrominated Biphenyls (PBBs)	↓ T and B cells	
Polychloro Dibenzodioxin	Severe lymphoid atrophy	TCDD, herbicides 2,4-D and 2,4,5-T
	↓ Adaptive immunity, ↓ CTL function	
Polycyclic aromatic hydrocarbons (PAH)	↓CMI, ↓ Humoral, ↓ resistance to infection	Dimethyl-benzanthracene, benzo [a] pyrene, ubiquitous
Nitrosamines	↓ T_H IgM, IgG; ↑ macrophage activity	Dietary diemthylnitrosamine
Organophosphates	↓ PMN chemotaxis; ↓ T_H immunity; ↑ upper respiratory infections	Malathione, parathion
Organochlorines	↓ T_H IgM, IgG;	DDT
Metals	↑ Sensitivity to infection	Lead (Pb), Arsenic (As), Mercury (Hg), Cadmium (Cd)
Solvents	Lymphocytopenia; ↓CMI	Benzene, Toluene, CCl_4
Mycotoxins	Inhibit protein synthesis	Metabolites of fungi: aflatoxin, ochratoxin, T-2 toxin
Cannabinoids	↓CMI, ↓ Humoral, ↓ CTL and NK activity	Recreational drug
Cocaine	↓CMI, ↓ Humoral	Local anesthetic, drug abuse
Opioids	↑ Sensitivity to infection	Pain medication, drug abuse
Ethanol	↑ Sensitivity to infection	Consumption

telangiectasia) can lead to immunosuppression. Exposure to a broad spectrum of chemicals in the environment are known to suppress the immune system with the most obvious feature the increased sensitivity to infectious disease. Further, a collection of drugs are used to intentionally suppress immune responses including steroids, azathioprine, and cyclosporine for preventing organ transplant rejection. The US population contains approximately ten million immunosuppressed individuals (3.6 percent of the population; Kahn 2008) - 1.3 million with HIV/AIDS, 0.15 million from solid organ transplant, and 0.31 million immune suppressed due to hemodialysis (Kunisaki and Janoff 2009) represent a few examples.

Indications of diminished immunity include (1) perpetual illnesses such as 4 to 6 colds each year, (2) prolonged stress, and (3) low energy/lethargy. Further, those with poor quality drinking water and exposure to insecticides, herbicides, cleaning solvents, and molds are at risk of being immune suppressed. One global consequence of immune suppression is greater sensitivity to infectious disease agents. Animal viruses become zoonotic infecting humans producing devastating diseases including pandemics.

Immune White Space

When planning a defense strategy one is obligated to look for what offensive threats are we missing, the white space. The innate immune system has a limited set of pattern recognition motifs that are generally associated with infecting organisms. The adaptive immune response is highly malleable but the obligation for co-receptors and interaction with antigen presenting cells means very small antigens are not likely to be recognized. Single organic molecules with molecular weight less than about 300 Daltons do not present sufficient information for immune defense recognition. These molecules can be foreign or xenobiotic and outside immune surveillance. Even if recognized, what would a T-cell do with them? Synthesis of a 150-kiloDalton antibody to clear a 300 Dalton chemical is so inefficient the energy lost from synthesis would be detrimental sapping the cell of energy.

A wide variety of naturally occurring mycotoxins and PAHs can suppress immune responses but antibodies to these molecules are not observed naturally. Hormones in the environment can mimic endogenous hormones disrupting host cell homeostasis. The immune white space does not mean our bodies are unable to clear smaller organic molecules. We have a parallel system to recognize and bring about clearance through metabolism, the subject of the next chapter.

The Defenses are Down: Adapt or Accept Pandemic

Nutritionally Immune Suppressed. Food insecurity sufficient to be associated with diminished immune capabilities and thus enhanced susceptibility to infection is found in 11 percent of the worlds population (795 million people). Most famine is located in developing nations with greatest regions in Africa.

Aging Population. The median age of the developed world is rising from 28 in 1950 to 40 in 2010 and expectation of 44 years by 2050. The percentage of the world over 65 years old rose from 5 percent in 1950 to 8 percent by 2015 and expected to reach 16 percent by 2050.

Stress due to Conflict. A record 65.5 million refugees (1 percent of the global population) were displaced from their homes, most due to wars. Conflicts in Africa include Somali, Nigeria, Darfur, Libya, Yemen, Sinai, Sudan, Ethiopia, Democratic Republic of the Congo, Mali, Central African Republic, and Angola.

Lack Access to Potable Water. Over 1 billion people lack access to safe water and 2.5 billion do not have access to adequate water sanitation. Poor hydration suppress the immune response. Africa is a dominant feature on the map of inadequate water for human consumption.

Chronic and Acute Infections. Most notable among the many chronic infections accompanied by immune suppression is HIV with 35 million infected globally. Bacteria produce toxins to reduce immune responses to survive in the host. Seasonal infections like influenza A also suppress the immune response resulting in secondary infections. Finally, parasitic infections like malaria infect significant portions of the global population.

Environmental Immune Suppression. The growing global production of carbon dioxide (CO_2) means increased release of products of combustion, which include polycyclic aromatic hydrocarbons. The increased appetite for chemical fertilizers, herbicides, insecticides, and solvents from manufacturing will lead to greater challenges to immune defenses.

Mycotoxins**.** One mold can produce many different mycotoxins capable of causing disease and suppressing the immune defenses. Mycotoxins include aflatoxins, ochratoxin, citrinin, ergot, patulin, and fusarium all of which contain a collection of specific toxins.

Summary. The infection threshold in an immune suppressed global population is unknown. We know the above threats to immune defenses are growing globally. Given the extensive history of global pandemics and thus evidence infection thresholds breached, the next global pandemic is inevitable. We are the captain of this global Kobiashi Maru and should consider adaptation as the option to cheat defeat.

Conclusion

The immune defense system is composed of a complex interaction of innate and adaptive responses that utilize both cellular and secreted mediators. This powerful resilience system walks a narrow path; too little response could lead to infection while too much response could mean an autoimmune response. Maintaining appropriate responses requires rapid feedback to fine tune the system.

Chemical exposures can cause immune system perturbations; either immune suppression or enhanced immune surveillance. Some people succumb without knowing the genesis of their discomfort. Most of us evade immune problems from daily environmental challenge without knowing we were challenged. This is likely to be coordinated by alterations in the immune system related expressed RNA.

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Chapter 9
Active Oxygen Defenses

Abstract Metabolic defenses against environmental chemical exposures are essential. The cytochrome P450 family of mixed function oxidases play a prominent role transforming lipophilic threats into more water-soluble metabolites that are cleared from the body. The promiscuous heme active site also generates radical oxygen and nitrogen species capable of enhancing defense against infection but also may present a liability in tissue damage. The transcriptional regulation of metabolic enzymes is complex and coordinated with immune responses. In this way, the metabolic defenses parallel immune defenses as both can recognize and respond to a very large array of foreign chemicals and organisms. Metabolism provides resilience through transcriptional plasticity and metabolites drive downstream transcriptional changes essential for coordinated resilience.

Keywords Reactive oxygen species · Cytochrome P450 · Genetic disease linkages · NADPH cytochrome P450 reductase · Transcriptome plasticity · Nuclear receptors

Introduction

Our best guess is that life began 4.28 billion years ago at the edge of a hydrothermal vent not long after oceans formed 4.4 billion years ago. Life was the result of a negotiation between water, a polar liquid, and hydrophobic organic polymers that seek to avoid water. The atmosphere filled with methane, ammonia, and hydrogen was very different from the atmosphere today. The result of the negotiation was a membrane barrier that enclosed a watery space that could isolate an environment different from that outside the membrane. Near infinite numbers of membrane bound vesicles, protocells, probably formed to be distributed widely across our newly assembled Earth, now 4.54 billion years old. Simple building blocks of life including amino acids and nucleic acids likely became concentrated in when vesicles dried in the primitive atmosphere. The conditions led to formation of larger organized molecules including ribonucleic acid (RNA). The RNA molecules can self-assemble into catalytic molecules now called ribozymes. Life from an RNA

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world can be attributed to Nobel Lauriat Walter Gilbert (Gilbert 1986). Thomas Cech shared a Nobel Prize with Sidney Altman for the discovery of ribozymes (Nobel Prize 1989). The details of early life are better left to experts to debate but it is the conflict between polar and non-polar molecules and the proposed role for RNA in resilience that are the subject of this chapter.

Life is a fight against entropy. Like any fight this requires energy and at the dawn of life, protocells decorated their membrane with electron transport systems. Chemical reactions inside the protocell consumed hydrogen by forming bonds with organic acids like conversion of the four-carbon fumarate into the four-carbon succinate. At the same time, the simplest organic acid formic acid degraded to carbon dioxide releasing hydrogen. A gradient of hydrogen developed with low levels inside the protocell and higher concentrations outside creating a membrane potential for hydrogen. The formation of chemical bonds inside the protocell and the hydrogen concentration gradient represent chemiosmotic coupling, an energy source to create order-supporting life. Ultimately, adenosine triphosphate (ATP) became an efficient molecule for energy storage within the protocell. A related NADH/NADPH molecule became the efficient molecule for storage of electrons and reducing potential, LEO GER (less electrons oxidize greater electrons reduce). Optimization of energy production led to "chains" of electron transporting units in an inexhaustible source of reducing power.

Hydrogen sulfide gas, H_2S, was also a very abundant molecule in the environment. Light provided energy to break H-S bonds releasing sulfur and hydrogen. The electron transport chains exploited the source of hydrogen atoms to reduce NAD/NADP to store energy as NADH/NADPH and photosynthesis began. The emergence of cyanobacteria began using H_2O to reduce NADH/NADPH producing oxygen as waste a product about 3.6 billion years ago. Oxygen accumulated in the atmosphere for the next 2 billion years.

Oxygen, a toxic environmental pollutant without parallel, divided the course of life a little over a billion years ago into forms that would exploit oxygen, aerobes, and those that would hide from it, anaerobes. Oxygen was present at about 1 part per million 4 billion years ago. The busy photosynthesizers lead raised atmospheric oxygen to a relatively stable level of 208,500 parts per million in 2 billion years. Sunlight played a key role by providing energetic radiation. Life simply found a photovoltaic mineral to convert energy to a useable form. A series of biosynthetic steps each with a product that would more efficiently convert the energy followed. Additional steps produce greater efficiency than the previous step (Granik 1965). Chlorophyll appeared about 1.1 billion years ago and heme followed dividing life into animals and plants. Plants consume carbon dioxide and water to create sugars and oxygen while animals consume oxygen and sugars to create carbon dioxide and water. A theme develops in which evolution results in conflict resolution and promotes diversity.

Our bodies use oxygen to defend against infection. Molecular oxygen (O_2) is converted to toxic radical forms of oxygen. Multiple forms of radical oxygen species (ROS) include singlet oxygen ($^1O_2$ an excited electron), supeoxide ion (O_2^-), hydroxyl radical ($^\bullet OH$), peroxyl radical ($RO_2^\bullet$), and perhydroxyl radical ($HOO^\bullet$).

The radical forms of oxygen can lead to formation of non-radical oxygen forms that cells use to kill invaders including hypochlorous acid (HOCl also known as chlorox bleach), ozone (O_3), and hydrogen peroxide (H_2O_2). A resting human inhales 1.8 to 2.4 grams of oxygen each minute to drive respiration in which oxygen and sugar are converted to water and carbon dioxide. A variety of enzymes manage oxygen: mitochondrial electron transport and the cytochrome P450 enzymes generate radical oxygen, xanthene oxidase makes hydrogen peroxide, phenol oxidases, NADPH oxidases (NOX) make superoxide ion, and myeloperoxidases (MPO) make hypochlorous acid. The reactive oxygen chemically bonds with nonpolar molecules in a pathogen converting them into polar molecules, often lipid peroxides that destabilize the membrane barriers of the invader. As a host we minimize damage to our self by localizing the site of reactive oxygen production such as within macrophages that engulf infecting organisms and by making radical inactivating mechanisms available to quench the spread of the reactive oxygen.

Reactive nitrogen accompanies oxygen as a defense mechanism. The reactive nitrogen species (RNS) include nitrogen monoxide ($^{\bullet}NO$), nitrogen dioxide ($^{\bullet}NO_2$), nitrosonium cation (NO^+), nitroxyl radical ion (NO^-), peroxynitrite ($ONOO^-$), nitrogen trioxide (N_2O_3), nitryl chloride (NO2Cl), nitrite (NO_2^-), nitrate (NO_3^-), and S-nitrosothiols. ROS and RNS are an excellent defense strategy because the release is instant and the action is very localized causing damage in the proximity of the pathogen. These reactive molecules chemically alter pathogen antigens forming natural adjuvants, create neoantigens that are more efficiently endocytosed, and lead to epitope spreading in chronic and autoimmune disorders.

The ROS/RNS defenses bring risk to benefit ambiguity. The ROS/RNS aggressively kill microbes but also cause collateral damage to host tissues. They signal the immune system that can control infection but also signal infecting pathogens to switch on mechanisms for protection against host defenses. An ongoing point-counterpoint dialog continues in the defense against infectious disease. The balance tips toward benefit with low concentrations of ROS/RNS and localization of their production.

Cytochrome P450

The cytochrome P450 (CYP) catalyze the metabolism of endogenous compounds such as steroids, bile acids, fatty acids, prostaglandins, leukotrienes, and biological amines. They are also responsible metabolism of exogenous compounds including drugs and an extensive list of xenobiotic molecules in the environment, which include chemical carcinogens. CYP enzymes numbering over 21,000 have been identified in all life forms including animals, plants, fungi, bacteria and viruses. These enzymes contain an iron-heme in their active site and use one or more electrons in combination with molecular oxygen (O_2) to oxidize a vast array of substrates. The mixed-function oxidase (MFO) reaction can be described as:

Mixed Function Oxidases

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

Where RH is an organic molecule substrate and molecular oxygen (O_2) is separated so that one atom is inserted into the RH resulting in ROH, and the other atom is reduced to water (H_2O). In addition to hydroxylation reactions, the CYPs catalyze the N-, O- and S- dealkylation of substrates. The CYP enzymes are embedded in the membrane of the endoplasmic reticulum where electrons are transferred from NADPH by either cytochrome P450 reductase or cytochrome b5 or in the mitochondria where electron transfer is facilitated by adrenodoxin reductase and adrenodoxin.

My third and final graduate school research rotation was in the laboratory of Dr. Michael Franklin, which focused on drug metabolism. A more biochemistry focused endeavor. The rotation project involved experimental treatments for epilepsy investigating their impact on drug metabolizing enzymes. The objective being to refine drug discovery for epilepsy by excluding those compounds that induce their own clearance, primarily be inducing cytochrome P450 enzymes. I was fascinated by the process of interpreting how different chemical structures can be optimized for benefit while avoiding adverse effects. Nearly all of the drugs were inactivated due to metabolism by cytochrome P450 (CYP) enzymes. At the time, only a crude understanding of the different CYP enzymes was appreciated and were referred to as a, b and c. The P450a were those CYPs present constitutively, P450b were CYPs induced by phenobarbital, and P450c were CYPs induced by 3 methylcholanthrene (3MC).

My thesis project involved a rapid method for separating CYPs found in the endoplasmic reticulum of a rat by high-pressure liquid chromatography (HPLC). I observed five different forms of CYP by HPLC, which we referred to as handprints (Iversen and Franklin 1985). I explored changes in the handprints with changes in the liver following CYP inducers, liver toxins, and regrowth of liver following partial hepatectomy (Iversen et al. 1985). I was most surprised by suppression of CYP expression when liver cells divide. This was clear evidence that CYP expression is regulated by endogenous mechanisms.

I wanted to gain experience in the emerging area of molecular biology for my postdoctoral fellowship at the Eppley Institute for Cancer Research. Dr. Edward Bresnick had distinguished himself as a pioneer combining studies in drug metabolism with molecular biology. Ed had become the director of the Eppley Institute and managed a large laboratory filled with graduate students, postdoctoral fellows, and technicians. He retained junior faculty and their labs in a highly interactive superlab and I followed the direction of Dr. Ronald Hines as my immediate supervisor and mentor. The project was to find the human P450c gene, clone it and determine its nucleic acid sequence.

Ron had recently cloned the rat P450c gene and we used that clone to probe the human genome for the human ortholog (Hines et al. 1985). The strategy involved

building a library of 20,000 base segments of the human genome in a bacteriophage. We made the mouse probe radioactive by incorporating ^{32}P into the nucleic acid sequence and then hybridizing the probe to tens of thousands of bacterial plaques created by our human genome library. We exposed the probed library to X-ray film to identify a plaque that retained the probe radioactivity. This identified plaque was created by a phage containing a segment of human DNA with sequence similar to the mouse P450c. The next year of my career involved cutting up the human DNA segment into smaller segments, mapping their relationship to each other and cloning the fragments. These fragments were then cut into segments 150 to 250 bases in size that were then cloned into smaller bacteriophage. I then used a technique of dideoxy sequencing to determine the nucleic acid sequence of these short segments. These short sequences were then assembled into larger contiguous sequences based on sequence overlap and association to the clone map. Persistence paid off and we determined the gene sequence of the first human drug-metabolizing enzyme. The emergence of molecular biology led to determination of Cyp1a1 gene structure followed by cloning and determination of the DNA sequence of the human CYP1A1, in 1987 (Iversen` et al. 1987a).

An early hypothesis linked the complexity of CYP gene expression with nuclear hormone receptors and the regulation of CYP gene transcription. Polycyclic aromatic hydrocarbons, 3-methylcholanthrene (3MC) as a representative, induce CYP1A1 through the AhR transcription pathway while phenobarbital (PB) induces CYP2B1 through the CAR transcription pathway. We hypothesized that administration of both 3MC and PB would simply induce their cognate transcription pathways. We observed synergy, the PB plus 3MC treatment enhanced expression of CYP1A1 mRNA by 14 times that of saline and up to four times that from 3MC alone. Cyp1A1/1A2 enzyme activity in saline controls set at 1.0 was lowered by 30 percent in PB treated rats to 0.7. 3MC treatment induced 1A1/1A2 enzyme activity to 5.25 times control but the combination of PB and 3MC induced activity to 8.32 times control, significantly more than 3MC alone (Iversen et al. 1987a, b). These studies revealed synergy between PB and 3MC for induction of Cyp1A1 enzyme activity but also revealed greater induction of Cyp1A1 mRNA transcription than the translated product. This reveals evidence of cross-talk in regulating expression of CYP1A1.

Antisense technology was utilized to separate regulation of transcription from CYP substrate interactions and to link a phenotype with the gene specific interactions with RNA. Metabolism of sedatives and anxiolytic drugs provided the basis for the "sleeping rat model" in which observable extended sleep times were observed with pentobarbital after inhibiting expression of CYP2B1 (Desjardins et al. 1995) and with midazolam after inhibiting expression of CYP3A2 (Desjardins and Iversen 1995). We also targeted expression of CYP1A1 to reduce DNA damage caused by metabolism of polycyclic aromatic hydrocarbons (Baird et al. 2002). We used rodent animal models to explore the feasibility of oral (Arora et al. 2002a) and transdermal (Arora et al. 2002b) delivery of oligonucleotides to inhibit CYP3A2. The success in mouse, rat, dog, and monkey studies targeting the orthologous

Table 9.1 Characteristics of human CYPs

Gene	Chromosome location	Splice variants	SNPs	Knock-out mouse	Prototype substrates	Genetic disease linkages
CYP1 (3)						
1A1	15q22	25	294	No phenotype	PAH's	Δ2E Ovarian Cancer
1A2	15q22	4	340	1a1/1a2/1b1 incomplete	Acetaminophen	
1B1	2	17	418	Embryonic lethal	DMBA	Glaucoma
CYP2 (16)						
2A6	19q13.1–13.2	12	464	9 gene KO 2a/2b/2f/2g/2s/2t	Coumarin 7-OH	Lung Cancer
2A7	19q13.1–13.2	11	493	Viable, fertile, no abnormalities	Tobutamide-OH	
2A13	19q13.2	3	432		Tobacco Nitrosamine	
2B6	19q12–13.2	9	1144		Nicotine	Liver/Colon cancer
2C8	10q24.1–24.3	33	822	$2c44^{-/-}$ K^+ Hypertension		
2C9	10q24.1–24.3	15	1422		Hexobarbital, warfarin	
2C18	10q24.1–24.3	18	1230	Primaquine failure	(S)-Mephenytoin	
2C19	10q24.1–24.3	6	2467	No Phenotype, No MTPT damage	Debrisoquine, Bufuralol	
2D6	22q13.1	40	488	No Phenotype, female hypertension	Propranolol	
2E1	10	21	33		Ethanol, Aniline	Parkinsons Dis
2F1	19	15	531			
2J2	1q32.1	10	747		Epoxidation Arach Acid	
2R1	11p15.2	15	281		Microsomal Vit D 25-OH	
2S1	19q13.2	14	498		Unknown	
2U1	4q25	5	515		Arachidonic Acid	
2W1	7q22.3	5	326		Lipids	
CYP3 (4)				$Cyp3a13^{-/-}$/$Cyp3a57^{-/-}$/ $Cyp3a59^{-/-}$		
3A4	7q22.1	27	716	Viable, fertile, no phenotype	Midazolam, Steroid 6β-OH, nifedipine	
3A5	7q22.1	40	690			
3A7	7q22.1	25	715			

Gene	Chromosome location	Splice variants	SNPs	Knock-out mouse	Prototype substrates	Genetic disease linkages
3A43	7q22.1	28	908			
CYP4 (12)				Cyp4a14$^{-/-}$		
4A11	1p33	22	455	Male hypertension	Lauric acid 12-OH	
4A22	1p33	10	493		Arachidonic Acid	
4B1	1p33	27	694		Fatty Acids	
4F2	19p13.12	12	744			
4F3	19p13.12	4	507		Leukotriene B4	
4F8	19p13.12	20	507	Cyp4f18$^{-/-}$ in neutrophils		Celiac and Crohn's
4F11	19p13.12	6	963			
4F12	19p13.12	19	1346		Epoxidation Fatty Acids	
4F22	19p13.12	3	1146		12(R)-lipoxygenase	
4V2	4q35.1-q35.2	19	779		n-3 polyunsat. fatty acids	
4X1	1p33	8	627			
4Z1	1p33	5	1092			
CYP5A1	7q34	30	5274		Thromboxane A2 synth.	
CYP7A1	8q11–q12	1	288		Bile Acids	
CYP7B1	8q12.3	3	3400		Steroid 7α-OH	Spastic paraplegia
CYP8A1	20q13.13	7	1580		PG-H2 to PG-I2	ΔE2 Hypertension
CYP8B1	3p22.1	2	172		Bile acids	
CYP11A1	15q23–q24	34	630		Steroid Biosynthesis	
CYP11B1	8q21–q22	16	535			Cong. Adr. Hypert.
CYP11B2	8q21–q22	4	470			
CYP17A1	10q24.3	13	179		Steroid 17α-OH	ΔE2 CA Hypert.
CYP19A1	15q21	47	2178	Progressive male infertility	Aromatase, Estrogen	ΔE5 Aromatase Def.

(continued)

Table 9.1 (continued)

Gene	Chromosome location	Splice variants	SNPs	Knock-out mouse	Prototype substrates	Genetic disease linkages
CYP20A1	2q33.2	21	1308			
CYP21A2	6p	30	91		Steroid Biosynthesis	
CYP24A1	20q13.2	17	711		Mitoch Vitamin D deg	ΔE9 Prostate canc.
CYP26A1	10q23.33	10	163		All trans Retinoic Acid	
CYP26B1	2q13.2	9	477	RA patterning defects	Inact. All Trans RA	ΔE2 Oral Cancer
CYP26C1	10q23.33	3	218		9-cis RA	
CYP27A1	2q33	19	730		Vitamin D Activation	ΔE4 CTX
CYP27B1	12q14.1	32	216		1α-OH 25-OH Vit D	ΔE4 Leukemia
CYP27C1	2q14.3	4	638			
CYP39A1	6q12.3	14	2129		7α-OH-24-OH cholesterol	
CYP46A1	14q32.2	11	952		cholesterol 24-OH	Int. 2 Alzheimer's
CYP51A1	7q21.2	18	388		Lanosterol 17α demthylase	Antley-Bixler

CYP3A led to the targeting inhibition of the human CYP3A4 (Arora et al. 2002c) and 5 subsequent phase I human studies.

CYP Diversity. The human genome contains 57 genes that encode proteins from the superfamily cytochrome P450 (CYP) that oxidize endogenous and environmental molecules (Table 9.1). The system effectively acts on molecules that are not recognized by the immune system as a parallel defense against xenobiotics.

CYP Activities

Transcriptional regulation of CYPs involved in metabolism of vitamins mediated by retinoic acid nuclear receptors and vitamin D binds to the vitamin D receptor (VDR) regulating expression of CYP24A1. These activities support the immune response. CYP creation of the biologically active form of vitamin D are expressed in circulating lymphocytes. Vitamin D binds the VDR NHR controlling T-cell antigen receptor signaling and activation (Geisler 2010). Vitamin A binds a collection of nine different NHRs (3-RAR, 3-RXR, and 3-ROR) and is involved in the growth, development, and maintenance of the immune system (Beijer et al. 2013). CYP26A1, 26B1, 26C1, and CYP2S1 are involved in retinoic acid (vitamin A) synthesis and degradation.

More than half (35 of 57 genes) of the cytochromes P450 (CYPs) 1A1, 1A2, 1B1, 2A6, 2A13, 2A7, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2 J2, 2R1, 2S1, 2 U1, 2 W1, 3A4, 3A5, 4A11, 4A22, 4B1, 4F2, 4F3, 4F8, 4F11, 4F12, 4F22, 4 V2, 4X1, 4Z1,, 5A1, and 8A1 play an integral role in metabolism of physiologically important eicsoinoids. Endogenous metabolism of ω-6 fatty acids to arachidonic acid and then on to prostaglandins (PG), prostacyclins (PGI), thromboxanes (TBX), leukotrienes, epoxyeicosatrienoic acids (EETs), hydroxyeicosatetraenoic acids (HETEs), dihydroxyeicosatrienoic acids (DHETEs), dihydroxyeicosatrienoic acids (HPETEs), lipoxins, and hepoxilins results in signal transduction to specific eicosanoid receptors. Endogenous metabolism of ω-3 fatty acids to eiscospentaenoic acid and docosahexaenoic acid and on to resolvins, docosatrienes, neuroprotectins, and eoxins also signal through eicosanoid receptors (Nebert et al. 2013). Leukotrienes increase production of pro-inflammatory cytokines and are chemotactic agents for neutrophils and monocytes. Prostaglandins and prostacyclins promote and facilitate resolution of inflammation. EETs modulate NFκB activity, HETEs are pro-inflammatory, HPETEs inhibit inflammation, and lipoxins inhibit signaling through toll-like receptors and TNF receptors.

CYP51A1 catalyzes the 14α-methylation of lanosterol leading to synthesis of cholesterol. In the skin, UV light converts cholesterol to cholecalciferol, which is metabolized by CYP2R1 to 25(OH)D, CYP27B1 to $1,25(OH)_2D$, and then CYP24A1 to $1,24,25(OH)_3D$ of active vitamin D. Cholesterol is the substrate for CYP11A1 conversion to pregnenolone leading to progesterone that is converted to 11-deoxycorticosterone by CYP21A2 and then on to aldosterone by CYP11B2. Pregnenolone may also be converted to 17-OH-pregnenolone by CYP17A1 and on

to cortisol by activities of CYP21A2 and CYP11B1. 17-OH-pregnenolone can be converted to dihydroepiandosterone (DHEA) by CYP17A1 and Cytochrome b5 and then on to estradiol by CYP19A1. These endogenous steroids include glucocorticoids, mineralocorticoids, androgens, and estrogens all of which signal through GR, MR, AR, and ER NHRs, respectively. These CYP activities are significant regulators of signal transduction of a vast array of genes, most with impact on the immune response.

Transcriptional and translational enhanced expression of CYPs can be induced by foreign compounds through interaction with nuclear horomone receptors (NHR). 3-methylcholanthrene (3MC), is representative of a wide variety of polycyclic aryl hydrocarbons binding to the aromatic hydrocarbon receptor (AHR) leading to induction of CYP1A1, 1A2, and 1B1. Phenobarbital is prototypic of barbituarates that bind the constitutive androgen receptor (CAR) inducing expression of CYP2B1 and CYP3A4. Pregnenolone and 16α-carbonitrile bind the pregnane X receptor (PXR) leading to the induction of CYP3A4. Steroid hormones induce CYP11, 1A2, 1B1, 2D6, and CYP3A4 via interactions with multiple NHR. Ethanol induces CYP2E1. Rifampicin interacts with PXR and CAR to induce CYP3A4/7 and CYP2D6. Chlofibrate is a hypolipidemic agent interacts with the peroxisome-proliferator-activation receptors (PPARs; Nishimaki-mogami et al. 1993) resulting in induction of the CYP2B1 and CYP4A family members. CYP induction is evidence of the role these enzymes play in adaptation to the environment.

CYP2D6 is exceptionally polymorphic with up to 13 copies of the gene, which can lead to an ultra-metabolizer (UM) phenotype (Lundqvist et al. 1999). People with UM phenotype rapidly clear up to 20 percent of all prescription drugs limiting the duration of their benefit and in the case of codeine, rapid conversion to morphine leads to adverse events resulting from elevated levels of morphine (Voronov et al. 2007).

CYP activities can be inhibited by some agents entering the active site. Ethyl isocyanide and carbon monoxide form a complex with the reduced heme-iron in the active site of CYPs, an interaction that produces an absorption peak at 450 nm responsible the the enzyme family name- a pigment body "cytochrome" that absorbs light at 450 nm P450" (Omura and Sato 1962). Metabolic intermediates of compounds metabolized by CYPs can form complexes with the CYP. These compounds were the focus of my thesis mentor, Michael R. Franklin who coined the term "MI complexes." Structurally related substrates were grouped into nonnitrogenous methylenedioxyphenyl compounds in which the insecticide synergy agent piperonyl butoxide is a representative. Nitrogenous compounds forming MI complexes were further subdivided into the amphetamine class including methamphetamine, SKF-525A class, the arylamine class including sulfanilamide and dapsone, and the oxidized alkylamine class (Franklin 1982). The MI forming compounds persist in the active site, which ultimately leads to induction of CYP expression. This general rule of CYP inhibitors points to the endogenous regulatory mechanisms governing CYP expression.

Numerous reactive metabolites are created by CYPs, most escape the active site without forming an MI complex only to form chemical bonds to biologically impor-

tant molecules of the cell. Several families of enzymes serve to inactivate these reactive metabolites through "Phase II" conjugating reactions. Phase II enzyme families include: methyltransferases, acetyltransferase, sulfotransferases, acyltransferases conjugate amino acids to reactive products, glutathione S-transferases, and UDP-glucuronyltransferases. Our body has a nearly unlimited capacity for CYP metabolism of foreign compounds some to reactive metabolites but the phase II capacity is limited. When phase II enzymes are exhausted, toxicity is inevitable. The concept of exhaustion is also observed in the T-cells which can lose function when overwhelmed by infections. Metabolic exhaustion and reactive metabolite toxicity generally leads to apoptosis, programmed cell death. Immune exhaustion often involves T-cells directed to apoptosis by inhibitory receptors, programmed cell death protein (PD1), TIM3, and lymphocyte activation gene 3 protein (LAG3).

CYPs are Redundant and Essential

Mammalian microsomal CYPs function by catalyzing the insertion of one atom of molecular oxygen into a substrate molecule while reducing the other atom to water. The two-electron transfer reaction is associated with two proteins: NADPH-cytochrome P450 oxidoreductase (POR; first electron and second electron) and cytochrome b5 reductase (Cyb5R; second electron). Loss of Cpr (POR) represents an approach to near global loss of CYP activity and results in an embryonic lethal phenotype but conditional organ specific "null" mice are viable and fertile with no anatomical abnormalities (Table 9.2). The Cyb5R knockout is viable, fertile with no anatomical abnormalities but do present a condition resembling autosomal recessive congenital ichthyosis (dry skin) and point to the Cyb5R role in saturated/ unsaturated fatty acid homeostasis. Loss of both POR and Cyb5R results in liver enlargement and enhanced expression of CYP isoforms but are lacking CYP metabolic activity but not enhanced mortality. The number of surviving CYP knockout mice is likely to be due to compensatory activity in organs with overlapping CYP activity.

The fact that CYP17, 26a1, and 51 are embryonic lethal provides evidence that these activities are essential and should reflect evolutionary selection pressure. The knockout mouse provides evidence for severe phenotypes that are unlikely to be observed in humans. The correspondence between some knockout mice and human genetic disease linkage points to many conserved functions between the two species. Evaluation of human genome wide associative studies (GWAS) represent another perspective on the role of CYP activities (Table 9.2). The GWAS identify a variety of cancers, hypertension, Parkinson's disease, and some autoimmune disease associations.

Table 9.2 Loss of CYP metabolism in knockout mice

Genetic loss of enzyme	Phenotype	Reference
$Cpr^{-/-}$	Embryonic lethal	
Cpr-null PT kidney	Fertile, no observed abnormalities	Liu et al. (2013)
$Cyb_5^{-/-}$ (complete null)	Viable, fertile, no anatomical abnormal	McLaughlin et al. (2010)
Cpr-null+Cyb5R null [liver]	Liver enlargement, steatosis	Henderson et al. (2013)
$Cyp1a1^{-/-}$	No obvious phenotype	Dalton et al. (2000)
$Cyp1a1/1a2/1b1(^{-/-})$	Incomplete embryonic lethal	Dragin et al. (2008)
	Hermaphroditic with cystic ovaries	
Cyp2a/2b/2f/2 g/2 s/2 t	Viable, fertile, no abnormalities	Wei et al. (2013)
	↓15-OH testosterone, ↓ pentobarb.	
$Cyp2c44^{-/-}$	High K^+ diet caused ↑SBP and Na ret.	Sun et al. 2012
$Cyp2d6^{-/-}$	Do not activate primaquine	Pybus et al. (2013)
$Cyp2e1^{-/-}$	Fertile, devel. Normally, no phenotype	Lee et al. (1996)
	↓MTPT damage, ↑2E1 assoc. PD	Vaglini et al. (2013)
$Cyp2j5^{-/-}$	No phenotype; female hypertension	Athirakul et al. (2008)
$Cyp3a13^{-/-}/3a57^{-/-}/3a59^{-/-}$	Viable, fertile, no physiologic abnormalities.	van Herwaarden et al. (2007)
$Cyp4a14^{-/-}$	Male hypertension; castration restores	Holla et al. (2001)
$Cyp4f18^{-/-}$	Viable, fertile, no phenotype	Vaivoda et al. (2015)
$Cyp4x1^{-/-}$	Viable, fertile, mild obese phenotype	Kharkwal et al. (2017)
$Cyp5^{-/-}$ [TXAS]	Defective hemostasis, resist AA-induced death	Yu et al. (2004)
Cyp7a1	Diminished bile synthesis	Schwarz et al. (2001)
Cyp8a1 [Prostaglandin I2 S]	Vascular wall thickening, interstitial fibrosis	Yokoyama et al. (2002)
$Cyp17^{-/-}$; $Cyp17^{+/-}$	Embryonic lethal D7; infertile	Liu et al. (2005)
$Cyp19^{-/-}$	Reduced spermatogenesis; germ cell A_0	Robertson et al. (1999)
$Cyp24a1^{-/-}$	Delay bone fracture repair/ mineralization	St-Arnoud (2010)
$Cyp26b1^{-/-}$	Embryonic lethal D11	Dranse et al. (2011)
	Severe limb defects; disrupt patterning	
$Cyp27B1^{-/-}$	Male growth retardation, hypocalcemia	Rowling et al. (2007)
$Cyp30^{-/-}$	Viable, fertile, no phenotype	Scheer et al. 2014
$Cyp46a1^{-/-}$	Impaired learning and memory	Meljon et al. (2014)
$Cyp51^{-/-}$	Embryonic lethal-heart failure; Antley-Bixler	Keber et al. (2011)

Transcriptome Plasticity and Genome Evolution

A trade-off between genome evolution and transcriptome plasticity highlights the importance of RNA recoding as a strategy for diversifying proteins. RNA editing is a possible mechanism of adaptation from an evolutionary perspective. This is particularly true for neural function in behaviorally sophisticated coleoid cephalopods

(Liscovitch-Brauer et al. 2017). RNA editing is affected by environmental stimuli (Duan et al. 2017; Rieder et al. 2015) and is different among cell types (Tan et al. 2017). ADAR is responsible for A-to-I catalysis and ADAR1 p150 is induced by interferons (George and Samuel 1999). In the octopus, adaptive RNA editing leads to a nonsynonymous site in potassium voltage-gated channel subfamily A member 1 (Kv1.1). The editing in this channel evolved in octopus in cooler water as a way to adapt by accelerating the closing of the channel in lower water temperature (Garrett and Rosenthal 2012). ADAR activity binding is les efficient and dsRNA structures are less stable at higher temperatures suggesting evolutionary selection for lower temperatures. Mice lacking ADAR2 experience seizures and die shortly after birth (Higuchi et al. 2000). Human diseases including ALS, autism, depression, epilepsy, and schizophrenia are associated with altered RNA editing activity. Human RNA editing is non-adaptive, editing is rarer in essential genes and genes under selection have lower editing levels. Human resilience probably utilizes other RNA diversifying events.

The CYP family must respond rapidly to recognition of environmental changes. While RNA editing may not be an adaptive strategy in humans, transcript variants may serve the same endpoint. Alternate exon use provides the transcriptome plasticity that supplants genome evolution. We interrogated several databases to establish a CYP transcriptome. The human cytochrome P450 family transcriptome contains over 965 different variant forms, many with common structural features sensitive to alternative splicing events that expand P450 protein diversity (Annalora et al. 2017). We conclude CYP variant transcript plasticity facilitates adaptation of the human metabolome to substrate burden. The response involves nuclear receptor recognition of small molecule burden and transcriptional signaling to adapt the CYP transcriptome. The CYP metabolic activity is the effector function combining promiscuous active site specificity with variant expression refinement to clear nonpolar chemical invaders from the body.

Infection Suppression of Drug Metabolism

An early observation that 11 children with asthma controlled by theophylline developed theophylline toxicity during the 1980 influenza B outbreak in King County WA (Kraemer et al. 1982). People infected with viral, parasitic or bacterial infections exhibit decreased capacity to metabolize drugs (Renton 1986). Drug metabolism is reduced in experimental models of tuberculosis (Batra et al. 1987). Endotoxin alone will suppress CYP activity (Gorodischer et al. 1986; Ghezzi et al. 1986). IL-1 is linked to the suppression. C3H/HeJ mice lacking TLR4 and Ghezzi mice that do not secrete IL-1 do not decrease CYP in response to LPS injection (Shedlofsky et al. 1987). IL-1 inhibited 7-ethoxycoumarin O-deethylase in primary murine hepatocytes. Purified endotoxin (E coli LPS 0127:B8) in isotonic saline injected intraperitonealy into 8–9 week old Sprague-Dawley rats at 30-100ug/kg suppressed CYP2C11 in males by 35% in 24 h but only 17% in CYP2C12 in females. The

CYP2C11 mRNA was suppressed by 90% within 12 h of exposure and remained suppressed for 3 days. The LPS suppressed both the oxidoreductase and cytochrome b5 in both male and female rats (Morgan 1989).

What is the effect of suppression of drug metabolizing enzymes? LPS suppresses CYPs but induce nitric-oxide synthase 2 (NOS 2). If LPS elevates NO and CYP generates OH radical then ONOO radicals may be formed which are particularly damaging. Perhaps suppression of CYP could be a mechanism to protect the cell from production of ONOO (Morgan 2001). CYPs may be suppressed so NO can reach a site of infection where it is then converted to ONOO by other enzymes. Alternately, CYP metabolism of arachidonic acid creates EETs which have anti-inflammatory activities such as inhibition of TNFα induced VCAM1 and NFκB activation (Node et al. 1999). Perhaps suppressed CYP2C and CYP2J prevent formation of anti-inflammatory activity and prevents hypotension. Suspects IL-1, IL-6, TNFα, TGFβ, and interferons as mediators. Adenylate cyclase activation and cAMP inhibit phenobarbital-induced CYP2B1 and CYP3A (Sidhu and Omiecinski 1995). LPS downregulation is blocked in PPARα$^{-/-}$ mice (Kono et al. 2009). Cytokines down regulate CYP expression in human hepatocytes (Sunman et al. 2004). Mice with null mutations in cytokine genes prevent down regulation of CYPs (Siewart et al. 2000). CYP clearance has been correlated inversely with plasma IL-6 levels in cancer patients (Rivory et al. 2002). CYP3A4 and 2C8 were down regulated by treatment with LPS, IL-6, TNFa, INFg, TGFb and IL-1. CYP2C18, a low expression isoform was not influenced by any cytokine treatment. CYP2C9 and 2C19 were down regulated by IL-6 and TGFb but not LPS, TNF, IFN, or IL-1. CYP2B6 was down regulated by IL-6 and IFN only. The influence on CYP expression is not equivalent across all isoforms (Aitken and Morgan 2007). Absence of either Cyp4a gene attenuated or abrogated the *C. rodentium* induced changes in spleen weight, colon crypt length, hepatic cytokine and acute phase protein mRNAs, and acute phase proteins. Changing expression in response to infection links CYPs to a response to infection. These data suggest Cyp4a10 and Cyp4a14 are involved in the regulation of host inflammatory responses to enteropathogenic bacterial infection (Nyagode et al. 2014).

Host–Pathogen CYP Countermeasures

When a host is infected by a pathogen accommodations are observed in both host and pathogen. Over evolutionary time scales, an immune system point counterpoint dialog is preserved in the genome. Emerging powerful proteome and genome sequencing tools continue to be refined to unravel the significant outcomes from the interactions. However, integration of the metabolome with immune responses is very limited. Does suppression of host CYP genes during infection reflect a benefit to the host or a manipulation by the infecting pathogen? Are the metabolic products of the 57 human CYPs influencing pathogen growth and survival or the human immune response to the pathogen?

Mimiviruses are the largest known viruses with genomes of 1.2 megabases. The *Acanthamoeba polyphaga mimivirus* infects amoeba and its genome encodes up to two CYPs. One is similar to the CYP4A family and the other is most like CYP51 found in bacterial, protozoal, and fungal organisms. Potential ancient CYPs may predate the appearance of oxygen (Wickramashighe and Ville 1975) and suggests a fundamental role in host:parasite interactions.

Bacterial CYPs function in camphor degradation (Davydov et al. 2013) and biotin synthesis (Lawson et al. 2004). *Campylobacter jejuni* encodes a single CYP, CYP1411c that is involved in pathogenicity and production of a capsualar polysaccharide (Alvarez et al. 2013). Bacterial CYPs are required for the synthesis of antibacterial drugs as streptomycetes produce numerous antibiotics such as erythromycin in *Saccharopolyspora ertherea* and vancomycin (Kelly and Kelly 2013).

The Mtb 4.41 megabase genome encodes 20 CYPs, a CYP density over 200 times that of the human genome (Cole et al. 1998). Mycolic acids are major components in the Mtb cell envelope essential for survival, virulence and antibiotic resistance (Barry et al. 1998). Inhibitors of mycolic acid biosynthesis includes isoniazid, ethambutol, and pyrazinamide. CYP124A1, CYP125A1, and CYP126A1 are likely α-terpineol hydroxylases and CYP143A1 is related to camphor hydroxylases (Ouellet et al. 2010). CYP51B1 was the first identified as a sterol 14α-demethylase but is not an essential gene for Mtb viability. Fluconazole and clotrimazole azole drugs form complexes with CYP heme iron preventing conversion of lanosterol to ergosterol. CYP128 is essential based on initial transposon mutagenesis studies (Sassetti and Rubin 2003) and CYP121 and CYP125A1 were determined to be essential in subsequent studies (McLean et al. 2008; Chang et al. 2007). CYP128A1 plays a role in sulfollipid biosynthesis. Mtb CYP121A1 is a cyclodipeptide synthetase enzyme that can form a complex with azole drugs opening new avenues to Mtb antibiotic drug discovery. CYP125A1 is required for survival in macrophages and for infection of mice (Rengarajan et al. 2005) and may oxidize cholesterol or other steroid like molecules to survive. CYP130A1 binds azole drugs econazole, clotrimazole, miconazole and ketoconazole and activities may lead to immune response release of INFγ and IL-10.

Protozoan parasites exploit CYP activities for survival and maintenance of infection. The *Leishmania donovani* genome encodes three CYPs. CYP5122A1 is essential for infecton, cell growth, and the sterol ergosterol synthesis in *L. donovani* (Verma et al. 2011). Azole antifungals can cure mice of parasite infection from the related protozoan parasite *Trypanosoma cruzi* through inhibition of CYP51 sterol 14α-memethylase (Roberts et al. 2003).

Parasitic trematode worms, schistosomes, infect over 600 million people resulting in 280,000 deaths each year. The *Schistosoma mansoni* genome encodes a single CYP3050A1 that is essential for worm survival. Exposure of *S. mansoni* to 10 μM miconazole kills between 78 and 90 percent of the worms after 7 days of exposure. The worms produce eicosinoids, which may suppress host immune anti-parasite functions. The CYP3050A1 may be involved in a variety of activities including: (1) cholesterol metabolism into steroids influencing egg production by female worms, (2) estrogen metabolism, (3) retinoic acid metabolism, and (4) hydroxylation of

ecdysone, a hormone known to be involved in growth and vitellogenesis of worms (Ziniel et al. 2015). The single CYP cannot accommodate all of the potential activities making the worms expression of nuclear hormone receptors for estrogens and retinoids as well as P450 reductase, ferredoxin reductase, and cytochrome b_5 reductase electron transport systems establish elements of functional context.

Carbon Monoxide Refines and Focuses Host Defenses

Carbon monoxide (CO) binds to bacterial NO reductases and other iron, copper and nickel sites in microbial proteins (Wasser et al. 2005) leading to inhibition of DNA replication (Keilin 1966). *Mtb* residing inside macrophages can induce host to express heme oxygenase-1 (HO1; Shiloh et al. 2008), the major source of CO in mammals (Morse et al. 2009). *Mtb* encode a CO resistance gene, *cor*, that is associated with virulence in a mouse model of infection (Kumar et al. 2008). CO rescues HO1 deficient mice from bacterial sepsis lethality (Chung et al. 2008). Molecules designed to release CO, CORMs, are antimicrobial agents (Nobre et al. 2007). CO has minimal effect on bacterial growth pointing to host responses for antimicrobial effect (Wareham et al. 2015).

Host response to CO induced by infection reduces CYP function, limit ROS generation, and limits immune response to infection. CO down regulates the innate immune response leading to impaired adaptive immune responses and improves transplantation success (Amano and Camara 2013). CO is anti-apoptotic, prevents programmed cell death, by preventing mitochondrial permeabilization and increased Bcl-2 expression and inhibiting cytochrome c oxidase. CO activates MAPK preventing TNF-induced cell death. CO induces macrophage phagocytosis. CO suppresses ROS generation leading to impaired lipid raft translocation and inhibition of TLR activation. CO reduces ICAM-1 expression in endothelial cells associated with reduced TNF and IL-1β production. These observations suggest CO is induced by microbes to favor microbe in the host:microbe interaction.

Hmox1 expression stimulated in macrophages by infection leads to phagocytosis and microbial clearance (Wegiel et al. 2014). HO-1 produced CO is the key mediator of macrophage defenses due to induction of ATP production and release by bacteria. ATP activates macrophages binding to the P2X7 surface receptor stimulating cytosolic NOD-like receptor, pyrin domain-containing 3 (NALP3) of the inflammasome inducing caspase-1 to release IL-1β. It is likely that immune suppressive events and CYP suppression are necessary to refine an effective macrophage recognition and clearance pathway for infecting microbes.

Defense Responses to Pregnancy

Pregnancy obligates immune tolerance to the paternal foreign antigens expressed by the products of conception. The immune system continues to maintain anti-infectious responses during pregnancy, vaccinations prior to pregnancy continue to protect. Pregnancy hormones including human chorionic gonadotropin (hCG), progesterone, estrogen, growth hormone influence the quality of the immune response. hCG expression during pregnancy induces immunoglobin class switching to non-effector function. Protection from exposure to new infections (neoantigens) is limited. Progesterone, which binds to progesterone nuclear hormone receptors (PR) regulates transcription of numerous genes. Progesterone also binds progesterone-induced blocking factor (PIBF) and the glucocorticoid receptor (GR) that leads to altered immune responses. Progesterone impairs macrophages production of NO and IL-1 and suppresses toll-like receptor activation of macrophages. Estrogen levels are elevated activating ERα and ERβ that regulates transcription in lymphocytes, macrophages, and dendritic cells (Schumacher et al. 2014). While data are somewhat limited in specific immune modulatory effects from pregnancy hormones, these hormones coordinate immune and metabolic activities through actions on transcription.

Human metabolism responds in a pathway specific manner as CYP2A6, CYP2C9, CYP2D6, and CYP3A4 activities are enhanced while CYP1A2 and CYP2C19 are suppressed (Anderson 2005; Hodge and Tracy 2007). Animal models of pregnancy generally present diminished CYP expression. In the rat CYP2A1, CYP2D2, CYP2C23, and CYP2E1 are down-regulated (He et al. 2007). Mice increase expression of Cyp3a16, Cyp3a41, and Cyp3a44 (Zhang et al. 2008) while expression of Cyp1a2, Cyp2c37, Cyp2d22, Cyp2e1, and Cyp3a11 are decreased and Cyp2a5 expression is unchanged (Koh et al. 2011). One problem in comparing mouse to human is the lack of orthologs for CYP2C, CYP2D (humans have only 2D6 while mice have nine isoforms), and CYP3A (humans have 4 isoforms while mice have 6). The changes responsible for pregnancy-induced changes include pregnancy hormones and elevated inflammatory responses. Growth hormone induces Cyp3a41 while estrogen down-regulates CYP2C19 in an ERα-dependent manner. The down-regulation of PPARα is expected to reduce expression of Cyp2e1 and Cyp2d22.

The metabolic defense responses to pregnancy are often opposite of the immune response. The driving forces for the changes appear to be pregnancy hormones. Progesterone binding to its cognate nuclear hormone receptor (NHR) enhances expression of CYP3A4 while suppressing immune IL-1 and nitric oxide activity. Progesterone also binds the glucocorticoid NHR that enhances CYP3A4 activity while suppressing a variety of immune responses. Estrogen binds to estrogen NHR suppressing CYP2C expression while enhancing immune responses (exacerbation of autoimmune disease). The expression of the peroxisome proliferation alpha NHR is up-regulated in pregnancy leading to suppression of CYP2E1 and CYP2D but enhanced immune response (associated with irritable bowel disease).

Nuclear Hormone Receptors Bridge Immune and Metabolic Defenses

Nuclear hormone receptors (NHR) regulate gene expression through interaction between their DNA binding domain and genomic DNA. They also carry a recognition domain in their ligand binding domains so they sense molecules by structural recognition and regulate transcription of specific genes. Like the CYPs, they bind lipophilic substances such as endogenous hormones but also xenobiotic compounds. Many of the 50 human NHRs regulate the transcription of CYPs so that NHR interactions include substrates and metabolic products.

NHR participate in selection of pre-mRNA splicing creating diversity in the expression of splice variants. NHR are involved in post-transcriptional regulation of exon 1 inclusion in the early response (protooncogene) gene *c-myc* (Maroder et al. 1990). The glucocorticoid receptor (GR) binds sequences in hormone-responsive genes leading to enhanced transcription. The GR ligand, dexamethasone, induces a human growth hormone (hGH) mRNA that is 100 nt longer than hGH mRNA through addition of poly(A) (Paek and Axel 1987). The GR influence alternate splicing of Slo potassium channels. Two splice variants are observed at splice site 5: BK and STREX (STRess axis-regulated Exon). Dexamethasone reduced inclusion of STREX and RU38486, an antagonist of GR restored STREX inclusion. Conversely, dihydroepiandrostenedione (DHEA) induced STREX inclusion thus opposing the effects of Dexamethasone (Lai and McCobb 2002). Glucocorticoids inhibit gonadotropin-releasing hormone (GnRH) expression in the hypothalamus by preventing excision of intron 1 of the GnRH pre-mRNA (Park et al. 2009). Glucocorticoid therapy effectively reduces neurodegenerative processes in the rare genetic disease ataxia telangiectasia by inducing exon skipping to exclude disease-causing mutations in the ATM gene while retaining ATM kinase function (Menotta et al. 2012). NHR interact with RNA polymerase as transcripts are created offering them the opportunity to shift splicing patterns in a ligand dependent fashion.

AhR is associated with tolerogenic dendritic cells (Quintana et al. 2010) and modulation of regulatory T cells (Funatake et al. 2005; Quintana et al. 2008). AhR$^{-/-}$ mice are hyper-responsive to inflammatory cytokines. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is an AhR ligand that is also a potent immunosuppressive compound (Kerkvliet 2012). Tryptophan is metabolized to kynurenine, an endogenous AhR ligand though to have immunomodulatory activity. Coal tar is a mixture of chemicals, some that bind AhR, is a current treatment for psoriasis, an autoimmune disease, and as an anti-inflammatory agent for seborrheic dermatitis (dandruff). It is safe and inexpensive, now on the list of the WHO essential medicines. Nancy Kerkvliet, a research friend, linked the AhR engagement to development of regulatory T-cells (Treg) and immune suppression of murine graft versus host disease (GVHD) (Funatake et al. 2008). These Treg cells suppress the development of cytotoxic T lymphocytes (CTL), a plausible mechanism for suppressing GVHD. The role of the AhR in developing immune tolerance led to screening compounds with drug-like properties that are ligands of the AhR for suppression of autoimmune,

allergy, and GVHD following hematopoietic transplant (Punj et al. 2014). The studies identified 10-chloro-7H-benzimidazol[2,1-a]benzo[de]so-quinolin-7-one (10-Cl-BBQ) as a potential drug candidate. Consistent with NHR function to coordinate transcription, AhR regulates both metabolism and the immune response.

NHR often mediate both expression of metabolic enzymes and immune responses. A few examples include: (1) The retinoid receptors (NR1B; RARα, RARβ, RARγ and NR1F; RORα, RORβ, RORγ and NR2B; RXRα, RXRβ, RXRγ) bind vitamin A and related retinoids leading to regulation of lymphocyte responses, T regulatory cell development and expression of CYP2D and CYP3A4 family enzymes. (2) The peroxisome proliferation associated receptors (NR1C; PPARα, PPARβ, PPARγ) bind ratty acids, prostaglandins and leukotrienes regulating TLR4 and CTLA4 immune responses and a expression of a variety of CYPs. (3) Biologically active steroid structures including estogens (NR3B; ERRα, ERRβ, ERRγ and NR3A; ERα, ERβ), androgens (NR3C; AR and NR1; CAR), as well as progesterone (NR3C; PR), corticosteroids (NR3C; GR, MR) all regulate immune responses and expression of CYPs. The role of small molecule recognition binding to NHR dimer permutations creates diverse defensive responses to xenobiotic exposure.

Metabolic Defenses

The immune system recognizes a portion of an antigen referred to as an epitope, often larger than 8 amino acids. These epitopes are significantly larger than molecules acted on by the CYP system which can metabolize molecules as small as two carbons, eg. ethanol. The active site and channels for entry and exit of molecules from CYPs limit molecular size, complementary to the immune system recognition. While the appearance of mutually exclusive defensive systems, these systems extensively overlap with activities coordinated by nuclear hormone receptors. The cells of the immune system evolved from stem cells that create blood while CYPs iron containing heme originated in red blood cells with original function to carry oxygen. Both systems utilize ROI/RNI but cells of the immune system are mobile travelling to a site of action while CYPs act at the sub-cellular level with diverse forms expressed in different tissues. Immune organs include the lymph nodes, bone marrow, the thymus and the spleen. In contrast, the CYP system is a subcellular system that is expressed in active metabolic tissues including liver, kidney, lung and steroidogenic organs.

Earliest forms of the immune system include development of protein degradation while the CYP system arose from electron transport systems. Electron transport and heme-binding oxygen appear to be the point of evolutionary divergence in metabolic and immune defenses (Table 9.3). Prokaryote defenses include DNA sequence recognition and CYP metabolism. Multiple cell types that can be mobilized to relocate at sites of invasion typify the immune system. The immune response systems elaborated pathogen-associated molecular patterns (PAMPs) to identify pathogens.

Table 9.3 Comparisons of defensive systems

Characteristic	Immune system	Metabolic system
Recognition	PRR- PAMPS	Promiscuous active site
	MHC, TCR, BCR	Nuclear hormone receptors
Adaptive response	Recombination; eg. VDJ joining	Transcriptome plasticity
	Elaboration of memory cells	No obvious memory
Effector mechanisms	MPO → •OH (ROS/RNI)	Heme-Fe → •OH (ROS/RNI)
	Antibodies	Metabolites: prostaglandins, prostacyclins, Eicosinoids, HEETs, EETs, glucocorticoids, Retinoids, VIt A, Vit D
	T-cells- T-killer, CTL	
	Systemic- cytokine release	
Response to infection	Activation of responses	Suppression of expression
		Carbon monoxide – HO-1
Pathogen countermeasures	Host mimicry	Microbe expression of CYPs
	Suppress immune effectors	
Pregnancy	Selective responses:	
	Tolerance to neoantigens	Induce CYP2D6 and CYP3A4
	Maintenance of memory	Suppress CYPs 1A2, 2C9, 2E1
Extreme challenge	Immune exhaustion	Induce expression- overwhelm phase II conjugation→Toxicity
Weak/loss of function	Immune deficiency	Slow metabolizers
	Numerous genetic diseases	Rare genetic diseases

In contrast, the CYP system relies on constitutive expression of enzymes with broad substrate specificity. The systems overlap as CYPs are expressed in immune cells and their precursors.

A hallmark of immune responses is the role of genome recombination. T-cell receptor and immunoglobin diversity driven by RAG1 and RAG2 gene recombination. The recombination of VDJ segments create on the order of 10^{18} different antigen recognition forms. The genome recombination gives the immune system memory. The CYP enzymes are unique in their promiscuous active sites with on the order of 10^5 substrates divided between 57 genes. Added diversity is driven by creation of 1000 CYP transcript variants shifting adaption from genome sequence to transcript. This reflects the CYP rapid adaptive response minimizing the need for memory responses and a key function in resilience.

Appreciation of the dual defense systems has been an emerging concept for decades. As the interplay between the systems becomes more sophisticated, improved therapeutic discovery and appreciation of environmental threat will emerge. Drug discovery has focused on immune responses and nuclear hormone

receptors for a variety of disorders from infection to cancer. Drug discovery targeting CYPs and related enzymes has been limited due to adaptive gene expression and transcriptome plasticity as well as radical products released by the heme-iron. The concept of personalized medicine will need to embrace novel approaches to drug discovery targeting CYP activities.

CYP Role in Cell Proliferation

My doctoral dissertation involved developing a method for separating the different forms of CYP found in the rat liver. The HPLC separated rat Cyps into five fractions revealing class specific changes in Cyp expression profiles in the regenerating liver. The first fraction represented 36 percent of Cyps in control livers but fell to 4 percent in the regenerating liver, a reduction of 90 percent. This fraction appears to represent Cyps in the 2B and 3A families. Insignificant changes were observed in fractions II, III, and V while a modest elevation in CYPs associated with fraction IV (Iversen 1984). The suppression of CYP expression was inversely proportionate to the frequency of mitotic hepatocytes, which was independent of the stimulus for liver regeneration. My thesis observations are consistent with more sophisticated studies of others using subtractive cDNA libraries from regenerating livers but reveal a consistent phenotype: suppression of Cyp2 family by 60 to 90 percent, a two to threefold elevation in expression of Cyp7 and prostaglandin D2 synthetase, and no significant changes in the remaining Cyps (Xu et al. 2004).

Liver regeneration after partial hepatectomy results in rapid hyperplasia, cell division, within 2 days of resection leading to a completely regenerated liver in 7 to 10 days(Higgins and Anderson 1931). The G_1-S cell cycle checkpoint gene p53 is highly upregulated during liver regeneration (Kren et al. 1996). We inhibited the expression of p53 in the regenerating liver with antisense oligonucleotides. Inhibition of p53 resulted in increased frequency of mitotic hepatocytes associated with marked polyploidy confirming previous reports of p53s role in the G_2-M cell cycle checkpoint (Waldman et al. 1996). Regeneration was more robust in p53-suppressed livers and suppression of Cyp3A2 was blocked (Arora and Iversen 2000). Earlier studies manipulating the expression of metallothionein, a radical oxygen scavenger, during liver regeneration resulted in greater oxidative stress, diminished hepatocyte regeneration, enhanced p53 expression and greater suppression of Cyp3A2 activity (Arora et al. 1998).

Further studies interrogating cell division and CYP expression confirmed the inverse relationship. The immediate early response gene, *c-myc*, is a regulator of cell proliferation, differentiation and apoptosis. *C-myc* is highly up-regulated during liver regeneration following partial hepatectomy (Kay and Fausto 1997; Michalopoulos and DeFrances 1997). We used an antisense oligonucleotide to transiently suppress *c-myc* during liver regeneration following two-thirds hepatectomy. The antisense treatment blocked expression of *c-myc* resulting in reduction of proliferating cell nuclear antigen (PCNA) and hepatocytes were arrested in G_0/G_1 phase

of the cell cycle. While inhibition of *c-myc* in control animals had no influence on CYP3A2 expression, inhibition of *c-myc* during liver regeneration suppressed expression of CYP3A2 (Arora et al. 2000).

Selective suppression of CYPs during DNA replication invites the current speculation linking the CYP active site with DNA polymerase. Aphidicolin is a tetracyclic diterpene made by the fungus, *Cephalosporum aphidicola*. Aphidicolin is a potent but reversible inhibitor of eukaryotic DNA polymerase. It mimics cytosine and will compete with the nucleobase for binding to DNA polymerase. In fact, aphidicholin might be a novel anti-cancer agent but it is rapidly metabolized by CYP3A4. Hence, the same molecule binds to the active site of both enzymes. Does the human metabolome contain an endogenous aphidicolin-like molecule? The presence of this molecule or class of molecules would represent a chemical checkpoint to entry into the cell cycle. Constitutive metabolism, metabolites, supports the checkpoint but lack of metabolism (loss of metabolites) permits progression into the cell cycle. Such a metabolite might serve to regulate stem cell proliferation as a failsafe against stem cell tumors. The emerging techniques in metabolomics hold the promise of confirming this highly speculative hypothesis.

Conclusions

Small molecules evade immune responses. Foreign chemicals are cleared from the body by metabolism in a functional parallel to the way the immune system clears foreign antigens. The two systems show overlap in size of molecule that is recognized but the CYPs focus on smaller molecules while the immune system is tuned to larger molecules. Both systems utilize radical oxygen and nitrogen but the improved distribution of the immune system offers more efficient delivery to a defensive site and CYP expression is suppressed. The coordination of CYP expression is evidence for functional linkage to the immune companion defenses.

The CYP family of enzymes are unique in their capacity to act on self-molecules as well as xenobiotics. Multiple CYPs act on substrates that are endogenous molecules such as mediators of inflammation such as arachidonic acid, prostaglandins, and eicosanoids. Conversely, CYPs act on endogenous molecules capable of immune suppression such as the glucocorticoids. These endogenous substrates and their products of CYP metabolism are ligands for nuclear receptors that regulate the quantity and quality of transcription, often involving immune response genes. Toxic xenobiotics often resemble/mimic endogenous molecules demanding precise recognition by CYP metabolism. I propose this demand is reflected in transcriptional plasticity in CYP expression.

The role of CYP defense as a companion to the immune system is underappreciated. Infections and pregnancy are events where foreign antigens challenge the immune system as well as influence transcription of CYP genes. Just as pathogens develop immune countermeasures, pathogens also carry CYPs capable of chemical countermeasures that support infection. This is an evolutionary point-counterpoint

involving both immune and metabolic responses. The emergence of new infections and expanse of chemicals in our environment demand metabolic resilience.

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Chapter 10
Analog Genetics

Abstract Analog genetics describes a continuum of outcomes, phenotypes, created by a discrete or digital set of base pairs in a genome. The concept is in contrast to discrete phenotypes created from discrete base pairs in the genome. The difference is that phenotypes are a distribution of character all arising from the discrete genome. The definition of a gene as the unit of heredity is disputed based on a spectrum of phenotypes resulting from a single gene.

Keywords Genetic disease · Gene splicing · Alternate exon · Alternate translation · Premature termination codon · RNA editing · Epigenetics · Xist · G-quartets

Introduction

We believe humans are capable of extraordinary physical feats. Comic book super heroes, X-men, and Olympians tell tales of fantastic conquests. Consider the exceptional people on this planet capable of impossible acts of biology without knowing of their daily conquest. One example is the recent examination of 589,306 human genomes of people in which signature evidence of rare genetic diseases with no disease manifestation were observed in 13 individuals (Table 10.1). They were unaware of the misery they should experience every day, or that they are not expected to survive. The study only included a fraction of known disease-causing mutations pointing to a greater potential number of cases and increased frequency so future studies are likely to expand the array of extraordinary people. Three of the 13 carry dual copies of mutated CFTR associated with cystic fibrosis. Only one in 88,000 cystic fibrosis patients carries two mutant copies of CFTR so finding 3 in 600,000 healthy patients is very rare (Saey 2016). Clearly, these individuals found a way to adapt, but how? I have seen evidence of these impossible acts in the laboratory by paradoxically finding functional proteins in cells from people genetically incapable of making these same proteins. I wanted to explore the multitude of molecular mechanisms contributing to exceptional non-genomic adaptation (Fig. 10.1).

People can live with only one lung and one kidney. People live without a spleen, appendix, gall bladder, adenoids, tonsils, a variety of lymph nodes, a uterus, ovaries,

P. L. Iversen, *Molecular Basis of Resilience*,
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Table 10.1 Disease-causing mutations with no phenotype

Disease	Mutated gene	Number with no phenotype
Cystic Fibrosis	CFTR	3
Smith-Lemli-Opitz syndrome	DHCR7	2
Familial dysautonomia	IKBKAP	1
Epidermolysis bullosa simplex	KRT14	1
Pfeiffer syndrome	FDFR1	1
APECED	AIRE	1
Acampomelic campomelic dysp	SOX9	1
Atelosteogenesis	SLC26A2	3

breasts, testicles and a prostate. People also live without the fibula bones in the leg and removal of up to six ribs. There are prosthetic replacements for the stomach, colon, pancreas, salivary glands, thyroid, bladder and the last kidney. Further, people live with amputated limbs, eyes, nose, ears, larynx, tongue, lower spine and rectum. In more severe cases, people live without a skull, heart and the remaining lung (BBC Focus 2017). In summary, people are remarkably adaptable to traumatic physical insults.

A surprising and uplifting phenomenon is the resistance of a population to become extinct. A population can adapt such as the great tit (*Parus major*), a bird population studied in England. These birds rely on a particular caterpillar as a feed source during the egg-laying season. Climate change over the past 47 years has led the caterpillar to emerge earlier and earlier each year thus threatening the survival and predictions of extinction of *Parus major*. However, the birds simply began laying their eggs a couple of weeks earlier to coincide with the availability of their caterpillar food source. Now these bird populations are stable and perhaps expanding (Blotkin 2017).

Gregor Mendel, the educated monk from the early 1800's, meticulously designed studies with pea plants to discover inherited characteristics are carried in discrete units we now call genes. Scientists wanted to understand and manipulate genes and by the mid-twentieth century, their efforts led to more granular characteristics. A large number of genes are made from DNA collectively called the genome. Determination of DNA structure (Nobel Prize 1962), was followed by solving the genetic code (Nobel Prize 1968). Gene structure progressed from a continuous block of DNA to split genes in which exons are separated by introns (Nobel Prize 1993). More recently, a world of non-coding RNA emerged to complicate mechanisms of gene expression (Nobel Prize 2006). In brief, an increasing appreciation for diversity accompanies the simple discrete unit of inheritance.

The genome is complex with unique structures associated with essential functions. The term telomere, Greek telos for end and meros for structures, was coined by Muller (Muller 1938) in 1938 from studies in Drosophila indicating the ends of chromosomes are specialized structures. The shortening of repeating telomeres is thought to be responsible for the "Hayflick limit" that cells have a defined replication

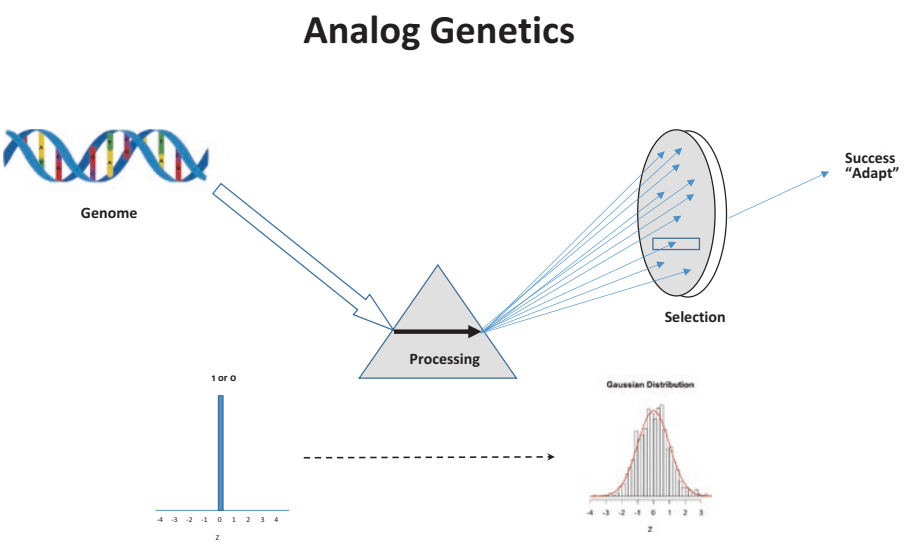

Fig. 10.1 Analog genetics

limit and are not immortal (Hayflick 1961). The limit is 50–70 cell divisions at which time the cells become senescent and cell division stops. Human telomeric DNA is 5–12 kb of highly repetitive dsDNA (5′-TTAGGG-3′/3’-AATCCC-5′) followed by 150–300 nucleotides of single stranded 3′-overhang DNA. Telomeric DNA shortens by 50–200 bases during each S phase due to the inability of DNA polymerases to begin de novo synthesis, known as the end replication problem (Olovnikov 1973; Watson 1972). Telomerase reverse transcriptase can overcome telomere loss through enzymatic (hTERT plus TERC and RNA hTR) telomere elongation adding repeats to the 3′-end of the chromosome. TERT is responsible for addition of 5′-d(TTAGGG)-3′ nucleotide segments to the ends of chromosomes which prevents degradation of chromosome ends (Nobel Prize 2009). Loss of hTERT (chromosome 5) is associated with *Cri du chat* a rare genetic disorder 1 in 50,000 live births with greater incidence in females by a 4:3 ratio. Alternative splicing is one mechanism of regulation of telomerase activity. Active telomerase is often detected in human cancer cells, a deleterious adaptation, but not in normal somatic cells making it an attractive target for the design of anticancer drugs.

The human genome is 44.8 percent moderately repetitive repeat sequences: (1) long interspersed nuclear elements (LINE) are 6 to 8 thousand base sequences that make up 21.1 percent of the human genome and mediate their transposition, (2) short interspersed nuclear elements (SINE) are 100 to 400 bp sequences that comprise 13.1 percent of the genome, (3) long terminal repeats (LTR) are sequences associated with endogenous retroviruses and comprise 8.3 percent of the genome, and (4) DNA transposons that make up 2.8 percent of the genome. LINEs are tran-

scribed into mRNA that is translated into a reverse transcriptase protein that makes a DNA copy of the LINE RNA, which is then integrated into the genome at a new site. LINE integration has the potential to regulate transcriptional activity of nearby genes, an adaptive role in gene expression. This process is blunted by DNA mutations in LINE DNA resulting in nonfunctional reverse transcriptase and through suppression by small interfering RNA (siRNA) encoded by some LINEs. The SINEs are also retrotransposons capable of integrating into new sites in the genome leading to exon shuffling thought to contribute to genetic diversity between species (Bohne et al. 2008). These repetitive elements collectively provide for exceptional genome plasticity, apparent adaptability, and regulate the genome size.

A male human has a smaller genome than human females. The human genome is 3,031,042,417 base pairs (bp) for the X-linked genome and 2,932,228,937 bp for the Y-linked genome. A female (XX) zygote (diploid genome) then has 6,062,084,834 bp and a male (XY) zygote has 5,963,271,354 bp. The occurrence of the 4 nucleotides is not equal, the GC (G + C) content of the human genome is 41% and not 50% as would be expected if the genome composition was equal. The underrepresentation of GC is observed throughout the animal kingdom. The relative mass of GC pairs is 616.3711 while the mass of AT pairs is 615.383 g/mole. The C-value is the pg of DNA in the haploid genome so a human C-value (3.031 MBP / 0.978 MBP/pg) is 3.099361 (2.9982 for the Y-linked genome). This is just a hint of genome plasticity, an apparent reflection of adaptability at the DNA level.

The concept that some genes are essential to survival of an organism means other genes are not always essential. Studies using directed deletion or random mutagenesis in bacteria identify 250–300 essential genes from approximately 4000 genes (Zhang and Lin 2009) implying over 90 percent of genes are non-essential. Similarly, heterozygous deletions covering 98.4% of the 4914 protein coding genes in yeast indicate 15–20% of all genes are essential (Kim et al. 2010). Studies in the nematode worm, (*Caenorhabditis elegans*), fruit flies (*Drosophila melanogaster*), and zebrafish (*Danio rerio*) tend to confirm the number of essential genes is smaller than non-essential genes. Studies in humans are more challenging but if we consider the 10,000 genetic diseases have been identified from 21,000 genes in the human, many of which are non-lethal, the chances are that humans also carry more non-essential genes than essential genes. Many genes are conditionally essential such that a non-essential gene in one environment may be essential in another environment. A substantial segment of the coding genome exists as a reservoir for adaptability to changing environments.

Our genome is not a firmly fixed blueprint. Chromosome loss and duplication provides cells with a rapid mechanism for adaption. Tumor cells acquire additional copies of oncogenes or lose tumor suppressor loci through mechanisms of chromosome instability (Holland and Cleveland 2009). I observed frequent changes in the chromosomes of patients with acute myelogenous leukemia (AML). We think this unstable genome is a driver of the progression of AML but what if we are observing

an adaptive response to the unexpected disturbances caused by the presence of the cancer.

What will happen if we isolate a single gene, insert it in a cell and observe of the expressed protein product? Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is an appropriate choice and the subject of a Nobel Prize in Chemistry for O. Shimomura, M Chalfie, and RY Tsien in 2008 based on the sort of question (Nobel Prize 2008). Insertion of the GFP gene into the genome of yeast, *Saccharomyces cerevisiae*, in specific sites associated with three different gene promoters (regulators of transcription) leads to promoter-specific rates of GFP transcription (Niedenthal et al. 1996). Yeast in which the GFP gene was inserted were selected for and positive clones were expanded from a single cell. Cells were expanded so that a population of cells expressing GFP could be examined. The amount of GFP in each cell of the population was then quantified by measuring the fluorescent signature by exciting the GFP with 488 nm light from an Argon-ion laser then detecting the characteristic 530 nm light emission in each cell by flow cytometry. The observations for the population of cells (mean fluorescent intensity ~30 a.u.) was about 10 times greater in GFP containing yeast compared to yeast with no GFP (wild type ~3 a.u.) but the range of expression covered a 10-fold range (10 to 100 arbitrary units), a distribution similar to the wild-type auto-fluorescence (0 to 10 arbitraty units; a.u.). One might expect a more narrow range in gene expression from a single gene in a single cell clone. Could this experiment reveal variations in GFP expression that encompass relatively rare events leading to boosting GFP expression to 100 a.u from the average of 30 a.u.?

Perhaps yeast are unusual so the experiment should be done in human cells. An example of a clonal HeLa Tet-On cells expressing mCherry-GFP-LC3 (microtubule light chain 3) a control protein to which an antibody will detect. Cells were transduced with a lentiviral pCW vector containing the Tet-On component (Takayama et al. 2017). Particular interest in the range of GFP (10^5 to $2*10^6$) and mCherry ($7*10^4$ to $2*10^6$) signal. Investigators are generally interested in mean channel fluorescence or AUC values. The question of interest is why the broad range in expression over a log range of expression from a clonal gene.

One Gene-One Enzyme

The definition of a gene as a hereditary determinant of a unit characteristic is over 100 years old (Johnannsen 1911). The relationship between a gene and an enzyme is based on heredity of eye color in *Drosophila*, fruit flies. Eye color was heritable and the color was the product of an enzymatic reaction (Beadle 1945). Extension of this concept held that all biochemical processes in all organisms are under genetic control and each biochemical reaction is under the ultimate control of a different

gene. Further, a gene mutation results in alteration in a single primary chemical reaction. The heredity concept is updated so that one gene is known to potentially make multiple proteins. Soon enzymes became a subset of proteins and the Central Dogma of molecular biology emerged: DNA is transcribed into RNA which is then translated into protein.

Central Dogma

The "Central Dogma" of molecular biology describes the transfer of information in DNA sequence to RNA sequence and then to protein sequence (Crick 1970). The Central Dogma involves a series of three processes beginning with replication, a process in which a DNA genome is copied to a daughter DNA genome. Second, transcription, a process in which specific segments of the DNA genome is copied into a single-stranded RNA including a small subset of messenger RNA (mRNA). Finally, translation, a process in which mRNAs serve as templates for synthesis of proteins. The initial version of the Central Dogma held that once information has passed into a protein it couldn't get out again, a directional flow from DNA to protein with no opportunity for information to flow from protein to protein or from protein to nucleic acid. Discoveries of viral reverse transcriptase, RNA → DNA (Temin and Mizutani 1970), and RNA dependent RNA polymerases, RNA → RNA (Koonin et al. 1989), update the Central Dogma.

Transcriptome Plasticity

The single gene as a pinpoint of genetic light clustered in a galaxy of an individual's genome may summarize "digital genetics." If each gene can be expressed in a diverse collection of variants then the pinpoint of light is actually a cloud. How this cloud is formed from a single gene is described in the following section.

1) **Imperfect Fidelity in Replication, Transcription and Translation**. Three polymerization reactions are fundamental to life; replication, transcription, and translation. DNA replication allows an incorrect nucleotide once in 10^8–10^{10} events (Kunkle and Bebenek 2000) but transcription allows an incorrect nucleotide once in 10^4 events (Rosenberger and Foskett 1981) and translation allows an error once in 10^3–10^4 events (Bouadloun et al. 1983; Edelman and Gallant 1977; Kramer and Farabaugh 2007; Laughrea et al. 1987). The ribosome uses local and remote conformational switches to govern induced-fit mechanisms to ensure accuracy in tRNA recognition, selection, and translation termination. Nucleotide pairing is the substrate for each reaction with dNTP for DNA replication and transcription and aminoacyl-tRNA for translation. Hydrogen bonding energy is insufficient to define selectivity; geometry of the base pair defines specificity such as the purine N3 and pyrimidine O2 distances.

The Net Effect of Altering Fidelity:
Replication. The replication fidelity of the human genome is 10^9 errors/base including proof reading. If the genome is 3.3×10^9 bases then each time the genome replicates there are 3.3 errors [$(3.3 \times 10^9$ bases$)(10^9$ errors/base$) = 3.3$ errors]. Hence, every cell in the body has a different genome sequence. There are 37.2 trillion cells in the human body, 3.72×10^{13} cells. Each with 3.3 independent base variants indicates the body is composed of a cellular quasispecies.

Transcription. The average primary transcript is 16,000 bases that is processed to an average 2200 bases when a mature mRNA. Translation fidelity is 10^{-4} errors/base and there is no proof reading. There are an average of 1.6 errors in each primary transcript [$(1.6 \times 10^4$ bases$)(10^4$ errors/base$) = 1.6$ errors]. The average mRNA has 0.22 errors per individual mRNA [$(2.2 \times 10^3$ bases$)(10^4$ errors/base$) = 0.22$ errors)] but 67% will be synonymous errors leaving 33% nonsynonymous. That means only 0.07 or 7% encode a protein with an amino acid error.

Translation. The error rate in translation is 0.1% or 1×10^3 and the average protein is 500 amino acids in length. Each protein has 0.5 errors due to translation fidelity [$(5 \times 10^2$ amino acids$)(10^3$ errors/amino acid$) = 0.5$ errors]. Hence, 50 percent of all proteins carry an error in amino acid sequence.

Summary. The human body has 3.3 errors/cell and 3.72×10^{13} cells resulting in 1.23×10^{14} DNA mutations overall. The mRNA coding for proteins are expressed as 1000 copies/transcript with 0.07 errors/transcript and in a given tissue, approximately 10 percent of the 22,000 genes are expressed. There are $(2.2 \times 10^3$ transcripts$)(70$ errors copies/transcript$) = 1.54 \times 10^5$ errors/cell. There are approximately 1000 copies of each protein/cell each with 0.5 errors/protein and 2,200 different proteins/cell [$(1 \times 10^3$ protein copies/cell$)(2.2 \times 10^3$ proteins$)(0.5$ errors/protein$) = 1.1 \times 10^6$ protein errors/cell]. Taken together each cell in the body has 3.3 DNA mutations, 1.54×10^5 mRNA errors, and 1.1×10^6 protein errors. Considering these errors are found in each of the 3.72×10^{13} cells in the body the diversity in gene expression is a distribution of outcomes and not a singular match to a prototypical genome.

Translation specificity relies on the 30S (40S eukaryote) subunit of the ribosome for codon-anticodon interactions and the 50S (60S eukaryote) subunit for peptidyl transfer and hydrolysis. Optimal growth and propagation can be diminished with too little and too much accuracy in translation (Kurland and Ehrenberg 1984). Mild defects in translation fidelity is associated with severe neurodegeneration and ataxia in mice (Lee et al. 2006). Low fidelity translation leads to unfolded proteins that activate apoptosis (Nangle et al. 2002) (programmed cell death or cell suicide). However, translational infidelity may provide increased phenotypic diversity pro-

moting adaptability to challenging environments (True et al. 2004; True and Lindquist 2000). Retroviruses exploit read-through by sequestering eRF1, a translation factor, to enhance expression of viral proteins (Orlava et al. 2003). Certain proteins are associated with mistranslation, which serves as a regulatory process such as a programmed frameshifting event encoded by *prfB* (Craigen and Caskey 1986). In this case, adequate RF2 leads to termination at a premature termination codon in *prfB* and full length protein is not made but limiting amounts of RF2 allows a read-through and *prfB* is made.

The most fundamental processes necessary for life are not perfect. It appears the environmental insults to living things over time selected for creatures not so rigid or perfect but not so error prone. The balance between high and low fidelity in these processes approaches a "just right" optimum tested repeatedly over time.

2) **Alternate Exon Use**. Consider the average human gene is composed of 8 exons and 7 introns with greater than or equal to three different splice variants. Every human gene will produce at least two different mRNAs (Lee and Rio 2015). This singular observation expands the human proteome from 21,000 to over 60,000 different proteins. The pre-mRNA transcripts average 16,000 bases that are processed by the spliceosome to an mRNA with average size of 2200 bases. Approximately 50 percent of human genetic diseases arise due to mutations that affect splicing (Siva et al. 2014). Examples of these genetic diseases are provided in Table 10.2 (Lewandowska 2013). Alternate splicing during pre-mRNA maturation is a process that results in a single gene coding for multiple proteins. In genes with more than one exon, each intron contains conserved elements with a 5′-exon $A_{(64)}G_{(73)}\backslash G_{(100)}U_{(100)}$-$(n)_x$UACUAACYYYYYYYYYYYYYYC$_{(80)}A_{(100)}G_{(100)}\backslash$ exon-3′ and these conserved sequences are recognized by a variety of snRNPs. The U1 snRNP binds the 5'-GU and recruits U2 and U2AF snRNPs. The U2 binds to a branch site A (underlined) and U2AF binds to the polyprymidine tract forming a spliceosome A complex. The sequence context of the exon junctional complex (EJC) defines an exon in as a range from weak to strong. Additional regulation of splicing involves cis-elements referred to as intronic splicing silencers (ISS), intronic splicing enhancers (ISE), exonic splicing silencers (ESS), and exonic splicing enhancers (ESE) and trans-acting proteins which are generally members of the serine rich (SR) proteins (Falanga et al. 2014). The four variables controlling alternate exon inclusion in mRNA are: (1) the presence and strength of *cis*-acting regulatory elements, (2) the rate of transcription elongation such that small exons are more likely to be excluded, (3) interactions between the C-terminal domain of the transcript and RNA polymerase II as well as recruitment of snRNPs, hnRNPs, and SR proteins, and (4) the kinetic equilibrium in U1 snRNP recognition of the 5′ splice donor splice site in the pre-mRNA (Murugan and Krieman 2012).

There are at least 12 known SR protein splicing factors are referred to as SRSF1 to SRSF12. Each SRSF protein has an RNA sequence Recognition Motiff (RRM) that bind degenerate pre-mRNA sequences (Table 10.3). The discovery of SRSF genes involved many different scientific groups and their function was not always obvious leading to several names for each gene as reflected in Table 10.3.

Table 10.2 Mutations influencing gene splicing

Gene	Disease	Mutation	Expression change
CFTR	Cystic Fibrosis	Intron 9 -1G > A	3′-splice acceptor Δ exon 10
GAA; acid alpha glucosidase	Pompe Disease	c.546 G > T	5′-splice donor Δ exon 3
			(still 10% wild type)
		c.1194 + 2 T > A	5′-splice donor Δ exon 7
		c.2646 + 1ΔTG	5′-splice donor Δ exon 18
CDKN2A	Melanoma predisposition	IVS2 + G- > T	Δ69 bp in exon 2
		c.457 G- > T	
LKB1	Leukemia	IVS2 + 1 A- > G	Intron switch to U12snRNP
FBN1/2	Marfan syndrome	Intron 30 T- > G	Branch point mutation Δ exon 31
COL5A1	Dystrohic Epidermolysis Bullosa	IVS32: T-25G	Branch mut. Δ 45 bp of exon 33
LCAT	Lecithin-cholesterol acetyl transferase		
NPC1	Neimann-Pick	c.882–28 A > G	Branch mut. Δ exon 7
KCNH2	LQT syndrome	−28 A > G intron 9	Branch mut. Δ exon 10
UROS	Cong. Ertythropoetic porphyria	c.661–31 T- > G	Branch point enhances wt expression.

Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked rare genetic disease in which dystophin expression is lost due to genetic mutations and deletions. The mdx mouse is a homologue of DMD caused by a point mutation in exon 23 of dystrophin (Bulfield et al. 1984; Sicinski et al. 1989). DMD patients and mice carrying mutations that ablate dystrophin paradoxically express dystrophin called revertant fibers. The incidence of revertant fibers in DMD patients ranges from 0–70 percent (Burrow et al. 1991; Klein et al. 1992; Fanin et al. 1995; Uchino et al. 1995) and around 1% in the mdx mouse (Hoffman et al. 1990; Nicholson et al. 1993a). These revertant fibers increase in frequency with age (Lu et al. 2000; Nicholson et al. 1993b) suggesting a selective advantage for a revertant fiber. There are numerous examples from rare genetic diseases in which out of frame mutations are associated with exon skipping (Table 10.4).

At least some of these splice variants represent an adaptive response. For example, the revertant muscle fibers for dystrophin in individuals with Duchene Muscular Dystrophy. The *mdx* mouse provides a genetic model in which a termination codon arises from a single base mutation in exon 23 of the dystrophin gene and the transcript is degraded by nonsense mediated decay (NMD). Exon skipping can restore an in-frame reading to an out-of-frame mutation can be extablished by careful

Table 10.3 SRSF RRM sequences

Name	Other names	Location	RRM sequences
SRSF1	SFRS1, ASF, SF2, SRp30a, SF2p33, MGC5228	17q22	TGAAGAAC; ACGCGCAAGGACAGAGC
SRSF2	SFRS2, SC-35, SC35, PR264, SFRS2A	17q25.2	AGCAGAGTA;GTTCGAGTAAGGAGAT
SRSF3	SFRS, SRp20	6p21	CATCA; GGTCCTCTTC
SRSF4	SFRS4, SRP75	1q35.3	GAAGGA
SRSF5	SFRS5, SRP40, HRS	14q24	GAGCAGTCGGCTC
SRSF6	SFRS6, SRP55, B52	20q12-q13.1	U(C/G)CG(U/G)(A/C)TGCGGC; TCAACCAGGCGAC
SRSF7	SFRS7, 9G8, ZCRB2, HSSG1, AAG3, RBM37	2p22.1	TCAACA; ACGAGAGAGGACGACGAG
SRSF8	SFRS2B, SRP46	11q21	Not determined
SRSF9	SFRS9, SRp30c	12q24.31	GACGAC; GTGGATTAAAGAGCTCGG
SRSF10	FUSIP2, FUSIP1, SFRS13A, TASR1, TASR2, SRp38, SRrp40, SFRS13, PPP1R149	1p36.11	AC(A/G)G(C/G)(GAA)n
SRSF11	SFRS11, p54, NET2	1p31.1	C-rich regions
SRSF12	SFRS11, SRrp35, SFRS19	6q16.1	Not determined

Table 10.4 Exon skipping associated with out-of-frame mutations

Disease/Gene	Reference
Hemophilia/Factor VIII	Naylor et al. (1993)
Fanconi Anemia/Group C genes	Gibson et al. (1993)
Marfan syndrome/fibrillin (FBN1)	Dietz et al. (1993)
Gyrate atrophy/(OAT)	Dietz et al. (1993)
Maple syrup urine disease/(BAKAD)	Fisher et al. (1993)

inspection of the resultant transcript. Indeed, in-frame deletions in dystrophin permit the synthesis of a shortened dystrophin protein with functional NH_2- and COOH- terminal domains are associated with a milder disease, Becker muscular dystrophy (BMD) (Love et al. 1991; Winnard et al. 1993; Mirabella et al. 1998) confirming the functional capacity of shortened protein. Revertant fibers in Duchenne muscular dystrophy arise from naturally occurring mechanism capable of alternate exon selection during the splicing of pre-mRNA. A variety of exon-skipping combinations are observed capable of by-passing the termination codon in exon 23 of the mdx mouse including splicing from exons 18 to 35 and exon 13 to 48 (Lu et al. 2000) but splicing from exons 13 to 30, 13 to 35, 18 to 24, 18 to 26, 18 to 30, 18 to 34, 20 to 26, 20 to 30, and 22 to 35 are also observed which are also in frame (Fall et al. 2006). These and earlier studies represent proof of concept for

developing Eteplirsen (ExonDys 51), the first therapeutic designed to induce skipping of targeted exons.

Pioneering studies by Dominski and Kole utilized synthetic oligonucleotides to restore correct splicing of beta globin for the treatment of thalassemia (Dominski and Kole 1993). Several years later we observed a synthetic oligonucleotide induced skipping of a portion of exon 2 of the *c-myc* pre-mRNA resulting in a truncated, dominant-negative *MYC* protein (Hudziak et al. 2000). Subsequent studies were aimed at using synthetic oligonucleotides to induce targeted exon skipping in the *mdx* mouse (Fletcher et al. 2007), the dog (McClorey et al. 2006a), and human muscle explants (McClorey et al. 2006b). The recent approval of a synthetic oligonucleotide that induces targeted exon skipping for the treatment of Duchenne Muscular Dystrophy generates enthusiasm for the exploration of this approach for thousands of rare genetic diseases. The FDA approval process was controversial but that is a story for another time.

What are the limits for oligonucleotide induced alternate exon use? The immune system employs alternate exon inclusion to refine immune responses (Mourich and Iversen 2009). We induced skipping of exon 3 in the T-cell co-receptor, CTLA-4, would lead to expression of a ligand independent transcript variant of CTLA-4 (LiCTLA-4) (Mourich et al. 2014). CTLA-4 signaling leads to immune tolerance and we found that inducing LiCTLA-4 in non-obese diabetic (nod) mouse dramatically reduced their development of diabetes. We have recently extended oligonucleotide induced exon skipping to create ligand independent forms of nuclear hormone receptors including the glucocorticoid receptor and the vitamin D receptor (submitted for publication). Exon skipping was used to qualitatively alter CD45 to identify its role in anthrax pathogenesis (Panchal et al. 2009) and reduce expression of IL-10 to identify its role in Ebola virus pathogenesis (Touriol et al. 2003).

Multiple transcripts created from a single gene is a natural event. The diverse transcripts often lead to proteins that are functionally distinct. This leads to multiple phenotypes emanating from a single gene. Currently, bioinformatics scientists catalog the diverse transcripts. Several key features need to be illuminated; What are the qualitative functional differences in these related proteins from a single gene? Do these proteins gain new signaling pathways, sub-cellular localization, or post-translational modifications? What is the relative production of each of these different proteins? Can a very small amount of some of these proteins dominate in function over the more abundant variations? What regulates the selection for one variation versus another? Finally, what is the role of the environment in shifting the regulation of alternate exon use?

Alternate Translation Start Sites

Translation of mRNA can be initiated at alternative sites leading to protein diversity by creating several variations in the amino-terminus of proteins from a single transcript (Touriol et al. 2003). If the alternate start is in frame, the resulting protein is

either truncated or extended qualitatively altering protein location or function. If the alternate translation start is not in frame then a different protein is created. Alternate in frame proteins tend to vary the subcellular localization as suggested by Blobel in his 1999 Nobel Prize lecture (Blobel 1999). For example, basic fibroblast growth factor (FGF) is translated from 4-CUG and 1-AUG initiation codons encoding 34, 24, 22.5, 22, and 18 kDa proteins (Prats et al. 1989). The larger CUG initiated proteins contain nuclear localization signals but the AUG-initiated 18 Da protein is secreted acting on the transmembrane receptor in an autocrine or paracrine manner (Arese et al. 1999).

Regulation of translation is determined by initiation events including the: (1) m7G cap, (2) the length and composition of the 5'-UTR, (3) the context of the AUG-initiation codon, (4) the poly(A) tail, and availability of translation initiation factors. The AUG sequence context prefers 5'-gccGCCACCAUGG-3′ but will bypass if a purine is lacking at −3 or no G at +4 relative to the AUG (Kozak 1995). If the 5'-UTR is short then an alternative Translation Initiator of Short 5' UTR (TISU), 5′- SAASAUGGCGGC-3′ can regulate initiation (Elfakess et al. 2011).

The idea of enhancing protein forms with different amino termini can be exploited for therapeutic benefit. Steric blocking nucleic acid analog oligomers targeting the AUG translation start-site can shift the translation start-site to downstream locations in an mRNA. A 2'-O-methyl phosphorothioate oligomer, OGX-11, targets the initial translation start site of clustrin mRNA which encodes a soluble form of clustrin (sCLU) leaving the downstream translation start site encoding a nuclear form of clustrin (nCLU) intact (Saad et al. 2011). We found a phosphorodiamidate morpholino oligomer (PMO-M1) targeting the initial translation start site of p53 shifted translation initiation to codon 40 (M40) expression and function of p44 (ΔN-p53) and p48 (p53β/γ) isoforms resulting in the expression and function of p44 (ΔN-p53) resulting in ΔN-p53 that lacks transcriptional activity at the p21 promoter (Ma et al. 2011).

Variations in the amino terminus of proteins created from a single gene is also natural event. An estimate that half of all genes have multiple translation start-sites. These proteins can have diverging function and are likely to be under selection pressure. This process also leads to multiple phenotypes emanating from a single gene. Unfortunately, these proteins cannot be detected looking at the DNA or mRNA sequences so expression arrays are not useful. Many of the same questions remain regarding expression and function. What regulates the selection for one variation versus another? Finally, what is the role of the environment in shifting the regulation of alternate amino termini?

Premature Codon Suppression

The central dogma of molecular biology starts with genetic information stored in DNA that is converted into proteins through the translation of mRNA. Translation involves initiation, elongation, termination and recycling steps as a sustainable

cycle of cellular activities. During elongation, the pairing of tRNA anti-codons to mRNA codons occurs within the ribosomal A site accompanied by stringent proofreading steps that ensure highly accurate protein synthesis, translation. When one of the three termination codons (UAA, UAG, or UGA) enters the ribosomal A site translation termination occurs. The eukaryotic release factor 1 (eRF1) recognizes all three stop codons. eRF1 forms a complex with eRF3-GTP that triggers ribosomal conformational shifts that position the eRF1 GGQ motif near the peptidyltransferase center. ABCE1 ATPase and eIF3, eIF1, eIF1A, and eIF3j facilitate dissociation and ribosomal recycling of the termination complex. In addition, the 3′-poly(A)-binding protein (aka CTP) plays a role in termination.

Elegant studies reported over 50 years ago revealed a novel ribosomal adaptive response. Streptomycin (Sm), a molecule made by fungi kills bacteria by blocking translation. *Escherichia coli* (*E coli*), a Gram-negative bacteria found in the human gut, is generally sensitive to Sm exposure. A genetic strain of *E coli* with a genetic mutation that impairs its ability to synthesize arginine (arg), an essential amino acid, requires dietary arginine to live, an arginine auxotroph. Gorini and Kataja found that adding Sm to the *E coli* arginine auxotroph lacking arginine (Sm^+Arg^-) paradoxically led to a conditional Sm dependence (CSD) (Gorini and Kataja 1964). Two lethal events, arg- in an arg auxotroph and the antibiotic Sm, taken together result in survival. They went on to show that in CSD, the putative Sm^r mutation is equivalent to a suppressor (a mutation that reverses the phenotype produced by a gene mapping at a different locus). They went on later that year to show Sm upsets the genetic code by interfering with ribosome-mRNA complex. Poly-U directed incorporation of phenylalanine is blocked by Sm but incorporation of isoleucine (UUA, UAA, CAU) and to a smaller extent serine (UUC, UCC, AGC) and leucine (UUA, UUC, UUG, UCC) are stimulated by Sm (Davies et al. 1964). Further, both magnesium and ammonium ion also influence translation accuracy and in the presence of Sm and lower Mg^{2+} stimulation is enhanced. Sm disrupts a train of ribosomes on a single mRNA, polysomes, but increases the appearance of individual 70S ribosomes suggesting the misreading hypothesis, as not the primary bacteriocidal mechanism of Sm but selective misreading of premature termination codons, PTC, may be the mechanism for CSD (Kogur and Prizant 1974). Low concentrations of Sm cause phenotypic suppression but high concentrations stop protein synthesis (Tai et al. 1978). These studies provide evidence for a natural environmental molecule capable of altering the fidelity of translation can enhance survival adaptability.

Suppression of termination occurs at a rate of 0.001–0.1 percent at normal stop codons and 0.01–1 percent at PTCs. Suppression of stop codons is mediated by aminoacyl-tRNA mispairing with a stop codon and amino acids tryptophan, tyrosine and lysine have been observed to be present at the PTC site indicating near-cognate mispairing can occur at any of the three codon positions. The 16S rRNA (eukaryotic 18S) bases A1492 and A1493 (eukaryotic A1754 and A1755) flip out of the helix to interact with the minor groove of the codon-anticodon helix in site A and G530 flips from *syn* to an *anti* conformation. These interactions confirm base pair fidelity and cause the 30S subunit to shift from open to closed conformation and GTP hydrolysis by EF-Tu. EF-Tu rapidly dissociates from the ribosome and the

peptidyl transfer from the aminoacyl-tRNA to form a peptide bond with the growing peptide occurs. An aminoglycoside binds near A1492 and A1493 inducing the shift in the absence of tRNA in the A site. PTC sites are more sensitive to aminoglycoside binding because termination is slower at PTC sites due to less interaction with the poly(A) binding protein (PABP) (Keeling et al. 2012).

Premature termination codon (PTC) sites differ from normal termination. Nonsense mediated decay (NMD) is one aspect of the difference invoking activity of UPF1, UPF2 (NMD2), and UPF3 genes. UPF1 interacts with eRF1 and eRF3 to form a surveillance complex and will activate NMD if UPF2:UPF3 heterodimer is bound to a downstream exon junction complex (EJC). The EJC is in place downstream of a PTC during pioneer translation in the nucleus and thus remains associated with the transcript, marking it as an aberrant termination site. The paused ribosome allows Smg-1 to bind to UPF1 forming the Smg-1-Upf1-Release complex (SURF) with Smg-8 and Smg-9. SURF interaction with EJC-Upf2:Upf3 leads to ATPase and helicase activity. Once phosphorylated, the complex promotes RF release and ribosomal association with Smg-5-7 to initiate NMD (Peltz et al. 2013).

Nonsense suppression is a natural process with some specificity in sequence context, cell type and developmental stage. An interesting note is loss of fidelity or catalysis of translation in misreading tRNA with similar codon sequences. Streptomycin and other aminoglycosides (binding 16S rRNA of prokaryotes) promote ribosomal misreading at high concentrations by binding to 18S rRNA of eukaryotes. The binding near the A site negates discrimination against near-cognate tRNAs allowing misreading and nonsense suppression.

There are over 1800 inherited human diseases caused by nonsense mutations leading to the development of assays that used to screen for therapeutic agents capable of suppressing nonsense mutations for treating these human genetic disease (Shiozuka et al. 2010). Screening of or 34,000 compounds of potential PTC inhibitors led to 12 candidates (Du et al. 2009). Screening PTC structural analogues of aminoglycosides with 3500 compounds led to active candidates with minimal cytotoxicity and UAA and UAG readthrough and ultimately PTC124, Ataluren (3-[5-(2-fluorophenyl)-[1,2,4]oxadiazol-3-yl]-benzoic acid).

Ataluren is a 284 Da achiral, orally bioavailable compound with no structural similarity to aminoglycosides. Readthrough is highest at UGA > UAG > UAA showing signal at 0.01–0.1 μM and maximal activity at 3 μM. The *mdx* mouse has a UAA codon in exon 23 (Glu-stop) and PTC124 maintained at trough levels of 2–10 μg/mL (5–30 μM) led to production of full-length dystrophin at 20% or wild type levels. In Duchene Muscular Dystrophy (DMD) patients, PTC124 led to decreased muscle fragility. In a cystic fibrosis (CF) mouse model, PTC124 restored expression of CFTR. In Miyoshi myopathy (lack of dysferlin), PTC124 induced 15% of dysferlin in patient explant samples. In Hurler syndrome, a disease characterized by lack of iduronidase (IDUA) and results in accumulation of glycosaminoglycan's (GAG's), PTC124 led to dose-dependent changes in GAG levels at 10 μg/mL (30 uM). Additional nonsense targets in carnitine palmitoyltransferase1A deficiency (CPT1A), Usher syndrome, and Batten disease (CLN1 gene encoding palmitoy-protein thioesterase-1, PPT1) benefited from 5–30 μM PTC124.

Ataluren failed in some nonsense models and revealed over tenfold variation in efficacy depending on sequence context of the PTC. Anticipated effective concentrations between 2–10 μg/mL are achieved with three times a day dosing (TID). Ataluren is bioavailable (F = 0.55) and single doses of 3 to 100 mg/kg are tolerated but repeat doses reveal a limitation below 50 mg/kg taken twice a day (BID). Phase IIb studies of Ataluren for the treatment of Duchene Muscular Dystrophy at 10–20 mg/kg revealed 31 meters difference relative to placebo in the six-minute walk test (6MWT) after 48 weeks of treatment but 20–40 mg/kg was not effective. A Phase III study for the treatment of cystic fibrosis (CF) was not effective due to a drug interaction with Tobramycin. Finally, Ataluren failed approval by the FDA in 2016 for the treatment of DMD.

The search for PTC compounds that are effective, potent and safe continues. A skeptical point of view is that companies seeking financial support for drug discovery refer to translation fidelity suppression as premature codon suppression. The difference is in the lack of evaluation of the rate incorrect amino acids are incorporated into proteins at non-termination codon sequences. Experimental evaluation of this non-specific outcome is challenging because a specific position for the amino acid error would be relatively rare, on the order of 0.1 percent, and hence likely to fall below the limits of detection. However, the outcome of such an event would be significant because in a 500 amino acid protein with a 0.1 percent error rate would mean no homogenous protein would be synthesized. While a mildly defective protein would likely function in a manner similar to a homogenous prototype, the immune system is likely to find such altered proteins as "not self."

Alternate Termination Codon Use

The termination codons include amber (UAG), ochre (UAA), and opal (UGA). Recently, the opal terminator was found to code for the amino acid selenocysteine (Sec) when in the proximity of a selenocysteine incorporation sequence (SECIS) (Papp et al. 2007). The amber terminator can be translated into pyrrolysine (Pyl) also regulated by proximity of cis elements.

The ribosome may "slip" during translation causing a frame-shift to +1 or − 1. This leads to premature termination from codons that become termination triplets when frame shifted. These codons might be selected for in organisms with relatively unstable rRNA. The translation stop-signal ratio (TSSR) between the three reading frames is conserved, suggesting selection pressure to preserve the position of "hidden stops" in transcripts.

Termination of translation is associated with an error rate often leading to stop codon readthrough (RT). Studies with each of the terminator codons and dual luciferase reporters downstream from the terminator reveal the opal (UGA) has a higher basal readthrough and ochre (UAA) has the highest fidelity (Longhran et al. 2014). The base following the termination codon influences the efficiency of termination, for UGA a C adds efficiency, for UAG a C or U influences termination, and for UAA a C adds efficiency. Viruses and some pathogenic fungi exploit the consequence of RT Li and Rice 1993).

Codon Bias

There are up to six codons linked to translation of a single amino acid, an inherent redundancy in the code. Genomes show a strong preference for certain codons over synonymous alernatives, a property that may serve as a barrier between different species. Rare codons are enriched at the N-terminus of essential genes (Chen and Inouye 1994). Further, codon use strongly influences translation efficiency leading to different protein levels in the cell (Sharp et al. 1993). Codon bias may provide for post-transcriptional translational regulation (Li 2015), alter translational fidelity (Hooper and Berg 2000), and coordinate with control of tRNA pools (Gingold et al. 2014; Von de Haar and Tuite 2007).

RNA Editing

Cells can edit RNA polymers after synthesis causing insertions, deletions and base substitutions. Deamination of nucleotides can shift cytosine (C) to uridine (U) and adenosine (A) to inosine (I). RNA editing of a primary transcript can use a guide RNA (gRNA) to facilitate insertions and deletions in the edited transcripts inducing a frame shift in translated mRNAs. RNA editing can initiate RNA degradation. Finally, RNA editing can alter RNA structures and potential protein binding sites.

Apolipoprotein B is expressed as Apo B100 in the liver but in the intestines, a CAA sequence is edited to UAA, a termination codon, leading to a truncated B48 version. Adensine (A) to inosine (I) editing is seen in double stranded RNA (dsRNA) acted on by adenosine deaminase (ADAR). The I can be read as a G in translation, miRNA or siRNA recognition, and endonucleolytic cleavage by Tudor-SN. A reverse editing from U-to-C and G-to-A mRNA are observed in WT1 (Wilms Tumor-1) transcripts. While U-to-C may be the result of transamination mechanisms, the G-to-A is likely created by apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3A (APOBEC3A).

Over 100 million sites within most genes of the human genome are cataloged (Bazak et al. 2014). RNA editing can alter RNA resulting in a nonsynonymous mutation and an amino acid change in a protein sequence. Knockout mice for ADAR1 ($ADAR1^{-/-}$) are embryonic lethal at day 12.5 and $ADAR2^{-/-}$ mice die shortly after birth. The RNA loss of A-to-I editing lethality may be associated with the function of the brain glutamate-gated ion channels (Sommer et al. 1991). Editing pre-mRNA or pre-lncRNA can alter a splice site resulting in altered information in the processed transcript (Rueter et al. 1999). Analysis of 445 human lymphoblastoid cell lines identified 1054 NA editing events associated with cis genetic polymorphisms, some associated with complex traits or disease (Park et al. 2017).

RNA editing may serve to regulate RNA catalytic functions by interfering with miRNA biogenesis (Blow et al. 2006), nuclear retention of miRNA, and alteration of miRNA seed regions (Kume et al. 2014). No known RNA editing control or regulatory mechanisms are understood. A novel metformin (1,1-dimethy-biguanide) association with multiple RNA editing sites in the 3'-UTR region of the ataxia telangiectasia (ATM) transcript. The GWAS SNP rs11212617 is associated with the effectiveness of metformin in treating type 2 diabetes (GoDarts GUDPS 2011).

Epigenetics

Heritable changes in gene expression producing a phenotype that are not due to alteration in DNA sequence are considered epigenetic. DNA methylation is the most prominent form of epigenetic regulators. When the promoter region of a gene is methylated that gene's expression is often suppressed as if the expression driver switch has been turned off. This sort of regulation is key in development, genomic imprinting, aging, cancer, and X-chromosome inactivation. In addition to methylation, histone proteins that are associated with the genome are acetylated further regulating gene expression.

A compelling example of epigenetic activity is the Dutch Famine Cohort Birth Study. A German blockade of food and fuel to part of the Netherlands in World War II led to food insecurity to approximately 4.5 million residents. The food rations fell below 1000 calories a day and 22,000 deaths due to starvation. The famine cohort study found that children of pregnant women during the famine had higher frequencies of diabetes (De Rooi et al. 2006; Ravelli et al. 1998) obesity (Ravelli et al. 1999), cardiovascular disease (Painter et al. 2006), microalbuminuria and other health problems. When children of the famine (F1) era pregnant women grew up and had their own children (F2), their children were also smaller than average (Painter et al. 2008). These epidemiological studies reveal heritable phenotypes associated with famine are epigenetic in nature.

Diethylstilbestrol (DES) was the first orally active synthetic estrogen administered to pregnant women from 1940 to 1971 to reduce the risk of spontaneous abortion (Herbst et al. 1971). DES causes a rare clear cell carcinoma vaginal cancer in young women exposed to the drug *in utero*. Expansion of epigenetic transgenerational health effects were documented for decades. A mouse model of DES exposure identified dysregulation of DNMT3A, MBD2, and HDAC2 likely due to activation of the estrogen receptor alpha (ERα) (Li et al. 2014). This example highlights the potential for environmental exposure to alter the human phenotype for multiple generations.

Nucleic Acid Methylation and Demethylation

Methyl groups added to cytosine (Evans and Evans 1970) and at times adenine (Ratel 2006) result in altered "activity" of a DNA region without changing the base sequence. Methylation is essential for development and associated with genomic imprinting, X-chromosome inactivation, repression of transposable elements, aging and carcinogenesis. The methyl group is added to the 5 position of cytosine creating 5-methylcytosine, the same pyrimidine location of the methyl addition to uracil to create thymidine. Spontaneous deamination of 5-methylcytosine creates thymidine leading to a T:G mismatch in the DNA genome (note: deamination of C leads to U which is rapidly recognized and repaired by the cell). If this mismatch is not repaired, replication of DNA will fix the mutation turning the C:G pair into an A:T pair. This spontaneous pathway to mutation may lead to selection against CpG dinucleotides in the genome as they occur at 21 percent of the expected frequency (Int Human Genome Sequencing Consortium 2001). The DNA methyltransferase enzymes almost exclusively act on CpG dinucleotide sequences creating 5-methylcytosine on both DNA strands. Non-CpG methylation of CpApC trinucleotide sequences is observed in embryonic stem cells (Haines et al. 2001), neural development (Lister et al. 2013), and hematopoietic progenitor cells (Kulis et al. 2015).

Oxidation of 5-methylcytosine mediated by the Tet family of enzymes creates 5-hydroxymethylcytosine (5hmC) (Tahiliani et al. 2009) which is abundant in the human brain (Kriaucionis and Heintz 2009) and embryonic stem cells. 5hmC is converted to unmodified cytosine by DNA demthylation enzymes suggesting a role in regulating levels of 5-methylcytosine. 5hmC destabilizes nucleosome structures leading to repositioning, an event associated with cell differentiation (Teif et al. 2014). Oxidation of 5hmC to 5-Formylcytosine is seen in embryonic stem cells (Pfaffeneder et al. 2011) which may be an intermediate in the demethylation process.

N^6-Methyladenosine (m^6A) is often found in mRNA, tRNA, snRNA and lncRNA. m^6A functions in mRNA include: (1) 3'-UTRs are associated with microRNA (miRNA) binding sites, (2) enhancement of stop codons (Meyer et al. 2012), (3) enhance selection of polyadenylation sites, and (4) enhance mRNA turnover (Wang et al. 2014). m^6A modifications in DNA have been mapped to over 7000 human genes at (G > A)m^6ACU motifs. The Mettl3 is an adenosine methyltransferase . The functions of the m^6A in DNA appears to modulate circadian periods and alternate splice site regulation (Fustin et al. 2013).

7-methylguanosine (m^7G) serves as the capping group on the terminus of mRNA providing a binding site for the eukaryotic translation initiation factor 4E (eIF4E) (Sonenberg and Gringas 1998). The 5′-end of GTP is added to the 5′-end of the pre-mRNA in the cell nucleus creating an unusual 5′-5′ triphosphate linkage followed by S-adenosylmethionine (SAM) methylation of N^7 of the guanine (Shatkin 1976). SAM creating cap 1 and cap 2 as 2'-O-methyl RNAs can also methylate the adjacent two riboses. Sm-class snRNAs have 5′-trimethylguanosine caps of m^7G and

Lsm-snRNAs have monomethylphosphate m^7G caps (Matera et al. 2007). Bacteria often use NAD+, NADH or 3′-dephospho-coenzyme A (Bird et al. 2016). The capped transcripts are generally resistant to nucleases, particularly 5′-exonucleases but the 5′-exonuclease Xrn1 specifically removes the cap in eukaryotes.

X-chromosome inactivation is seen in most female mammals. The have two X-chromosomes but are cellular mosaics in that they only use one in any given cell. Inactivation begins during gastrulation of the embryo when methylation of a long non-coding RNA, Xist, suppresses expression in the active X-chromosome, Xa. The inactive X-chromosome, Xi, continues to express Xist which essentially coats the Xi leading to suppression of transcriptional activity. The X-inactivation explains curious phenotypes such as the female only three-color cat, either a calico or tortoise shell phenotype. The X-inactivation also explains the offspring of a horse and a donkey in which if the male is a donkey the resulting offspring is a mule but if the male is a horse, the offspring is a hinny. This is also seen in the lion-tiger hybrids if the male is a tiger the offspring is a tygon, but if the male is a lion the offspring is a liger. The mule does not look like a hinny and a tygon does not look like a liger.

Duchenne muscular dystrophy (DMD) is an X-linked recessive condition caused by mutations in the dystrophin gene. DMD usually affects males because they only inherit one copy of the dystrophin gene so if it carries an inactivating mutation the boy will have the condition. Female carriers are heterozygotes and often thought to be asymptomatic but if one X-chromosome is inactivated, they should manifest the condition. The initial estimates of symptomatic carriers was in the range of 2.5 to 7.8 percent (Moser and Emery 1974; Norman and Harper 1989) but when cardiac presentation were evaluated, the carrier proportion increased to 22 percent (Hoogerwaard et al. 1999). The frequency of less than 50 percent points to non-random X-inactivation. This is plausible based on different patterns of X-inactivation in pairs of monozygotic female twins heterozygous for dystrophin gene mutations (Pena et al. 1987). A more detailed investigation into X-inactivation patterns revealed skewed X-inactivation in 2 of 6 symptomatic carriers (expect 0/6) and 5 of 11 asymptomatic carriers (expect 11/11) and 6 of 17 carriers were symptomatic (expect 8.5 if random Xi) suggesting the two mechanisms, patterns of X-inactivation and transcriptional behaviors of DMD are regulated independently (Brioschi et al. 2012). Creating human induced pluripotent stem cells, iPSC, from a symptomatic carrier lost X-inactivation and when differentiated into myotubes they were XaXa (Miyagoe-Suzuki et al. 2017). Questions remain concerning how epigenetic marks are manipulated for therapeutic purposes and by environmental exposures will be discussed later.

Histone Acetylation and Deacetylation

Genomic DNA is assembled into nucleosome structures composed of 147 base pairs of DNA coating a core of 4 pairs of histones, H2A, H2B, H3, and H4. The histone tails lie in the minor groove of DNA and the amino groups of lysine amino acids are

acetylated leading to increased transcription and deacetylated leading to decreased transcriptional activity. Histone acetyltransferase (HAT) enzymes are add two-carbon acetyl groups changing the net charge from positive to neutral disrupting histone association with DNA. Conversely, histone deacetylase (HDAC) enzymes remove the acetyl groups restoring the net positive charge and enhancing associations with DNA.

Epigenetic Drugs

Epigenetics is defined as heritable alterations in gene expression and chromatin structure due to chemical modifications but not changes in the primary nucleotide sequence (Nightingale et al. 2006). Epigenetic inheritance is mediated by: (1) DNA methylation, (2) histone modifications, and non-coding RNAs (Feinberg and Tycko 2004). Core histones, H2A, H2B, H3 and H4 form an octamer structure in which 146 base pairs of DNA wrap around the pairs of 4 basic proteins forming a nucleosome. Histone H1 coordinates the repeating nucleosomes into higher order structures. The N-terminal nucleosome protein tails are then modified by acetylation, methylation, phosphorylation, ubiquitination and ADP-ribosylation with acetylation the most extensively investigated (Nebbioso et al. 2012). Different histone modifications act combinatorially as a "histone code" leading to unique functional outcomes (Sahl and Allis 2000; Jenuwein and Allis 2001).

Histone and DNA modifying drugs are classified into chemical structure categories (Table 10.5):

G-Quartets

Guanine quadruplexes (G-quartets) are four-stranded nucleic acid structures that can form in DNA and RNA. They are emerging as versatile regulatory elements of transcription (Agrawal et al. 2014), translation (Beaudoin et al. 2010), genomic stability, cellular signaling, and alternative splicing. G-quartets are associated with chromatin remodeling; (Hansel-Hertsch et al. 2016) 5'-UTR region translational regulation of genes including the estrogen receptor (Balkwill et al. 2009), TGF-β (Agarwala et al. 2013), FGF (Bonnal et al. 2003); 3′-translational regulation of PITX1 (Ariyo et al. 2015); and are adjacent to mitochondrial deletion breakpoints (Dong et al. 2014).

G-tracts are overrepresented in RNA near splice sites (Xiao et al. 2009). The G-tracts form G4-RNA structures that contribute to alternate pre-mRNA splicing. G4 structures located within introns have been observed as splice enhancers as seen

Table 10.5 Summary of epigenetic drugs; investigational to approved

Histone deacetylase Inhibitors HDACi			
Hydroxamic acids	Trichostatin A (TSA) Yoshida et al. (1990)		1990
	Suberoylanilide hydroxamic acid (SAHA) Richon et al. (1998)		1998
	Pyroxamide Butler et al. (2001)		2001
	Vironostat	Adv T-cell lymphoma	FDA 2006
	Panobinostat (LBH589)	Non-Hodkin's lymphoma	2008
	CHR-3996	Advanced Tumors	2010
	CHR-2845	Heme & Lympoid malig.	
	SB939	Solid tumors, MDS	2010
	ITF2357 (Givinostat)	Hematoligic malignancy	2010
	PXD101 (Belinostat)	DMS, MM	2011
	JHJ-26481585	Leukemia, MDS	2009
Cyclic tripeptides	2S,9S)-2 amino-8-oxo-9,10-epoxy-decanoyl (trapoxin A)		
Cyclic tripeptides	Depsipeptide (Romidepsin)	T-cell lymphoma	FDA 2012
Short-chain fatty acids	4-phenylbutyrate	Lymphcytic leukemia, AML, MDS, melanoma	2005
	Valpric acid		2009
	Pivaloyloxymethyl butarate (AN-9)	CLL, MM, NSCLC	2007
Benzamides	MS-275 (Entinostat)	Heme Malig., MDS, Mel	2004
	MGCD0103 (Mocetinostat)	Leukemia, lymphoma	2008
Sirtuins (Sir2)	Splitomicin	Neurodegenerative disease (ALS)	
	Tenovins		
		Inflammatory disease	
	AGK2	Autoimmune disease	
	Sirtinol		
	Suramin		
	EX-257		
	Salermide		
	UVI5008		
	Quercetin		
	Piceatannol		
	Resveratrol		
Miscellaneous	Depudecin		
Histone Acetyltransferase Inhibitors (HATi)			
GNAT (Gcn5 N-acetyl)		None in clinical trial	
MYST		None in clinical trial	
P300/CBP	Curcumin	Atopic asthma, COPD	1997
Histone methyltransferases (HMTi)			

(continued)

Table 10.5 (continued)

S-adenosylmethionine	SAM- nonspecific		2009
Chaetocin			
BIX-01294			
UNC0224			
3-deazaneplanocin A			
2-PCPA	Tranylcypromine	Inhibit LSD1	2007
DNA Methylransferase inhibitors			
Nucleoside analogs	Decitabine (5-aza-2'-deoxycytidine)		2002
	Vidaza (5-azacytidine)		FDA 2011
Small Mol. DNMTi	Hydralazine		FDA app
	Procainamide		FDA app
	RG108		2005
Natural DNMTi	Psammaplins (marine sponge) Tea polyphenols (EGCG)		
	Bioflavonoids (fistin, genistein)		
Antisense DNMTi	MG98		2013
Non-coding RNA			
miRNA	MiR-29b		

in Pax9 intron 1 (Ribeiro et al. 2015), TP53 intron 3 (Marcel et al. 2011), alpha/beta tropomycin (Sirand-Pugnet et al. 1995), and thyroid hormone receptor (Munroe et al. 2015) as well as splicing silencers as in proinsulin intron 1 (Kralovicova et al. 2014). G4 structures located within exons may lead to exon exclusion from mature mRNA as seen in FMRP exon 15 (Didiot et al. 2008) as well as slicing enhancement as in BACE1 (Fisette et al. 2012). The splicing influence is mediated by recruitment of the ss-RNA binding proteins hnRNP F/H containing three quasi-RNA recognition motifs (qRRMs) specifically recognize G4 cis-acting elements. The qRRM bind RNA in a single-stranded form preventing the formation of a G4 structure (Samatanga et al. 2013).

The CYP3A5*1 is a functional allele but CYP3A5*3 alleles have a 6986A > G mutation in intron 3 that leads to a cryptic splice acceptor and nonsense mediated decay of the aberrant mRNA (Lamba et al. 2002; Xie et al. 2004). The common variant CYP3A5*3/*3 is thought to be null for functional protein but individuals can express wild type CYP3A5 (Lin et al. 2002). Our observations indicate a G-quadruplex in CYP3A5 intron 3 can restore reference protein expression in CYP3A5*3/*3 genotypes by altering splicing.

Therapeutic exploration of G-tracts and G-quartets has been ongoing for nearly 25 years. Initially, an oligonucleotide aptamer was found to bind thrombin as a novel anticoagulant (Griffin et al. 1993). The G-quartet was fond to have anti-proliferative activity as an untargeted activity of antisense oligonucleotides (Burgess

et al. 1995). Indeed, numerous proteins selectively bind to G-quartets in DNA and RNA (Brazda et al. 2014) expanding the potential diverse activities of G-quartet therapeutics. Antisense oligonucleotides have been developed to disrupt G-quartets leading to translational switches (Rouleau et al. 2015). One of the most advanced therapeutics, Imetelstat (GRN163L), inhibits telomerase for the treatment of malignant neoplasia. A variety of small molecules bind G-quartets or disrupt their four stranded structure including BRAC019, HXDV, Telomestatin, and TMPyP4 (Table 10.6).

Table 10.6 G-quartet/telomerase inhibitors

Compound	Strengths	Limitations
Imetelstat (GRN163L)	Allosteric telomerase inhibitor	Limited efficacy in tumors with short telomeres.
	IC_{50} = 50–250 nM	
	Clinical efficacy against breast cancer (Ph II), NSCLC (Ph II), multiple myeloma and pancreatic cancer (Ph I)	Limited dose escalation; MTD 9.4 mg/kg
5′-d(TpTpApGpGpG)-3’	Natural DNA	G4 nonspecific (Δ transcription, pre mRNA splicing)
AS1411 [5′-d(GGTGG)$_4$–3′]		TLR inhibition-Δ immune response
		Nuclease sensitive- unstable
5′-d(TsTsAsGsGsG)-3	Nuclease resistant	G4 nonspecific (Δ transcription, pre mRNA splicing)
	IC_{50} = 300 nM	TLR inhibition-Δ immune response
		Limited dose escalation; MTD ~8 mg/kg
BIBR1532	IC_{50} = 100 nM for telomerase	EC_{50} = 30–80 μM
		Stabilize G4 – TLR inhibition?
		G4 nonspecific (Δ transcription, pre mRNA splicing)
Telomestatin	IC_{50} = 5 nM	Stabilize G4 – TLR inhibition?
	Activate p53 ± induce p16/RB	G4 nonspecific (Δ transcription, pre mRNA splicing)
BRACO19	IC_{50} = 100 nM; CC_{50} = 10–25 μM	Stabilize G4 – TLR inhibition?
	Combinations with Flavopiridol (PhII)	G4 nonspecific (Δ transcription, pre mRNA splicing)

The G-quartet structure overlaps in function with epigenetic regulators, alternate translation start-sites, and alternate exon use. The structures are encourages to form in the presence of potassium and sodium ions but lithium ions tend to disrupt the formation of the structures. This may shed light onto the mechanism of action of lithium for the treatment of bipolar syndrome. It may also represent a cellular monitor of physiological stress and pain sensation with shifting potassium concentrations. The potential for heme interactions remains to be explored in detail. In each case, the role of chemicals in the environment and their interaction with G-quartets deserves attention. The guanine components of a G-quartet are especially sensitive to formation of oxidative products, 8-oxo-guanine, which is likely to disrupt G-quartet structure. Reactive chemical compounds form guanine adducts that are likely to influence the structure formation and disrupt existing G-quartets. Sensitivity of guanine to chemical modification may point to the G-quartet as a component of an adaptive response.

Conclusion

The environment redefines the individual. All living things are exposed to an environment. Most environments are constantly changing. The genetic tools available to adapt to the changing environment must accommodate small and large environmental changes, which may span short or long durations. The evolutionary mechanism in which the most fit offspring will survive favors rapidly dividing viruses and bacteria over humans in a rapidly changing environment. This may explain the profound threat of emerging infectious disease.

The historic drug discovery process has exploited molecules in the environment capable of interfering with genome integrity, translation fidelity, epigenetic regulation, and protein binding. Re-evaluation of how chemicals in our environment bring about benefit or toxicity often leads to a new appreciation of the flow of information from the genome to phenotype. One example is the “endocrine disruptors” such as the introduction of potent estrogens into the sewers leading to environmental impact of birth control treatments. A curious observation that with increasing diversity of potent man made pollutants entering the environment since the 1960s has been accompanied by the greatest extension in life expectancy. One of the bountiful explanations is that we are capable of remarkable and relatively rapid adaptation.

Consider

Information from a single base pair to a genome. Quantitative information content in a message is inversely proportional to the occurrence of the message, *p*. An infrequent message would be associated with larger informational content so that $p(x_i)$ is the probability of a message with occurrence of x_i. The informational content of that message content is I_i so that the message content in bits is given by:

$I_i = \log_2(1/p(x_i))$

If we consider the human genome with 3 billion base pairs composed of four bases, A, C, G, and T the probability of any base at a specific position in the genome is $(0.25)^{3 \times 10e9}$ or $p(x_i) = \log 0.25 * \log 3 \times 10^9$ or

(-0.602 * 9.477) = -5.706 antilog value is $1.968 * 10^{-6}$. Hence, the informational content is $1/1.968 * 10^{-6}$ or the $\log_2$ of $5.082 * 10^5 = 18.955$ using a web based calculator. So the informational content of the genome is about 19 bits. The equivalence of 1 bit of information to entropy is given by kln2, where k is the Boltzman constant. The bit is given by Eg ~ kT ~ hc/R where R is defined in terms of the size of the Universe. Since the Universe is expanding at an ever-increasing rate, the enthalpy and thus informational content of a single base pair is decreasing. Best estimates are that 1 bit is also the minimum energy in the Universe equivalent to $\sim 10^{-45}$ ergs and an equivalent mass of $\sim 10^{-66}$ g and $\sim 10^{-29}$ °K. The enthalpy of a human genome is then $19 * 10^{-29}$ °K or about $19 * 10^{-29} = 1.9 * 10^{-27}$ °K.

While this seems like an insignificant number, the critical point is the human genome has quantifiable information. Further, this is 19 times greater than the cosmic background radiation. Consider a human population of 6 billion genomes ($6*10^9$). The equivalent is $1.14*10^{-17}$ °K.

Seeking a mechanism for rapid adaptation tends to exclude evolution embodied by a collection of DNA mutations over generations. The proposed adaptation mechanism is in the processing events that occur after DNA is transcribed. The adaptability process is "analog genetics" in which a single gene encoded in our genome can produce an extensive variety of products and a nearly infinite collection of parallel phenotypes. This is a logical extension of the progress of science given the recent emergence of next generation sequencing technology.

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Chapter 11
Eteplirsen

Abstract Once we observed alternate exon use in the mouse model of Duchene Muscular Dystrophy, the basis for molecular resilience was established. The application of antisense oligomers to enhance resilience was set in motion. Manipulation of exon exclusion/inclusion for therapeutic benefit in individuals with rare genetic diseases holds broad potential.

Keywords Duchenne Muscular Dystrophy (DMD) · Induced exon-skipping · Orphan products development · Prescription Drug User Fee Act (PDUFA) · Food and Drug Administration Safety and Innovation Act (FDASIA)

Introduction

I feel compelled to write the story of a drug with a generic name that includes my initials and most of my name.[1] The story typifies arrogance in science, shifting agendas in drug development, and the fact that it takes a large team with diverse skills to extend discovery to drug approval. The history of Duchenne Muscular Dystrophy is littered with names of discarded discoverers and so I am honored to join this distinguished group of the forgotten.

The Discovery of Duchenne Muscular Dystrophy

Charles Bell (1774–1842), an Edinburgh surgeon-anatomist, is best known for describing paralysis of facial nerve or Bell's palsy. Bell published a study in 1830 of an 18 year old man with progressive muscle wasting and weakness (Bell 1830) that began at age 10. This is the first published report of what was likely Duchenne muscular dystrophy.

[1] This is an inside joke since generic drug names should not contain recognizable references. The naming committee acknowledged my role in creating the project while recognizing my role being edged out by new management.

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Gaetano Conte with the help of Dr. L Gioja described cases of two boys with severe pseudo-hypertrophic muscular dystrophy in a paper published in the Neapolitan Medical Journal (Conte and Gioja 1836) in 1836. The two boys were brothers with characteristic enlarged calf muscles in whom disease onset occurred at age 8. The article, written in Italian, was overlooked due to a lack of social co-operation among scientists at the time.

Edward Meryon, a physician trained at the University College in London, reported the first systematic study of pseudohypertrophic muscular dystrophy. The name referred to enlargement, pseudohypertrophy, of the calf muscles that was consistently observed in the early stages of the disease. Meryon reported his observations of eight affected boys and their nine healthy sisters from three families at the Royal Medical and Chirurgical Society in December 1851. Mr. Partridge presented the case of the oldest brother in the second family to the Pathological Society of London in 1847. A necropsy revealed widespread fatty degeneration of the muscles. The first family had four affected boys and six healthy girls, the oldest boy died at age 16. Meryon described the autopsy where, "The chief structural change existed in the system of the voluntary muscles, which was throughout the entire body atrophied, soft, and almost bloodless; and, although the muscular fibers appeared to exist, yet were they not of that deep red colour as seen in the healthy and natural state." Meryon extended his observations to include six families publishing a monograph (Meryon 1864) in 1864. Meryon's significant contributions to the greater understanding of this disease has been acknowledged.

Dr. W J Little at the London Hospital reported in 1853 of brothers aged 12 and 14 that had been unable to walk from age 11. The older brother died at age 14 and necropsy examination showed muscle tissue had largely been replaced by fat (Little 1853).

Guillaume Benjamin Amand Duchenne described in detail the disease that would become associated with his name in 1861 (Duchenne 1861) and 1868 (Duchenne 1868). He claimed Meryon confused his cases with progressive muscular atrophy, an error Meryon corrected but credit had already shifted to the Frenchman. The first 13 cases described by Duchenne included 2 young girls. Duchenne invented the "harpoon," a needle designed to perform percutaneous muscle biopsies to improve diagnosis and understanding of the disease.

Griesinger described the histology, microscopic anatomy, of pseudo-hypertrophy (Griesinger 1865) in 1865 gaining recognition in the disease discovery. The microscopic view confirmed muscle destruction, repeated attempts of repair and fat accumulation within the muscle.

The hereditary basis of the disease was described by Gowers (Gowers 1886). "The disease is thus transmitted by women who are not themselves its subjects, thus the congenital tendency is exclusively due to the maternal element in the embryo. This is also shown by another fact, that the children of the same women, by different husbands, have been affected." The characteristic movements' children display when raising themselves from the floor are known as Gower's maneuver.

Peter Emil Becker and Franz Kiener (Becker and Kiener 1955) described a late onset and more benign course of progressive symmetrical muscle weakness and

atrophy often associated with calf hypertrophy and weakness of the quadriceps. They reported on five family members that may not require a wheelchair and if they did, this was not observed before the age of 16. This disease became known as Becker muscular dystrophy.

Louis M. Kunkel and his associate, Eric P. Hoffman, identified the 427 kDa dystrophin (Hoffman et al. 1987) protein and the gene that if mutated leads to DMD as well as its location on the X chromosome Xp21.2 (Kunkel et al. 1989). Kunkel also found mutations/deletions in dystrophin produced both Duchenne and Becker's muscular dystrophies with the differentiating feature being that Becker's mutations leave the mRNA reading in frame so a protein is produced (Hoffman et al. 1988).

The patient perspective of DMD begins between ages 1 to 6 with developmental delay. Symptoms are not definitive at this stage, perhaps just a little late transitioning from a crawl to upright walking. A little later in life a child has difficulty in standing from a position on the floor. The first symptom that is likely to lead to a diagnosis between age 3 and 5. Progressive changes in walking ability follow ending in requirement for a wheelchair generally near age 12. The loss of ambulation is followed by limited use of the arms within a year or two. The muscles of the diaphragm weaken in the late teens, which leads to the need for ventilation at night followed by ventilation 24 h a day. Supportive medical treatments can extend life well beyond the twenties into the thirties but the heart muscle fails leading to death.

I had read of these clinical facts when we first considered the DMD project in 2005. Over time the patient reality came into focus as I met parents and boys with DMD. The boys have trouble keeping up with their friends between 3 and 5 years old. Abnormal jumping and hopping accompanied by use of hands to climb up themselves when they get up from the floor, the Gower's maneuver. Muscle weakness in the legs may mean leg braces to support standing and walking. Progression to a wheelchair is a milestone event in the disease but much more. Boys are well aware of their fate by this time and would do anything to delay this event, a point of diminished hope. Sitting in a chair all day makes joint contractures rigid and progressive scoliosis can lead to surgery to cut functional muscles that cause curvature of the spine because the rate of muscle degeneration is not always equal on both sides of the spine. It isn't enough that activity is diminished, pain is an obligate part of progression. Life with a ventilator is associated with increased frequency of respiratory infections. The more familiar one becomes the more this disease seems unfair. What deed could mean a person should be destined to this hell on earth. I developed a sense of pride in simply being associated with a project to help boys with DMD.

DMD Genetics

DMD effects one in 3500 male births resulting in a prevalence of 35,000 to 40,000 cases in the United States. Dystrophin is one of the largest genes in the human genome spanning 2.5 million base pairs (0.08% of the human genome). The gene is

divided into 78 exons with 11,031 coding bases that are translated into 3677 amino acids and a 427,000 Dalton protein. Mutation frequency leading to DMD involves *de novo,* newly created, mutations in two of three cases so genetic counselling is not effective in eradicating the disease. The mutations are not distributed across the gene uniformly or in a random pattern as might be expected. The region from exons 46 to 53 carry the highest frequency of mutations, the reason for this is unknown. Roughly, 70% of mutations are missense or frame-shifting mutations, 15% are nonsense mutations resulting in a premature termination codon, and 15% are insertions and duplications of the dystrophin gene segments.

Duchenne muscular dystrophy (DMD) is an X-linked recessive condition caused by mutations in the dystrophin gene. DMD usually affects males because they only inherit one copy of the dystrophin gene so if this single copy carries an inactivating mutation the boy will have DMD. Female carriers are heterozygotes and often thought to be asymptomatic. However, X-inactivation is a random process that occurs in the first 2 cell divisions of an embryo. If the X-chromosome carrying the healthy copy of dystrophin is inactivated, the active X-chromosome carrying dystrophin mutations should manifest DMD in the female. The initial estimates of symptomatic female carriers was in the range of 2.5 to 7.8% (Moser and Emery 1974; Norman and Harper 1989) but when cardiac presentation was evaluated, the carrier proportion increased to 22% (Hoogerwaard et al. 1999). The frequency of less than 50% points to non-random X-inactivation or perhaps X-inactivation involved both dystrophin carrier X and healthy X in different daughter cells of the zygote division. This is plausible based on different patterns of X-inactivation in pairs of monozygotic female twins heterozygous for dystrophin gene mutations (Pena et al. 1987). A more detailed investigation into X-inactivation patterns revealed skewed X-inactivation in 2 of 6 symptomatic carriers (expect 0/6) and 5 of 11 asymptomatic carriers (expect 11/11) and 6 of 17 carriers were symptomatic (expect 8.5 if random Xi) suggesting the two mechanisms, patterns of X-inactivation and transcriptional behaviors of DMD are regulated independently (Brioschi et al. 2012). Creating human induced pluripotent stem cells, iPSC, from a symptomatic carrier lost X-inactivation and when differentiated into myotubes they were XaXa (Miyagoe-Suzuki et al. 2017). Questions remain concerning how epigenetic marks are manipulated for therapeutic purposes and by environmental exposures.

Discovery

Science is a process that builds on scientific accomplishments of the past. Often those accomplishments arise out of simple curiosity and other developments are solutions to problems. The DMD project involved multiple threads of scientific discovery.

One path of discovery has been richly rewarded with Nobel Prizes. Watson, Crick, and Wilkens reported the structure of DNA involving a helical twist two strands of nucleic acids paired by hydrogen bonds in 1953 and a 1962 Nobel Prize

(Nobel Prize 1962). The discovery launched an era of subsequent discoveries and stimulated philosophies of life. The relationship between DNA and proteins led to work by Tatum and Beadle suggesting one gene for one protein in 1958, an observation eclipsed by advancement of science (Nobel Prize 1958). Polymerase enzymes capable of synthesis of DNA and RNA won a Nobel Prize for Arthur Kornberg in 1959. Jacques Monod explored the regulation of gene expression to explain events that trigger DNA to make RNA in sharing a 1965 Nobel Prize (Nobel Prize 1965). The solution to the genetic code came from Holley, Khorana, and Nirenberg in 1968, in which three nucleotides operate together to encode a single amino acid and the role of transfer RNA (tRNA) required to translate each of the 64 possible triplet codes into 20 amino acids and 3 termination codons (Nobel Prize 1968). I remember the surprise when Roberts and Sharp won the Nobel Prize for finding split genes in 1993, the discovery of introns and exons as well as pre-mRNA processing (Nobel Prize 1993). These accomplishments provided the base of science leading to the modern era of molecular biology.

Robert Letsinger created building blocks for solid support DNA synthesis enabling automated DNA synthesizers in 1969 (Letsinger and Ogilvie 1969). I was fortunate to have been involved in installing our first DNA synthesizer at the Eppley Institute for Cancer Research as a postdoctoral fellow in 1985. We made short 20-base oligonucleotides as primers necessary for the polymerase chain reaction pioneered by Kary Mullis and rewarded by a Nobel Prize in 1993 (Nobel Prize 1993). I collaborated with Mario Stevenson to synthesize DNA conjugated to peptide delivery molecules promoting cell uptake to block human immunodeficiency virus (Stevenson and Iversen 1989). Chemists then began innovation leading to modified nucleic acids with a notable discovery of phosphorothioate (PSO) DNA by Gerald Zon and his team in 1985 (Matsukura et al. 1987). The PSO modification is subtle in that one of the non-bridging oxygens bound to the phosphate backbone is replaced by sulfur. This single atom switch triggered the era of oligonucleotide therapeutics because the PSO is resistant to degradation in biological systems.

My training as a pharmacologist led to interest in the "drug-like" properties of the PSO and a chance meeting with Jerry Zon in 1987. Jerry shared my interest and had recently left the FDA to take a position at Applied Biosystems to explore PSO as therapeutics. Jerry conducted studies using PSO to block the emerging human immunodeficiency virus and was on the cutting edge of therapeutic development. The initial question was how PSO would behave in an animal. We initiated pharmacokinetic studies by first developing a method to synthesize radioactive PSO using ^{35}S (Stein et al. 1990). Our first report of the pharmacokinetics of a PSO came in the early 1990s and the favorable "drug-like" properties advanced candidate PSO toward clinical development (Iversen et al. 1994). We explored the limiting toxicities of PSO as we considered an anticancer PSO targeting p53 finding the compounds bind alpha-adrenergic receptors causing a transient loss of blood pressure following bolus intravenous injections (Cornish et al. 1993). We also found a variety of off-target activities of PSO attributed to the strong ionic character of the sulfur (Egan et al. 1991). We could avoid hypotension by using slow intravenous infusions and proceeded to prepare the first investigational new drug application (IND) to the

Food and Drug Administration (FDA) for systemic use. Our clinical trial with acute myelogenous leukemia began in 1992 (Bayever et al. 1992) and encouraging observations reported in 1993 (Bayever et al. 1993). The era of therapeutics designed to interfere with RNA function began.

Alternative splicing was first reported in adenovirus transcripts in 1977 (Berget et al. 1977). Viruses often create maximum diversity from genomes of limited size so alternate splicing leads to one gene with multiple mRNAs encoding different proteins. Calcitonin was the first human gene found to be alternately spliced reported in 1981. Calcitonin is encoded in exons 1–4 of the genes 6 exons but an mRNA with exons 1–3 joined to exons 5 and 6 (excluding exon 4) encodes calcitonin gene related peptide (CGRP) (Rosenfeld et al. 1981). Closer examination reveals every multi-exon gene in the human genome will make alternate spliced mRNA (Lee and Rio 2015).

Decades of studies revealed a splicing structure, the spliceosome, composed of small nuclear ribonucleoprotein (snRNP) complexes with enzymatic activity cutting and joining RNA. A specific splice site is defined by the net effect of cis-acting regulatory sequences located in introns and exons with either positive (splicing enhancers) or negative (splicing silencers) influence on splice site selection. In this way, each splice site is associated with a relative splice site potency that can be influenced by tissue specific factors and chromatin modifications.

A single base mutation referred to as a single nucleotide polymorphism (SNP) can influence alternative splicing. The genetic blood disorder, β-thalassemia, is caused by SNPs that alter normal splicing of β-globin pre-mRNA (Weatherall 1994). Mutation in the G at the first position in intron one, also known as the splice donor site, activates cryptic splice acceptor sites within the intron. Mutations within intron 2 will create an active splice acceptor and insertion of intron sequence in the mature mRNA that leads to either nonsense mediated decay of the mRNA or synthesis of an aberrant or diminished expression of the β-globin protein. These cryptic splice sites gain relative splice site potency as a result of the SNP.

Ryszard Kole created a 2'-O-methyl PSO targeting the β-globin cryptic splice-site in intron 2 to shift splicing back to the appropriate splice acceptor in exon 3 in 1993 (Dominski and Kole 1993). These studies demonstrated an antisense oligonucleotide could interfere with splicing factors recognizing cryptic splice sites in the pre-mRNA within the nucleus and restore correct splicing. In 2001, Steve Wilton and his team adapted the antisense strategy to shift a normal splice site in the dystophin pre-mRNA to induce an abnormal splicing event. They used 2'-O-methyl PSO to induce exon skipping to exclude exon 23 of dystrophin from the mdx mouse that contains a termination codon to permit synthesis of a dystrophin protein (Mann et al. 2001). The seeds of discovery now assembled so time to grow.

Bench to Bedside

The Wilton team included scientists from a European consortium charged with therapeutic discovery for DMD. Terry Partridge joined a group from the Netherlands to form Prosensa, a company created to commercialize the antisense therapy for DMD. The group in Leiden had created a database of DMD and BMD mutations and were funded to progress to the clinic.

Steve Wilton came to Portland on February 7 in 2005 to partner the DMD antisense therapy between our company, AVI BioPharma, and Wilton's lab in Perth Australia. He had arranged for support from Glaxo Smithkline (GSK) in a nonprofit effort to find treatments for rare genetic diseases. We had gained experience with our compounds shifting splice sites with the discovery of AVI-4126 targeting *c-myc* (Hudziak et al. 2000). We found our binding site at the AUG translation start-site was near the exon 2 splice acceptor site shifted the splice acceptor to a cryptic location within exon 2. Our *c-myc* antisense agent excluded the translation start site so initiation of translation began at a site about halfway inside the mRNA. The resulting protein lost function as a transcriptional regulator and gained function as an inhibitor of transcription, a dominant negative protein. Wilton requested that we participate in his exploration of therapeutic candidates for DMD that could lead to clinical trials in Australia. We agreed to send compounds to Perth supporting Wilton's research effort.

This new research program added to our existing funding from the Department of Defense investigating medical countermeasures for Ebola and Marburg hemorrhagic viral infections and countermeasures for Ricin toxin and Anthrax. We were also discussing a partnership with Cook Cardiology to make a stent capable of eluting AVI-4126 for preventing coronary restenosis. In addition, we had encouraging results from our anticancer program in a collaborative study with Harvard targeting the androgen receptor to be used in patients with prostate cancer and Exelixis targeting tumor associated kinases. We had far too many disparate projects but the strategic plan was to find multiple partners to stabilize the future of our company.

I left Corvallis at 3:00 pm on June 27, 2005 driving to Portland for a flight to San Francisco. My following flight to Sydney Australia was delayed by 90 min to 11:30 pm and I arrived in Sydney on Thursday June 29th at 7:00 am. The transit through passport control, baggage claim and customs took an hour so I missed my flight to Perth. Rescheduling my Perth flight involved a long line because torrential rains were causing travel system delays displacing hundreds of travelers to new flights. I was able to fly to Melbourne and after another delay; I caught a connecting flight to Perth arriving at 3:00 pm on Thursday. Steve collected me and we went to his home in Perth only to drive to his beach house in Peppermint Grove. Finally, I was able to sleep through the night waking Friday ready for action. A Sydney physician, John Rasko, joined me for a day of wine tasting in the Margret River region and a day to lessen the clouds of jet lag.

We held our meeting at the Cape Vale Winery beginning at 10:00 am on July 2nd. Professor Frank Mastaglia opened the meeting pointing to the UK consortium, the

Dutch consortium, and the now formed Australian consortium for the treatment of DMD using antisense exon skipping. Our meeting was attended by a group from Sydney including John Rasko, Monique Ryan, and Kathy North, a group from Perth including Steve Wilton, Sue Fletcher, and Phillip Lamont, and Andrew Kornberg from Melbourne, Woon Chee Yee from Singapore, and Rakesh Patel from Aukland. While my travel distance was acknowledged, these people are accustomed to long flights several times a year. We addressed three key questions: (1) Are we ready to consider human clinical trials? (2) What will the requirements from regulatory bodies involve? and (3) What is the optimal clinical trial design?

A survey of observations involved comparison of multiple oligomer chemistries, peptide nucleic acids (PNA), 2'-O-methyl PSO, and PMO. The PMO we had shipped to Wilton's group were effective in the mdx mouse model (M23D(+7–18)) but the PMO conjugated to an arginine rich peptide ($(RXR)_4XB$)to enhance cell delivery was significantly more impressive. We had little information regarding the peptide toxicity but had observed some weight loss in animals treated with peptide PMO (PPMO) not observed with PMO alone. I presented our experience with PMO in various cardiovascular, viral and cancer model systems including data from intra-arterial, intravenous and oral delivery. The relative merits of plain PMO versus PPMO were not resolved and debate continued for years to come.

John Rasko presented perspectives on the Australian Therapeutic Goods Agency (TGA). He expressed concern for review by the Office of Gene Technology in the Department of Health and Aging because they may require significantly more pre-clinical information. He also pointed to approval committees from both the Health Research Ethics Committee (HREC) and the Gene Therapies Related Advisory Panel (GTRAP). Further, an Institutional Biosafety Committee (IBC) will also need to approve our plan. Optimism for progress came from the Australian version of the IND process involving CTN/CTX (Clinical Trial Notification/Clinical Trial Exemption) in which the drug sponsor submits an application to the TGA. The medical principal Investigator provides experience, ethics, and biosafety components. The studies require Good Manufacturing Processes (GMP) and Good Clinical Practice (GCP) assurances, which would also be expected in the US as well as the EU. The study would require monitoring by a clinical research organization (CRO) and a data management organization (DMO) but permission to conduct studies could proceed after a two to 3 month review process.

The clinical trial design began with a discussion of which exon should we start with and what chemistry should be employed. The chemistry question was resolved by distributing different trials to each of the three consortia; Prosensa representing the Dutch group would use 2'-O-methyl PSO, the AVI BIoPharma would support the Australian group funded by GSK philanthropy would use PMO, and the EU group would be free to choose. Concern for an immune response to the exon skipped dystrophin arose because the unique protein might be recognized by the human immune system of a boy with DMD as a foreign neoantigen. Resolution of this concern was to limit the physical region of dystrophin expression. We chose to inject a small muscle in the foot, the extensor digitalum brevis (EDB) because if an immune response manifested, the muscle could be removed surgically without

seriously affecting the patient. Another option was to inject the foot of healthy volunteers but this was quickly discouraged. A tentative plan was set in motion; the meeting was a success.

I spent July 4th 2005 alone in Sydney and while I missed celebrating the holiday with my family, I enjoyed walking to the Sydney Opera House, the harbor bridge, and the park along the harbor. That evening I experienced the cultural melting pot of Sydney and the festive evening atmosphere. I returned to Corvallis energized about the DMD project. I wanted to confirm the reported observations of Wilton and others as well as explore enhanced cell delivery strategies. We initiated cell culture studies in our laboratory to explore exon skipping and delivery to muscle cells in culture. We also provided various PMO and PPMO to Wilton's group to explore activity in the mdx mouse model (Fall et al. 2006), the dog model of DMD (McClorey et al. 2006a), and in human muscle explants (McClorey et al. 2006b). We also investigated treatment of young mdx mice (Fletcher et al. 2007) as well as chronic treatment of the mdx mice (Wu et al. 2008). One approach involved identification of a muscle specific peptide to conjugate to the PMO so that skeletal muscle would be targeted for delivery (Yin et al. 2009).

DMD is a fool's errand in the fall of 2005. Our management team acknowledged the DMD project as an excellent opportunity to partner with a larger pharmaceutical company. The board of directors was not optimistic citing; the 3000 boys that would benefit from a drug developed to skip exon 51 will fail to return value to AVI BioPharma investors. Given the standing investment by GSK in the Wilton lab, we felt GSK was the best first partner opportunity. We travelled to Philadelphia to meet with a business development group from GSK. Stakeholders from manufacturing, clinical development, and business collaborating were some of the GSK meetings. The take-home message; a development project involves development meetings attended by leaders from multiple business units. Each business unit has an account that is charged for time expended in each meeting. An overview of account expenditures for all projects by a group of pharmacoeconomists will lead to termination of projects that are projected to cost more than the estimated return from drug sales. The assessment that our DMD project would be terminated before any clinical studies or manufacturing efforts would begin. In short, the DMD project is a NO GO for GSK.

The DMD climate began to warm early in 2006. I attended a DMD conference in Monaco sponsored by a GSK non-profit division. The first objective was to focus the development effort. Select on group with the optimal development plan to support. My presentation was one of many each with a proposed plan of attack. A scientific panel from GSK then led the meeting through targeted questions. A linear path of logic reached a bottleneck at cell culture studies. The panel proposed comparing the transport of our PMO versus Prosensa's 2'-O-methyl PSO into cells in culture for selection of the best oligomer chemistry. I protested, transport properties of cells in culture do not accurately reflect transport in an intact human. Further, I was concerned for criteria in selecting the laboratory where the comparison would be conducted, what would the experimental design utilize as the accepted outcome, and what input would AVI have in the experimental design. Finally, I felt safety

should be an early stage concern for the decision process. Sadly, the panel operated under an immutable series of accepted development steps and ignored my protest. Ultimately, GSK invested over $100 million in the Prosensa project only to drop the partner after phase II clinical studies.

January 8, 2007 I attended the Wicker Project Workshop at Children's National Medical Center in Washington DC. The National Institutes of Health acknowledged exon skipping as a tangible therapeutic approach the treatment of DMD. The Welstone Fund was interested in exploration of optimal chemistry and delivery methods to bring exon skipping into the clinic. Qi Lu from South Carolina presented a brief history of exon skipping publications and the need for a battery of model systems to evaluate competing technologies. Myoblast cultures from the mdx mouse and human muscle were treated with both 2'-O-methyl PSO (Mann et al. 2001) as well as PMO (Gebski et al. 2003) chemistries. Observations from *in vivo* studies with systemic delivery in the mdx mouse have been encouraging (Lu et al. 2005) but systemic treatment with PMO were most effective (Alter et al. 2006). The diaphragm and heart muscle groups are key areas of concern since improvement of strength in the extremities will not offer sufficient meaningful benefit. Toshifumi Yokota reported on studies with the canine DMD model in which three PMO compounds were administered as a "cocktail" leading to impressive reductions in creatine kinase (CK) blood levels, a marker of muscle damage, from 120,000 pretreatment to 48,000 post treatment Yokota et al. 2009). This was impressive because the dog is a larger animal and the disease more closely parallels the human disease than small animal models. Francesco Montoni, a physician with the MDEX consortium in London, spoke mid-morning about our upcoming investigation of PMO injected into the EDB. The review by the gene therapy advisory committee (GTAC), the ethics committee, and medical health research authority (MHRA) had been recently completed. The local injection trial would begin in March. Tan Nguyen from the Office of Orphan Products Development at the FDA spoke after lunch. He emphasized the need for adequately well-controlled studies, suggested a fast track designation for priority review, and accelerated approval. He also recommended a pre-IND submission as a means of gaining FDA consultation before submitting the legally binding IND document.

Late in the summer of 2007, I submitted a proposal to a foundation created to support the development of a PMO designed to skip exon 50. The proposal was supported and I viewed my role as an arbiter of studies conducted in three well-established and expert outside laboratories. The first goal was to identify the optimal PMO to skip exon 50 from a collection of compounds provided by AVI BioPharma. I held a teleconference meeting held within 4 months of the project beginning to present a synthesis of observations to conclude the optimal sequence goal. Two laboratories screened compounds; each identified a different optimal sequence. I down-selected one of the two sequences based on a property of self-binding, an unfavorable trait resulting in poor pharmacokinetic properties. The teleconference ended with all in agreement for the optimal target sequence. Unknown to me, the head of the laboratory that had identified the down-selected compound contacted the foundation to protest the selection process. This triggered the third outside

laboratory to contact the foundation to lobby for a peptide conjugated version of the PMO. The peptide conjugate PMO had favorable properties but an optimal peptide had not been identified and no safety data were in hand. I recommended we move forward with the optimal PMO with no peptide conjugate. I felt the peptide represented a potential toxicity liability that should not be the first step forward in young boys with DMD. The investigators dissent led to delay and no exon 50 skipping compound has progressed to clinical development in more than a decade.

The DMD project had progressed to a phase Ib human clinical trial at the Great Ormond Street Hospital in London under the direction of Francesco Montoni. The January meeting in Washington DC was highly informative encouraging progress to systemic treatment regimens. Documents were prepared for the FDA, IND 77,429, in preparation for trials in the United States. Our clinical director decided to ignore the pre-IND submission in favor of a formal IND. We had impressive independent confirmation of the efficacy of exon-skipping with our PMO compounds. We also had years of clinical experience with the PMO chemistry in other trials, primarily as antivirals, indicating an impressive safety profile. He considered pre-clinical toxicology with our exon-skipping PMO, AVI-4658, to be unnecessary and possibly adding risk to the program. This was based on the fact that skipping exon 51 in a healthy individual would actually shift the transcript out of frame thus creating DMD. While I suggested we use the mdx mouse for our toxicology studies, he argued this would not be a validated toxicology model and unacceptable. We filed the IND with the FDA by fall of 2007 and a request for an orphan indication in August of 2007. The FDA promptly reviewed the IND putting us on clinical hold until we submitted a complete preclinical toxicology package. The clinical hold could have been avoided by submitting a pre-IND to gain the FDA advice but the clinical hold meant no progress toward systemic trials in the United States.

The MDEX clinical trial actually started in December of 2007 and was completed by December 2008. The study involved seven boys with DMD aged 10 to 17 divided into two dose groups (0.09 and 0.9 mg) with AVI-4658 injected into the EDB on one side and saline injected into the other side. Both EDB were biopsied after 3 weeks. Each dose of AVI-4658 was divided into 9; 0.1 mL injections into the EDB at the top of the foot. Each subjects fibroblasts transfected with MyoD to make myoblasts were treated with AVI-4658 to confirm the exon skipping would result in dystrophin expression. The AVI-4658 injections were well tolerated with no inflammatory infiltrates and no evidence of anti-dystrophin antibodies. This was the primary purpose of the study and an enormous relief that expression of dystrophin in a boy with DMD was recognized as self. The secondary endpoints of the study involved evidence of exon skipping and protein expression in the AVI-4658 treated side compared to no effect on the saline treated side (Kinali et al. 2009). The dystrophin expression in all treated muscles was more than 20% of that in the contralateral saline-injected muscles. A similar study using local EDB injection conducted by Prosensa and their partners from the Netherlands injected PRO051, a 2'-O-methyl PSO designed to skip the same exon 51 resulted in 17 to 35% dystrophin expression but comparison to the contralateral EDB was not a component of the protocol (Van Deutekom et al. 2009).

The success in the phase Ib study removes all doubt that exon skipping can restore dystrophin expression in a boy with DMD. A systemic delivery study then becomes and ethical obligation and we had not cleared the clinical hold placed by the FDA so continuing in London remained an active option. The Phase 2 dose-escalation study (0.5, 1, 2, 4, 10, and 20 mg/kg) involved 19 boys aged 10 to 13 with DMD mutations amenable to treatment with Eteplirsen (Cirak et al. 2011). The study also involved intravenous injection and systemic delivery of the oligomer. Like most dose-escalation studies, this one included pharmacokinetic (PK) analysis of the PMO in the blood. Key PK observations included a relatively short plasma half-life of 1.6 to 3.6 h and elimination by the kidney into the urine. Eteplirsen was well tolerated and no drug-induced adverse events were observed following single doses of up to 900 mg and cumulative exposures exceeding 10,000 mg. The percent of a dose that is eliminated in urine increases as the dose increases such that at 0.5 to 4 mg/kg dose 32.3 to 46.2% of the dose is eliminated in the urine but at 10 to 20 mg/kg dose 60 to 64% of the dose is eliminated in the urine. AVI-4658 induced exon 51 skipping in all dose groups with associated dystrophin protein expression.

We conducted toxicology studies to satisfy FDA concerns and clinical hold, which enabled systemic clinical trials in the United States. As might be expected, we replaced the head of clinical development so toxicity studies could be viewed from a fresh perspective. First, AVI-4658 safety pharmacology and genotoxicity evaluation (Sazani et al. 2010) followed by studies in nonhuman primates in which no drug-related adverse events were observed at doses including the maximum feasible dose of 320 mg/kg (Sazani et al. 2011a). Finally, the study proposed 5 years earlier, toxicological evaluation in the mdx mouse (Sazani et al. 2011b). The mouse studies revealed no drug-related adverse events up to 960 mg/kg. These studies confirm the unprecedented safety profile of the PMO chemistry and the clinical hold ended.

The pivotal clinical trial was conducted under the direction of Jerry Mendell at the Nationwide Children's Hospital in Columbus Ohio. The 3 year progression of DMD in 12 boys randomized into three treatment groups; a placebo, a 30 mg/kg dose and a 50 mg/kg dose administered intravenously once a week for 24 weeks at which time the placebo group was shifted to the 30 mg/kg dose. The pivotal endpoint was the 6 min walk test (6MWT) of how far can the boys walk in 6 min. The boys age averaged 9.4 years old at the beginning of the study and walked 363 meters in the 6MWT. After 3 years the boys treated with Eteplirsen walked 151 meters farther than age and mutation matched historical controls, a highly significant difference (Mendell et al. 2016).

Musical Chairs

A radio program today discussed the emerging problems with the game musical chairs for young children. It seems the game has some mind bending and perhaps warping properties for children. When the music stops we all sit down but one of us

gets left out. The lack of a chair instills a lingering sense of not belonging and may encourage future anti-social behavior. A conservative radio talk show host proceeded to bash the sensitivity in this interpretation and pointed out that this never caused him any problems. I wonder if being a conservative invalidates his perspective.

In 1960 I was a preschool genius. I attended Grand Canyon Elementary School located on the south rim of Grand Canyon National Park, a natural wonder of the world located in northwestern Arizona. My family lived just outside of town, in the suburbs if you wish, in the park employee housing area. We didn't consider the interesting diversity of the people in the neighborhood because there was a limited diversity; all Caucasian middle class (perhaps lower middle class). My parents were second generation Danish and their friends included second generation German and Italian families. Just outside the park employee housing the prominent ethnicity was cowboy and beyond that perimeter Native American Indians.

The male residents wore cowboy boots, old and weathered for day-to-day wear and polished black for Sunday Church boots. The daily uniform consisted of blue jeans and long sleeved cowboy shirts with the tails out. The subject was not discussed but all men wore white jockey shorts and white T-shirts. White cotton socks at the toes and white cowboy hats at the top. The key measure of a man's character was how he prepared the hat brim to provide a comfortable fit. I regret that I was too young to have developed deep insights into the relationships between hat shape and a man's character. In the summer, men frequently wore red and blue bandanas either around their neck or in the rear pant pocket. Men all walked as if their feet hurt and with poor posture which could be due to riding horses or their pickup trucks did not have comfortable seats.

Women wore blouses and skirts. Women at work wore short sleeved white shirts with blue jeans and most wore cowboy boots. Most women worked at the gift shops, restaurants, and hotels, which include the Bright Angel Lodge and the El Tovar Hotel. Virtually all of the teachers at Grand Canyon Elementary School wore a small sweater attached with a small connector around the neck and placed over the shoulder like a cape. These women of education also wore jewelry on their lapel, neck and ears. My memories reflect proper women, always kind and in good disposition. The more I learn in retrospect, there was much impropriety but I was a little kid so was oblivious to the gossip of the day.

Summers were hot and dusty, winter was cold with snow and the spring and fall were perfect. The fair weather population was tremendously swelled in response to the extensive tourist trade but winter populations were greatly reduced. My fellow classmates included children of park employees, local businessman, and Hopi Indians. There were 18 students in my preschool/kindergarden class.

Fall of 1960 approached and it was time for a Thanksgiving Holiday Party. This holiday was coincident with an important harvest of pinon pine nuts collected by local Hopi Indians. How similar to the original celebration of America's European settlers with the fall prosperity to be shared among the grade school social melting pot. An interesting personal insight is that I was a bit clueless about my classmates, I knew their names but did not invest much time in getting to know them. My key

interest was making a dash for available swings at recess, if you do not run to get one you will not get to swing during recess. A second area of interest was practicing my letters and ad hoc spelling efforts. I spent a lot of time attempting to write objectionable words for some reason. This out of the box effort included phonic spelling of "poop" as I was unable to come up with how to come up with the "sh" sound so spelling "shit" was beyond my capabilities but I was interested in learning. Perhaps most 5-year-old children are focused on small activities but these two activities summarized my first quarter of pre-school.

The holiday party began with punch, cookies and cake for all, as much as you could eat. Just as we were ready to kick back rubbing our bloated bellies, the teacher accompanied by volunteer mothers, decided we should play some games. I can't remember most of the games but unfortunately musical chairs is seared into my memory. We cleared the desks from the center of the room, moved our chairs into a linear array with chair back to chair back in a row. Music was provided by a square box that was plugged into the wall and opened to reveal an armature with a needle. Sound came out of a speaker on the front of the device. A record played "Pop goes the weasel" which was interrupted by a volunteer mom who lifted the needle from the record to indicate time to find a chair. The room was hot and the game continued endlessly, as I was one of the final four. I found myself next to Esther, a Hopi girl that did not understand English very well. I arrived at this position in a surprising way in that the previous person next to me did not want to sit down, leaving an easy open chair. The next cycle of music was long and a curious "poop" odor became prominent in the room. As the music stopped, Esther sat but then moved ahead by a chair leaving me an easy opening. I looked at the chair to find a generous smear of "poop" leaving a drama in the mind of a 5 year old, do I go for the seat anyway or should I get bumped out to only to leave some other kid to sit in the shit. I opted to stand and as it turns out so did the other chair candidate. The teacher was perplexed, as children do not leave open chairs in the game so she prodded us to sit. She became somewhat animated in "what is the matter with you kids" version when one of the volunteer moms decoded the mystery. Once the evidence was revealed, the perpetrator was soon identified, poor Esther. The small classroom was filled with remorse, finger pointing and retching. I can honestly say I think musical chairs is a piece of shit game.

On March 27, 2007, the board of directors asked the chief executive officer (CEO) of my company to resign. They did this because they felt the CEO had misled shareholders in a few selected presentations to the investment community. While the CEO is a good friend, this action was not expected to cause him great concern since he had indicated he would retire in 3 months. I was saddened by the action but as part of the action, he was given an 18-month severance package. If they had waited for his retirement, he would have no salary extension. The irrational behavior of the board seemed a little humorous if not overly dramatic. A game of CEO musical chairs followed complete with 6 different CEOs in the next 5 years. Changes in the CEO was accompanied by changes in our executive team with 3 changes in the chief medical officer (CMO) position, 3 changes in the head of regulatory affairs, 3 changes in the chief financial officer (CFO) position, 3 changes in the head

of business development (BP) and 2 different leaders of the manufacturing group. Indeed, I was the last of the original executive team to find a chair and wouldn't you know it, the chair got covered in poop.

I joined AVI BioPharma in 1997 as the company completed their initial public offering (IPO). We were 25 employees in a building with room to expand. We had no product pipeline, no animal data, and no lead compounds ready for clinical development. My job as head of research and development was to seek the limitations of our proprietary PMO technology, find a feasible therapeutic to discover, and then quickly move this into the clinic. The task demanded innovation, organization, and communication. The PMO chemistry was known in the emerging antisense industry but the group in Oregon was considered a fringe element. The compounds were considered "hard to make" and unlikely to scale up to needs for clinical trials. The neutral character was interpreted as unlikely to offer drug-like pharmacokinetic properties; rapid elimination and poor distribution into tissues. The mechanism of action was not catalytic so potency was expected to be so poor that an effective dose would not be feasible. Our board of directors was populated by our executive team, including myself, and a couple local real estate developers that had been angel investors earlier in the life of the company known as Antivirals.

Fewer than 1% of new biotechnology companies survive more than one year and 90% of those will not survive a second year. We were always scrambling to find sufficient money to complete the work required to move the company forward. Our small executive team worked well together to focus on critical PMO synthesis at larger scale, measured expenditures, hard work, and charisma of our CEO. We embraced the "we will do well by doing good" philosophy and cherished every employee. The transitions of 2007 to 2012 reflected corporate growing pains and the success of our early efforts but resulted in a company with a new name, Sarepta, a new management team, and a product in late stage development. Innovation was no longer felt welcome, the company moved to Boston, and teams of employees were coordinated by project managers. My position as senior vice president of research and development transitioned to senior vice president of research and innovation and finally to distinguished scientist. I was asked to leave the company in 2016 in the same month as the FDA advisory meeting to approve Eteplirsen.

The board of directors greeted the DMD project with resistance in 2005 but by 2007 opinion had transitioned to DMD as the only project the company should support. Initially, the DMD project was to be exploited as a vehicle for big pharma collaborating but by 2011 the business plan was to take Eteplirsen to approval and never partner the DMD project. The development of Eteplirsen to FDA approval was like most drugs by requiring 12 years from discovery to approval. Estimating cost is difficult but the cost would be between $650 million to $1 billion. The market capitalization of AVI BioPharma/Sarepta rose from $150 million to over $4 billion.

Ad Comm

The DMD community anxiously awaited an approved drug to treat their boys that suffer daily from this genetic disease. Late in 2015, there were three companies with advanced drug products ready for review by an FDA advisory committee (AD COMM). The investment community interest was reaching a climax, as these products would change the game. An approval would mean drugs could be developed to treat rare genetic diseases, an expansive emerging market and the test bed for personalized medicine. Most academic scientists were not aware of the high stakes game about to unfold but would benefit from a new age ready to reward innovation. Hopes were high because odds favor at least one of the three products would be approved.

The group from the Netherlands had championed Drisapersen (PRO-051), a 2'-O-methyl PSO designed to skip exon 51 of the dystrophin pre-mRNA. Their company Prosensa had collaborated with big pharma giant GSK for a sophisticated phase 2 clinical trial with marginal outcome. GSK passed on advanced development so Prosensa collaborated with a mid-sized California pharma company, BioMarin. They assembled NDA documents for FDA review with their AD COMM meeting on November 23, 2015. I watched their public meeting on my computer with mixed anxiety as I had reviewed their published results that included injection site reactions in 79%, renal abnormalities in 61%, and anti-drug antibodies in 29% of the patients. I felt they did not demonstrate efficacy in either the 6MWT or the biochemical evidence for exon skipping or dystrophin protein expression. The risk to benefit balance fell on the risk side for this drug in my opinion but their product closely mirrored our own Eteplirsen. I was surprised by the lack of curiosity by the AD COMM in that they seemed unaware of the history of PSO toxicity. The BioMarin team exploited the marginal background understanding of the AD COMM reviewers by leaving toxicity issues as open ended and uncertain if serious adverse events were just anomalies. The AD COMM rejected Drisapersen (Kyndrisa) and the FDA prescription drug user fee act (PDUFA) new drug application (NDA) decision, required within 60 days of the AD COMM, formalized the rejection in January 2016. The decision lowered expectations for all rare disease therapeutics.

A second candidate, Ataluren, a premature termination codon suppression therapeutic developed by PTC Theraeputcs was requesting an AD COMM review. Ataluren had already gained market authorization by the European Medical Authority (EMA) in August 2014. However, the FDA declined to approve as the data were "insufficient to warrant review" in February of 2016. While discouraging to the rare disease community, I felt this was the appropriate response from the FDA based on my review of the published data related to Ataluren. The premise for selective premature codon suppression was a favorable interpretation of a diminished translation fidelity phenomenon. I had also collaborated with a lab that reported the discovery of Ataluren was based on a false positive result in the early discovery process. This drug was developed on the hope that the efficacy bar would be low for FDA approval based on the desperate need for a drug to treat DMD.

Sarepta submitted final NDA documents to the FDA so that a January AD COMM meeting was scheduled. A severe snowstorm hit the Washington DC area the day before our scheduled meeting, which was postponed. What drama, the last hope for rare disease therapeutics and the survival of Sarepta forced to wait for a re-scheduled AD COMM. The delay forced Sarepta to re-evaluate their financial projections leading to rapid re-organization. The Corvallis facility would be closed and most of the employees terminated, including me.[2] Consolidation in Boston meant all resources were required to support the Eteplirsen review by the FDA.

The AD COMM met on April 25, 2016 beginning at 8:00 am EST. Janet Woodcock, director of the Center for Drug Evaluation and Review (CDER) described the process. The goal is to produce a truncated dystrophin protein but the threshold level has not been established. The panel was asked to consider the impact of an erroneous approval of a drug that doesn't work but also the impact of an erroneous rejection of a drug that works. The Food and Drug Administration Safety and Innovation Act (FDASIA) sets breakthrough therapy designation for treatments for serious diseases. Robert Temple, deputy director of CDER, reviewed the history of FDA standards of approval citing peer reviewed published articles emphasizing potential bias in that comparisons to historical controls always do worse than randomized trial controls. While scholarly, the presentation lacked sophistication in that he did not address the rare disease aspect, the lack of historical experience with rare disease, and finally no examples came from musculoskeletal system drugs. The FDA invited companies to develop drugs for rare disease with legislation, FDASIA, specifically designed to facilitate review by the FDA in cases where large clinical trials are not feasible.

The meeting then moved to Ashuhosh Roa, director of biotechnology, and Farkas, the FDA chairman of the musculoskeletal AD COMM. They addressed the biochemical endpoints that were used to support claims of efficacy. First, they discounted the value of the mRNA data that confirm skipping of exon 51, the mechanism of action of Eteplirsen. The rationale was that the mRNA is not stable, particularly for genes with multiple exons like dystrophin. This conclusion is not supported by scientific literature, in fact the opposite is reported, multi exon transcripts are actually more stable. The fact that a decade of mRNA data have been used to define exon skipping in the peer reviewed scientific literature confirms adequate mRNA stability. Sarepta did not contest this claim and accepted the FDA decision to ignore the most convincing evidence supporting Eteplirsen. Second, they pointed to the lack of dystrophin protein quantitation citing weaknesses in protocol and inconsistent observations reported in the literature. This concern was justified but the protocol had been improved based on the advice of the FDA so that quantitation of dystrophin protein in the pivotal study would meet FDA standards and were reproducible. The concern is further diminished by the fact that no one

[2] The closure of the Corvallis site and termination notices for the staff was done by teleconference from Boston. The CEO wanted to wish well by saying, "Go Buckeyes" as a reference to Ohio State University. He apparently did not know that Oregon State University, also OSU, located in Corvallis mascot is the Beavers.

knows how much dystrophin is necessary for a muscle to function. Finally, they pointed to the immunofluorescence data localizing the dystrophin expression to the appropriate site in the muscle fiber and the number of dystrophin positive fibers was inadequate. The concern is the same as for the dystrophin protein expression and the fact is no one knows how many positive fibers are required to produce a functional muscle or as evidence that the disease has progressed or not.

A negative tone established by the AD COMM preceded the Sarepta presentation and specific questions. The AD COMM expressed disappointment that historical controls were used and the study was not blinded and that the placebo group had been crossed over into a treatment group. This violates FDA recommendations that pivotal trials be "well controlled" that could bias interpretation of the clinical observations. When Sarepta was asked why they did not include a larger placebo control group, Sarepta responded that they did not have enough drug.[3] This was the most observed AD COMM meeting in the history of the FDA and neither the FDA nor Sarepta brought their "A-game."

The most convincing evidence supporting Eteplirsen came during the public comment phase of the meeting, which extended through the afternoon. Louis Kunkle, a member of the National Academy of Sciences, discoverer of the gene responsible for DMD and the world's authority on dystrophin, testified that he was impressed that any level of dystrophin had been observed following treatment with Eteplirsen. A physician from UCLA testified that he had surveyed most cases of DMD in the United States finding boys with DMD can no longer walk beyond age 12.9 which he published in the peer-reviewed literature. This mitigated the limitation of the historical controls and shed light on the 6MWT data in which treated boys age 14 were still walking. Testimony that there is no potential for bias in loss of ambulation came from prestigious physicians mitigating the need for a blinded study. Public speaker 52 read a letter from US Senator Joe Donnely of Indiana and four additional US Senators urging approval of Eteplirsen. All 12 boys that were treated in the pivotal trial testified, some included video of their progress during the treatment. I was emotionally moved by their presentations and as I felt tears rolling down my cheeks, I looked around at my colleagues in Corvallis, all were crying. We could see people crying in the audience at the AD COMM meeting site as well. The testimonials came from a who's who in muscular dystrophy, political leaders, and pateints.

The duration for this AD COMM meeting had been extended into the evening when the AD COMM voted. Instructions to the panel revealed two possible approval pathways. The first vote for accelerated approval was rejected with 3 in favor, 7 apposed, and 3 declined to vote. The second vote pertained to approval under the Food and Drug Administration Safety and Innovation Act (FDASIA) was also rejected with 6 in favor and 7 apposed. A visible shrug moved through the audience when one of the treated boys ran to the podium shouting, "You are so arrogant, you think we don't understand this disease but we are the experts." Our future had already been determined when we were informed we would not continue to work at

[3] An embarrassing response since no drug is required for a placebo group.

Sarepta. Now it seemed the future of Sarepta had been determined. More significantly, the future of drug development for the treatment of rare disease would suffer from a three-time loser at the FDA. The scheduled PDUFA decision was expected on May 26th but the decision was delayed due to internal conflict at the FDA. The final decision came in September 19, 2016 when Janet Woodcock reported Eteplirsen was approved!

No Good Deed

The FDA agency staffers had singled Eteplirsen (commercial name Exondys 51) for destruction but the drug was approved. Janet Woodcock approved the therapy after a behind closed doors brawl with agency staff. The agency insiders demanded a placebo trial considered unethical and impossible to fulfill for a rare pediatric disease (WSJ 2016). The agency released 100 pages of documents to disparage data supporting Eteplirsen. They indicated clinical benefit was not established in conflict with the decision to approve indicating the effect is reasonably likely to predict clinical benefit. Curiously, Dr. Aaron Kesselheim wrote a dissenting opinion the Journal of the American Medical Association. Kasselheim has no experience treating DMD, misinterpreted the disease severity, and was incorrect in thinking Eteplirsen treated a nonsense mutation in the dystrophin gene. This counter approval rhetoric led Anthem to decline insurance coverage for Eteplirsen treatment, Aetna to limit treatment to boys under 14 years old, and Humana to only reimburse treatment for ambulatory boys. Some view, "… the bureaucracy's toxic culture of political control and contempt for private innovation" a priority for change. The dose is based on weight but can cost $300,000 a year and an incentive for insurers to seek a way out of reimbursement.

Backlash over the emotional appeal of parents' stories at the AD COMM meeting persisted well beyond the September 19, 2016 approval. The AD COMM was not advised about the role advocacy groups played in the development of Eteplirsen. The FDA has been extending a larger role to patient families and advocacy groups. Jennifer McNary, mother of two DMD boys in the pivotal trial, said "the drug works." After 4 months on eteplirsen, her son Max could open a milk carton and needed a wheelchair less often (Pullman and Mullins 2017). Catherine Collins, mother of a DMD boy in the pivotal trial acknowledges parents helped push the regulatory approval but felt Sarepta was "trading on our desperation." In February 2013, Ms. McNary brought a petition with more than 170,000 signatures asking the FDA to expedite approval. In June of 2015, Sarepta initiated three trials with 110 boys and Christine McSherry, mother of a 21 year old DMD boy, was enrolled. Elis Unger, director of the Office of Drug Evaluation, reported in July of 2016 that the FDA review team unanimously rejected approval of Eteplirsen. They concluded eteplirsen was a "scientifically elegant placebo" giving false hope. When Janet Wookcock overruled the decision, the dispute was sent to FDA commissioner Califf.

He deferred to Woodcock's judgement and authority. The Trump administration stated desire to improve the culture at the FDA.

The approval process put financial community on a roller coaster. Sarepta had been warned by the NASDAQ that its share listing might be dropped from the exchange for trading below $1. In 2012, a reverse 1 to 6 stock split accompanied by a name change from AVI BioPharma to Sarepta raised share price and the NASDAQ maintained listing on the exchange. A July 24, 2012 press release reporting significant benefit at 36 weeks of treatment raised share price to $8 a share. A follow-on press release at 48 week treatment on October 3, 2012 boosted share price to $45. November 12, 2013 GlaxoSmithKline PLC completed three trials with Drisapersen involving 290 patients. The results were not persuasive and the FDA met with Sarepta to inform the study raised doubts about Eteplirsen. Sarepta shares fell to $13. On October 27, 2014 Sarepta reported the FDA had found "marked disparities" in data at Nationwide Childrens Hospital causing Sarepta share price to fall 30% overnight. The FDA approval on September 19, 2016 moved Sarepta share price to $60.

Conclusion

Exon skipping is here to stay, at least for a while. Sarepta now has Golodirsen, a PMO targeting skipping of exon 53, and Casimersen, a PMO targeting skipping of exon 45, in phase III clinical trials. A PMO designed to skip exon 52 is completing preclinical testing and the first PPMO targeting exon 51 is in phase I trials. Success with the PPMO will lead to replacement of Eteplirsen. The PMO platform approach is promising if we are going to create personalized medicine for the over 4000 genetic diseases with no currently approved treatment.

The multiple transformative technologies currently available is a palate with many colors. The investment community will flutter from one technology to another urged by the irrational exuberance of analysts seeking attention. These technologies will experience a shorter and shorter interval of enthusiasm in this era of explosive information. The greatest limitation to success is the quality of scientific and clinical review. The most highly paid pharma executives have limited scientific insight and will predictably pursue financial reward over benefits to patients. The FDA revealed mixed agendas and the pull of political influence bringing their review process into question. The bright light to remember is that a project succeeded in the face of many obstacles.

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Chapter 12
Regulating Resilience

Abstract Transcriptome plasticity is an evolutionary surrogate in humans and serves to counter the profound threats imposed by continuously evolving infectious diseases, chemical exposures, and a changing environment. The race to adapt between infections agents and host cells pits very high genome plasticity of viruses and efficient horizontal gene transfer of bacteria against human cell transcriptome plasticity. The human alternate exon use as a "plug and play" strategy creating real time transcriptome plasticity represents a differentiating feature from bacteria and most viruses. Biology always seems to find a way and our resilient transcriptome plasticity can be hacked by infectious agents as they can influence alternate exon use in their host cells as part of their remarkable ability to manipulate their host.

Evolution operates through natural selection. In the case of infectious disease, the human host responds to defend against the infection, most frequently by an immune response. The response imposes natural selection on the infectious pathogen that evolves to evade the host response. Infectious pathogens are at a significant evolutionary advantage due to their relatively short replication interval. If a single-stranded RNA virus is capable of doubling every 10 min then entirely new populations can arise to evade selection pressure in an hour. If bacteria can double every 60 min then an entirely new bacterial population can arise to evade selection pressure within an average workday. Human evolution is much slower such that a generation time of 20 years means a human population can arise to evade selection pressure only after a 100 years or more. An evolutionary competition between humans and their infectious pathogens favors the infectious pathogen and numerous pandemics support this observation.

Keywords Splice-site affinity · Nuclear receptors · Transcription factors · Small nuclear ribonucleoproteins · Resilient metabolism · Placebo effect · Aromatherapy

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Introduction

Resilience embodied in transcriptome plasticity represents a response mechanism capable of operating within an individual in a timeframe capable of matching the infectious pathogen evolution. This transcriptome plasticity relies on the modular exon structure of the human genome and the alternate exon use process. This benefit establishes a lower limit of alternate exon use to ensure infectious resilience.

Cancer cells use alternate exon use in ways that differ from their noncancerous tissue counterparts. The tumor cell is competing to survive within our bodies using alternate exon use to gain benefit. In ductal carcinoma of the breast, alternate exon use includes dysregulation of cell cycle control including differential exon use in Cyclin D3 (CCND3), cell division cycle 25B, and cyclin dependent kinase (CDK1) among others (Bjorklund et al. 2017). Some exons are not included, they are ultra-conserved and referred to "poison cassette exons" because they contain early in-frame stop codons resulting in nonsense mediated decay (NMD). The liability from the lethal consequence of cancer establishes an upper limit for alternate exon use.

The tendency for transcriptome plasticity to remain between limits is encoded in the genome. Each exon junction must retain a conserved sequence that can be recognized by splicing factors, a 5′-splice donor and 3′-splice acceptor AG/GU with flanking sequence defining a spectrum of spliceosome affinity (Fig. 12.1). A collection of features including splicing enhancers/inhibitors, intron length, and available SRFs in a particular cell ultimately define the frequency of a specific splice site and thus define the exon. The resilience model allows for alternate exon use but enhancing the frequency that a lower affinity splice site will be the splice site and thus redefining the exon. The model further proposes environmental influences act to

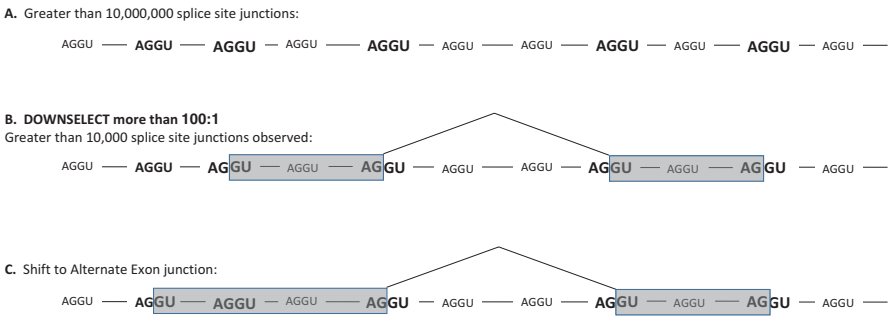

Fig. 12.1 Alternate exon resilience model. (**a**) A four base recognition sequence occurs with probability $(0.25)^4 = 0.0039$; the number of four base sequences in the genome is $3*10^9 * 0.0039 = 11{,}718{,}750$. If the average gene has 7 exons, each with two junctions, then there are $20{,}000 * 14 = 280{,}000$ exon junctions. This means the excess number of potential exon junctions is $11{,}718{,}750/280{,}000 = 418$. (**b**) The affinity of an exon junction is defined by flanking sequence splice enhancers/inhibitors as well as availability of splicing factors in a particular cell (greater affinity indicated by larger text size and bold letters). (**c**) Environmental influences can shift the relative affinity to an alternative exon junction

influence the structure of the transcription complex that redefine splice site affinity.

Resilient Transcription

Cellular resilience is mediated by quantitative and qualitative changes in gene expression. Our genome offers a variety of strategies for regulating gene expression but in humans, transcriptional regulators, the determinants of transcription, are the key drivers. The quantitative enhancement or suppression of specific gene transcription is the first layer of cellular resilience. Regulating the quality of transcription through alternate exon use represents a second layer. The following discussion links a changing environment to molecular events that create a resilience response.

Transcription involves a DNA-dependent RNA-polymerase in which a DNA template is copied into a single stranded RNA by an RNA polymerase. In humans, the RNA polymerase II-dependent transcription begins with the assembly of a transcriptional complex that includes six transcription factors (TF) numbered as TFIIA, TFIIB, TFIID, TBP, TFIIE, TFIIF, and TFIIH. This RNA polymerase complex binds to a specific DNA sequence called the promoter. The RNA polymerase-promoter complex activity is regulated by a collection of positive influences activators and coactivators as well as negative influences repressors and corepressors. The net effect of positive and negative influences is the transcriptional activity for each gene. Once RNA synthesis begins, the RNA polymerase begins to move along the DNA template and abortive initiation begins allowing the complex to escape the promoter. The growing RNA has a $5' \rightarrow 3'$ polarity that is opposite the DNA template strand $3' \rightarrow 5'$ polarity, an anti-parallel orientation. The RNA polymerase proceeds along the DNA template at a variable rate depending on local features such as DNA methylation, histone acetylation, and overall chromatin structure. The termination of transcription involves addition of adenines to the terminal $3'$ end in a process called polyadenylation.

The 20,000 to 21,000 sequences in DNA that encode proteins are divided into approximately 200,000 exons, modular segments that encode proteins. Exons tend to encode structural features of proteins so that the final protein structure is determined by the assembly of several structural blocks. When an exon is skipped during the pre-mRNA maturation process (splicing), a structural block is removed and the resulting alternate protein may have an altered function. Similarly, additional exons may be included during pre-mRNA maturation leading to an additional structural block that may also alter protein function. Alternate exon use can increase the range of protein functions across the genome resulting in a dynamic phenotype composed of hundreds of thousands of functioning proteins from just 21,000 genes.

Splicing must be a tightly controlled process. Early appreciation of exons and splicing came from adenovirus-2 lytic stage in mammalian cells as it expresses non-contiguous DNA segments (Berget et al. 1977; Chow et al. 1977;). The segments included in mRNA are exons and excluded segments are introns (Gilbert 1978).

Alternate exon use in endogenous genes was first recognized when alternate calcitonin transcripts expressed in rat medullary thyroid carcinoma originated from the same gene that resulted in different proteins (Rosenfeld et al. 1982). At the same time small nuclear ribonucleoproteins (snRNPs) association with pre-mRNA (Lerner et al. 1980; Krainer and Maniatis 1985) led to realization they were part of the splicosome (Rody and Abelson 1985; Butcher and Brow 2005). The *cis* elements that define the exon-intron junctions and the intronic branch points sequence were identified (Reed and Maniatis 1985). Appreciation of cis elements that enhance or silence splice-site utilization and their *trans* elements including RNA-binding proteins (RBPs), heterologous nuclear ribonucleoprotein partilces (hnRNPs) (Gallinaro et al. 1981), and serine-arginine rich proteins (SR proteins) (Krainer et al. 1990) emerged to define a spectrum of splice site avidity. These trans-acting factors can either promote or inhibit alternate exon use based on the aggregate effects of their location, presence of opposing regulatory factors, and concentrations of the trans-acting factors (Goren et al. 2006). Alternate splicing is further influenced by sequence modifications from RNA editing and epigenetic marks (Coelho and Smith 2014). Finally, alternate splicing can be tissue-dependent leading to tissue specific transcriptome diversity (Merkin et al. 2012). The mammalian nervous system is a tissue with more abundant alternate splicing than other organs (Ule et al. 2006). Neuronal alternate splicing differs within neuronal populations, perhaps even synapse-specific (Traunmuller et al. 2016). Neuronal differentiation is associated with polypyrimidine tract binding proteins (PTB) including PTBP1, PTBP2 and SRRM4.

Knockdown of the RNA-editing ADAR1 enzyme leads to increased circular RNA expression (Ivanov et al. 2015) that compete with alternate splicing for spliceosome recruitment (Ashwal-Fluss et al. 2014) probably because circular RNA is created by spliceosomes. Exclusion of exon 9 in human PTBP2 alters the regulation of 1500 alternate splicing events (Gueroussov et al. 2015). Loss of the RBP RBFOX1 isoform leads to defects in corticogenesis in the brain due to diminished axon growth and dendrite development (Hamada et al. 2015). Alternate splicing of microexons (3–27 nucleotides) is misregulated in autism spectrum disorder (Chabot and Shkreta 2016). Retaining intron 5 in the acrosome RNA binding protein (ACRBP) creates a variant protein that participates in formation of the acrosome on the apical surface on the head of sperm (Kenemori et al. 2016).

Myotonic dystrophy is characterized by cardiac conduction defects and insulin resistance that are associated with a CTG repeat sequence in the 3'-UTR of dystrophia myotonica protein kinase (DMPK). The CTG repeats for a hairpin structure that sequesters the muscle blind RNA binding protein MBNL and leads to an alternate splicing pattern in skeletal muscle. Aberrent splicing of skeletal muscle-specific chloride channel 1 (CLCN1) leads to a transcript with a premature stop codon that is associated with myotonia (Lee and Cooper 2009). Lack of MBNL can also lead to an increase in skipping exon 11 of the insulin receptor 1 (IR1) and insulin resistance (Pistoni et al. 2010).

Alternate splicing and alternative polyadenylation regulate the innate immune signaling Toll-like receptors (Carpenter et al. 2014). The medullary thymic epithelial

cells (mTEC) are responsible for self-antigen presentation to immature T cells to define self. These mTEC express more alternately spliced genes for many splice junctions in most genes in the genome in an attempt to represent all the host's self-antigens (Danan-Gotthold et al. 2016). B cell alternate splicing in the Ig heavy chain produces two isoforms at the 3′-end: a membrane-bound antigen receptor and a secreted antibody (Early et al. 1980; Rogers 1980). T cell effector function is modulated by alternate splicing as inclusion of MALT1 exon 7 (a more active protein) is negatively regulated by hnRNPU and induced TCR signaling (Meininger et al. 2016). Inclusion of exons 4, 5, and 6 of CD45 is expressed in naïve T cells while these exons are excluded in T cells resulting in lower TCR signal transduction (Beverly et al. 1992). This appears to de due to dimerization of the shorter form of CD45 and less phosphatase activity.

Infecting organism can intervene in our transcriptome plasticity. Bacteria produce nucleomodulins that actively manipulate host gene expression. The *Helicobacter pylori* induces changes in gastric epithelial cells phosphoproteome in which nearly one-third are proteins associated with the spliceosome or RNA splicing (Holland et al. 2016). The Shigella type III secretion protein, IpaH9.8, disrupts splicing activity of host cells by binding to the U2AF35 splicing factor resulting in reduced expression of chemokines and cytokines involved in neutrophil recruitment (Okuda et al. 2005). Both *Salmonella enterica* and *Yersinia pestis* express SspH1 and YopM, respectively, which are orthologs of IpaH9.8.

Viral proteins can modulate splicing of cellular pre-mRNAs. Herpes simplex virus type 2 (HSV-2) ICP27 protein modulates splicing in promyelocytic leukemia (PML) (Nojima et al. 2009). HPV16 infection upregulates the host splicing factor CELF3 which is associated with MAPK and VEGF signaling (Xu et al. 2016).

Transcription produces a pre-mRNA that is processed, specific marks remain after processing, folded, and assembled into an RNA-protein complex (mRNP) that

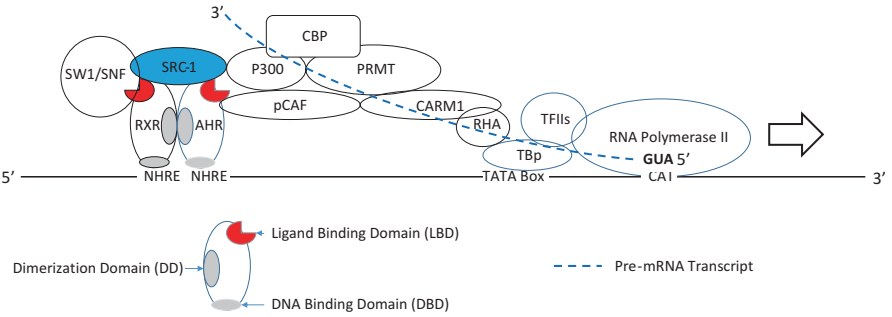

Fig. 12.2 Transcriptional complex conformation shift enables alternate exon use. The transcriptional complex is a large structure assembled at sites on a DNA template strand (black line) based on a combination of sequence specific DNA binding proteins. In this example, nuclear hormone receptors bind endogenous or environmental chemical ligands to a ligand binding domain (red) as a signal to bind DNA. The growing RNA transcript (dashed blue line) passes by coactivators that may serve to both facilitate transcription and participate in transcript splicing. The large arrow at the right indicates the direction of progression of the transcriptional complex

are all essential for export to the cytoplasm. During transcription by RNA pol II a variety of proteins and snRNPs bind in an ordered process to facilitate 5′-capping, splicing, 3′ cleavage and polyadenylation. Once released from the gene, the mRNA enters an interchromatin channel where they join pools of gene-specific mRNPs waiting for encounters with the nuclear pore complexes (NPCs) (Mor et al. 2010). Those mRNPs that are not export competent will add to the pool size (Ma et al. 2013). Successful transport through NPC the mRNPs are dissociated into the cytoplasm by export factors Gle1, IP6, and DDX.

The spliceosome is assembled on an emerging transcript (Bauren and Wieslander 1994; Ameur et al. 2011) in close proximity to RNA pol II (Fig. 12.2). Splicing leads to deposition of the exon junctional complex (EJC) composed of eIF4AIII, Mago bound to Y14, and Barentsz. The EJC can recruit export adaptors, translation initiation factors, and assembly of the nonsense mediated decay (NMD) complex. Polyadenylation at the 3′-end is required for mRNA export including isoforms with different polyadenylation sites directed by RNA pol II CTD (Tian and Manley 2017).

DNA damage triggers the DNA damage response (DDR) which disrupts pre-mRNA splicing of genes involved in DNA repair, cell-cycle control and apoptosis (Lenzken et al. 2013; Naro et al. 2015; Dutertre et al. 2011). ATM, ATR, DNA-PK, and p38/MK2 can detect DNA damage created by reactive oxygen species (ROS), alkylating agents, crosslinking agents, ioizing radiation, telomere dysfunction, UV exposure, genotoxic drugs, replication stress, and R-loop formation. Poly(ADP-ribose)-ylation of chromatin components is associated with DNA damage and serves to recruit DNA repair factors (Haince et al. 2008). The PARylation provides a binding site for splicing factors NONO, hnRNP A1, and hnRNP G.

Nuclear Receptors Activate Transcription and Influence Splicing

Vitamin D. Drink milk, it contains all the building blocks you need including calcium, phosphate, potassium, vitamin A, riboflavin, vitamin B12, and vitamin D. On the other hand, women who drank three glasses of milk a day were twice as likely to die during a study period than women who drank less than one glass a day. The vitamin D response system encompasses diet and environmental exposure to the Sun integrating physiology and the immune response for optimal resilience.

Lack of vitamin D can lead to rickets, a childhood disease in which growing bones lack sufficient calcium and phosphate. Vitamin D was named by Elmer McCollum in 1922 because it was the fourth vitamin to be named (Wolf 2004). Harry Steenbock observed foods and other organic materials increased their vitamin D content following exposure to ultraviolet light in 1923. His patent irradiating food, particularly milk, was lucrative for the Wisconsin Alumni Research Foundation (WARF) but also led to elimination of rickets in the United States by 1945. In 1925,

Alfred Fabian Hess found vitamin D is not really a vitamin as sunlight could induce synthesis of vitamin D in humans. Sunlight is so efficient that adequate amounts of vitamin D can be produced by exposure of the face, arms and legs to the sun for 5 to 30 min a week. Ten minutes of strong sunlight are equivalent to drinking two hundred glasses of milk. The indicator of vitamin D status in the blood is the 25-hydroxyvitamin D (25(OH)D) as it is stable with a circulating half-life of 2 weeks. Our robust response to sunlight is one of the best examples of how we respond at the molecular level to the environment.

People in temperate regions are exposed to less intense UV light from the Sun because of the lower Sun angles. People living at higher latitudes receive as little as 10 percent of the UV light experienced by our more equatorial ancestors. Our ancestors that made the transit to higher latitudes adjusted by reduced melanin content in the skin. The consequently lighter skin meant efficient synthesis of sufficient vitamin D in the reduced Sun. However, people with high melanin content in their skin living at high latitudes, increased time spent indoors out of the Sun, and sunblock to prevent sunburn means a larger percent of the population are vitamin D deficient. The consequences of vitamin D deficiency are controversial because the recommended daily amounts are conservative at 600 to 800 international units (IU) but some recommend 3000 IU daily and a poll asking researchers what they themselves take daily was an average of 5500 IU. Could higher vitamin D reduce the incidence of cancer, autism, and a variety of infectious disease?

Vitamin D binds a nuclear receptor, vitamin D receptor (VDR), a transcriptional regulatory protein (Haussler and Norman 1969). The VDR forms a heterodimer with the retinoid-X receptor (RXR) and together they bind the vitamin D response element (VDRE) in DNA resulting in transcription or transrepression of specific genes. One function of these transcribed genes is to promote calcium absorption. Three VDR isoforms have been observed with VDRB1 (477 amino acides) translation initiation in exon 1, the VDRA isoform (427 amino acids) is synthesized from a translation start site in exon 2, and the third is a shorter form created by alternate splicing, the FokI variant (424 amino acids) (Gardiner et al. 2004). The longer VDRB1 enables a different reaction to calciferol or lithocholic acid while the short splice variant has higher transcriptional activity than full length VDR (Jurutka et al. 2000).

Steroid and secosteroid hormones regulate gene transcription by binding to nuclear receptors (NR). The hnRNPC1/C2 binds pre-mRNA as a splicing regulator but also binds dsDNA functioning as the VDRE-binding protein regulating transcription. Nuclear receptors bind ligands and then bind specific DNA response elements to regulate transcription. Once bound to a response element, the NR recruit coregulatory proteins that coordinate assembly of the transcriptional unit (Lonard and O'Malley 2012). NR coregulatory proteins share structural similarities to RNA splicing proteins suggesting the coregulatory proteins can couple transcription and RNA splicing. The NR share homology so bind similar coregulatory proteins suggesting a broad relationship between NR activation and coupled RNA transcription-splicing. The dual role of NR and their coregulators are responsible for both magnitude and correctness of gene expression Auboeuf et al. 2002).

The hnRNP family of RNPs can bind to both single- and double-stranded DNA (McKay and Cooke 1992; Takimoto et al. 1993) and are associated with pre-mRNA have multiple functions in RNA processing (Venables et al. 2008). hnRNPs can act as transcriptional suppressors (Miau et al. 1998) as well as splice site suppressors. A key feedback loop involves 1,25(OH)2D binding to the VDR thus activating association with the VDRE and transcription of CYP24A1. CYP24A1 hydroxylates the active VDR ligand, 1,25(OH)2D, into a poor VDR ligand, 1,24,25(OH)3D, completing a negative feedback loop. However, interactions between VDR and hnRNPC1/C2 as well as numerous other genes can lead to CYP24A1 splice variants, one missing exons 1 and 3 (CYP24A1-SV) and another skipping exon 10 (CYP24A1 var. 2). The CYP24A1 var. 2 is a heme-binding domain-deficient and consequently inactive enzyme for creation of 1,24,25(OH)2D but is likely able to bind substrate 1,25(OH)2D and transient reduction in availability for VDR binding. Loss of hnRNPC1/C2 leads to an increase in CYP24A1 var. 2, a role in suppressing splice variants (Zhou et al. 2016).

Aryl Hydrocarbon Receptor (AHR). I propose re-tasking of the aryl hydrocarbon receptor (AHR) from understanding mechanisms of toxicity to a resilience mechanism from xenobiotic agents.

Derivatives isolated from coal tar in the 1930s provided the precursors of diethylstilbesterol (DES) (Dodds et al. 1938) as well as the polycyclic aromatic hydrocarbons (PAH) exemplified by benzo[a]pyrene (BP). A curious relationship in which DES, an estrogen mimic, used in hormonal therapy for breast cancer led to the 1966 Nobel Prize in Physiology and Medicine for Charles Huggins while the same animal models revealed PAHs including 3-methylcholanthrene to be mammary carcinogens (Huggins et al. 1961). Indeed, steroids like estrogen share striking structural similarities to PAHs. Estrogens were found to bind to the estrogen receptor (ER) but the PAH dimethylbenzeanthracene (DMBA), a potent mammary carcinogen, does not bind to the ER (Keightley and Okey 1973). The pesticide dichloro-diphenyl-trichloroethane (DDT) is a mixture of *o,p'*-DDT, an estrogenic compound, and *p,p'*-DDT, an antiestrogen, protects female rats from DMBA-induced mammary cancer (Silinskas and Okey 1975). Protection is associated with DDT induction of metabolic clearance of DMBA.

The PAH were known to induce their own metabolism, the aryl hydrocarbon hydroxylase (AHH) eventually linked to cytochrome P450 1A1. Oliver Hankinson found Hepa-1 cells (mouse hepatoma cells) exposed to BP in culture resulted in cell death from bioactive metabolites of BP but rare mutant cells survived due to their lack of production of BP bioactive metabolites (Hankinson 1979). These mutant cells revealed three genes necessary for BP-selection; CYP1a1, the AHR (Legraverend et al. 1982), and the aryl hydrocarbon receptor nuclear translocator (ARNT, also known as HIF-1β). The cloning of ARNT and AHR led to the discovery of the bHLH/PAS family of transcriptional regulators and sensors of environmental signals (Gu et al. 2000). The link between AHR binding to a ligand and transcriptional activation of gene expression, termed induction, came with the use of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD was 30,000 time more

potent than other PAHs in inducing AHH while remaining chemically stable bind to the AHR resulting in gene expression (Poland et al. 1976).

The AHR response element (AHRE) sequence is conserved across mammalian species has been identified as 5'-GCGTG-3′ (Harper et al. 1992). This pentanucleotide sequence occurs frequently in the genome as expected.

The AHR binding TCDD is seen in a wide range of vertebrate species but the protein does not bind ligand in insects. AHR expression is nearly ubiquitous in vertebrate tissues suggestive of important biological function but the AHR knockout mouse is viable. However, knockout mice are resistant to TCDD-induced toxicities of immunosuppression, hepatotoxicity, carcinogenicity, and teratogenesis (Lahvis and Bradfield 1998). Conversely, a ligand independent AHR lacking the ligand binding domain results in shortened life-span and frequent development of stomach cancer, thymic atrophy, and liver enlargement (Brunnberg et al. 2006). Deletion of the trans-activation domain (TAD) of AHR in rats confers resistance to TCDD toxicity (Pohjanvirta et al. 1998) but does not interfere with AHR-mediated induction of CYP1A1, CYP1A2, CYP1B1, or UGT1A1. Compounds that induce CYP1A1 do not cause dioxin-like toxicity pointing to diverse AHR responses to TCDD.

A search for an endogenous ligand for AHR returned arachidonic acid metabolites including prostaglandins, bilirubin, and tryptophan metabolite indole-3-pyruvate. 7-ketocholesterol (7-KC) is an endogenous compound that binds AHR but inhibits CYP1A1 induction by TCDD. Low-density lipoproteins (LDL) in the hydrodynamic sheer stress of blood flow becomes an AHR activator (McMillan and Bradfield 2007). Given the role AHR plays in vascular development, LDL activation of AHR under conditions of blood flow may facilitate vascular development (Lahvis et al. 2005).

Potentially, structural features of AHR may account for varied functions. Guinea pigs are highly susceptible to TCDD lethality while hamsters are exceptionally resistant. The affinity of TCDD to the mouse AHR is 10 times greater than to human AHR, which is in good agreement with lower dose induction of CYP1A1 by TCDD in mice (Okey 2007). Replacing the mouse AHR with human AHR in transgenic mice leads to mice less susceptible to TCDD-induced cleft palate. The chemical diversity of AHR agonists reveal some high-affinity ligands but not all high-affinity ligands produce dioxin-like toxicity. Agonists produce different conformation of the AHR protein than antagonists (Henry and Gasiewicz 2003). Selective AHR modulators elicit diverse AHR-mediated responses Safe and McDougal 2002). AHR will form protein-protein interactions with ARNT but will also form a complex with the retinoblastoma protein (RB) resulting in arrest of cell cycle (Huang and Elferink 2005).

The structural similarities between AHR ligands and ER ligands result in cross-activation of ER and AHR. Even ARNT can participate as a coactivator of ER-dependent transcription. In fact, AHR recruits ERα to the promoter region of CYP1A1 explaining the estrogen-dependent nature of CYP1A1 induction (Matthews and Gustafsson 2006).

Resilient Cells of the Nervous System

Behavior defined by a collection of molecular, gene-expression events awaits the light of clarity. Resilience is often associated with the integrated psychological adaption to a set of good or bad circumstances. Characterized by protective factors that allow people to adapt to adverse conditions such as maltreatment, catastrophic life events, or urban poverty. This psychological resilience is the result of an individual's interaction with their environment and the processes that promote well-being. These processes originate in the central nervous system with dynamic changes in gene expression leading to molecular responses.

Worms and flies have a similar number of genes as humans but what separates these species is that primates, especially humans, have the most complex alternate splicing capability. Further, alternate splicing is different between different types of cells found in different tissues including kidneys, heart, and brain. The nervous system makes extensive use of alternate exon inclusion. The role of enhanced protein diversity clearly plays a critical role in neurogenesis, brain development and elaboration of neural circuits but we can speculate that neuronal alternate splicing may facilitate creativity and imagination.

Alternate splicing modulates the interactions between the neuronal synaptic cell-adhesion, synaptogenesis. These neuronal contacts define neuronal circuits of the brain. Adhesion molecules neuroligin (NLGN) and neurexin (NRXN) hold the synapse in shape facilitating the connection of pre- and post-synaptic neurons (Taniguchi et al. 2007). Inclusion of short alternative exons within NLGN1 at three sites and NLGN2 at two sites account for variability in their affinity to NRXN ligands (Bolliger et al. 2008). Three human NRXN genes use alternate promoters and alternate splicing to generate diverse proteins. Alternate exon use in these adhesion molecules may provide the basis for necessary diversity of synaptic connections.

Neurons rely on transport channels for their activity. These membrane channels are activated by addition of phosphorous by various kinases. Both the kinase and the membrane channel transcripts are alternately spliced to create alternate proteins and refined channel activity.

Splicing involves multiple regulators, the splicing regulators often have paralogues, and different neuronal cell types express different combinations of splicing regulators. The RNA-binding proteins, polypyrimidine tract binding protein 1 (PTBP1) and serine/arginine repetitive matrix protein 4 (SRRM4) influence neuronal fate and neuronal differentiation. PTBP1 suppresses exon 10 (exclusion 10 exclusion) of PTBP2 mRNA resulting in nonsense mediated decay and reduced expression of PTBP2, a driver of neuronal differentiation. The PTBP1 knockout mouse leads to loss of ependymal cells of the lateral ventricles and embryonic lethality (Suckale et al. 2011). Depletion of PTBP1 from cultures fibroblasts is sufficient to induce their *trans*-differentiation into neurons (Xue et al. 2013). SRRM4 appears to antagonize PTBP activity and suppression of SRRM4 expression in the developing mouse is associated with embryonic lethality.

Loss of splicing regulators including neuro-oncological ventral antigen 2 (NOVA2) and fox-1 homologue 2 (RBOX2) disrupts neuronal migration resulting in cortical and cerebellar lamination defects. *Nova2*$^{-/-}$ mice show abnormal inclusion of exons 7b and 7c in Dab1 in the Reelin-signaling pathway (Ayala et al. 2007). *Rbfox2*$^{-/-}$ mice show increased motality and reduced size of the cerebellum due to loss of Purkinje cell migration to their proper location in the internal granule layer (Gehman et al. 2012).

The rare genetic disease myotonic dystrophy is linked to CTG triplet repeats that sequester the muscleblind (MBNL) splicing factor. Hundreds of exons are misspliced but fetal isoforms of the calcium-activated potassium channel lead to abnormal sleep patterns, memory loss, and pentylenetetrazole-induced seizures.

Splicing misregulation is now associated with neurodegenerative disease. Alterations in the transactivating response DNA binding protein (TDP43) are linked to amyotrophic lateral sclerosis (ALS) and frontal temporal lobar disease (FTLD). Neurodegeneration may arise due to loss of TDP43 from the nucleus and loss of splicing function (Ling et al. 2013).

Approximately 20 percent of the energy generated by respiration in the human body is used for brain metabolism. Most of the energy consumption by the brain is involved in maintaining neuronal membrane potential. Cells that demand high-energy utilization also show resilient features of alternate exon use.

Resilient Immune Responses

Genome recombination involving a variable region (V), a diversity region (D), and a joining region (J) define the adaptive immune response. The discovery of the generation of antibody diversity through V(D)J recombination is attributed to Susumu Tonegawa and the 1987 Nobel Prize I Physiology and Medicine. Antibodies are composed of a heavy chain and a light chain. The human immunoglobin heavy chain contains 2 constant gene segments, 44 variable gene segments, 27 diversity gene segments, and 6 joining gene segments. The human light chain also has 2 constant gene segments and multiple V and J segments but no D segments. The assembly of an antibody through DNA rearrangement can lead to approximately $3 * 10^{11}$ different combinations. The specific rearrangement is determined by a combination of presentation of an antigen but self-antigens are removed so that only environmental antigens result in active antibodies.

Lymphocytes also gain specificity through recombination of V(D)J segments with D-to-J recombination in the β-chain of the T cell receptor (TCR) followed by V-to-DJ rearrangement. The α-chain of the TCR recombines V-to-J segments in a manner similar to immunoglobin light chains. The TCR assembly involves association of the recombined α- and β-chains to create antigen specific TCR. Self-antigenic T-cells are removed during thymic selection early in life so that only environmental antigens result in functional T-cells.

The immune response is energetically demanding invoking a metabolic shift to obtain energy from storage and nonimmune processes. The activated immune system can consume 2000 kJ/day and chronic inflammatory diseases can lead to anorexia, cachexia, and contribute to metabolic syndrome (Straub et al. 2010). Extracellular adenosine (e-Ado) acts as a sensor released from metabolically stressed cells or leakage out of damaged cells to switch energy availability to the immune response (Bajgar et al. 2015).

The dynamic immune activation and enhanced energy demand during immune response is accompanied by transcriptome plasticity and alternate exon use. The TLR signaling regulates immune responses of the cell. Macrophage infection by M tuberculosis leads to hundreds of alternate splicing events, which show temporal and infecting strain specificity (Kalam et al. 2017). Alternate splicing is observed in hundreds of genes during T-cell activation and B-cell stimulation (Martinez and Lynch 2013). Alternate transcripts encoding cell surface receptors (CD3, CD28, CD45, CD46, CD6, CD8, CD83, CD96, CTLA-4, Fas, and ICAM-1), cytokines (IL-4, IL-7Rα, TLR8), transcriptional regulators (ATF2, FOXP3, GATA3, HIF1α, ILF3, IRF3, IRF1, RUNX1), RNA processing (U2AF, RBM25, EIFG2, AUF-1), cell signaling (IRAK1, MAP 2 K7, MAP 3 K7, MAP 4 K2, TRAF3, VAV1), ion channels (ITPR1, TRIM26, TRIM65), cytoskeletal elements (EVI5, CCDC14, CLASP1, FAM21C, SLMAP, Tau), and mitochondrial transcripts (ARMC10, FAM36A, TID1, and TIMM50). Clearly, foreign antigens induce resilience through alternate splicing in addition to DNA rearrangements associated with the adaptive immune response.

Resilient Metabolism

The cytochrome P450 (CYP) superfamily of 57 genes represent a front line defense against environmental exposures as discussed earlier. We recently reviewed metabolic resilience finding 965 alternately spliced transcripts from the 57 genes (Annalora et al. 2017). While several CYP splice variants are associated with pathology, we have not adequately investigated the potential for alternate splicing to adapt or provide benefit. In addition to CYP tissue-specific splice variants, we found

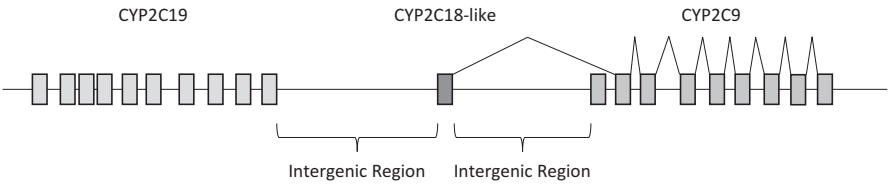

Fig. 12.3 Trans-splicing. Complex splicing of exons from two different genes, termed trans-splicing, leads to chimeric transcripts. Intergenic transcripts containing a novel human cytochrome P450 2C exon 1 spliced to sequences from the CYP2C9

CYP splice variants that direct subcellular trafficking within the cell from the endoplasmic reticulum or mitochondria to the nucleus, peroxisome, plasma membrane, cytosol, or vacuole compartments. The modular exon structure means splicing a portion of the CYP2C18-like gene can be spliced to the CYP2C9 gene creating a chimera of the two genes by trans-splicing (Warner et al. 2001) (Fig. 12.3).

The processing of CYP pre-mRNA is coordinated within the nucleus by multiple factors including nuclear receptors including the peroxisome proliferator-activated receptors (PPAR) which interact with the PGC-1α transcriptional coactivator to regulate oxidative metabolism and mitochondrial biogenesis (Wu et al. 1999). Numerous steroid substrates of CYPs also bind nuclear receptors, which interact with coactivators that modulate the assembly of the spliceosome complex that acts on CYP pre-mRNA splicing (Auboeuf et al. 2005). Vitamin A metabolites bind nuclear receptors including the retinoic acid receptors (RAR) and retinoid X receptors (RXR) that promote assembly of the SC35 coactivator regulating alternate splicing of protein kinase delta (PKCδ) and other pre-mRNAs (Apostolatos et al. 2010). The pattern of chemical substrate/ligand interacting with CYP active site and nuclear receptors leading to alternate splicing and transcriptome plasticity is central to the proposed molecular basis of resilience.

Resilience Implications

Therapeutic Management of Resilience Transcriptome plasticity explains some complex disease states such as cancer, metabolic syndrome, and diabetes. Conversely, therapeutics that influence alternate splicing have mechanisms of action that are not precisely understood. Alternate exon use may offer deeper insights into the disorders as well as therapeutic agents used in treatment. Development of resistance to treatment may also be the product of molecular resilience.

Cancer therapies include chemical agents with steroid and retinoid structures that are ligands to nuclear receptors thus capable of contributing to transcriptome plasticity. These steroids include: (1) androgens that offer some palliation in postmenapausal, hormonally sensitive advanced breast cancers and in stimulating prostate cancer cells growth to potentiate cytotoxic effects of radiophosphorous on bony metastases. (2) Corticosteroids for their anti-lympholytic effects in acute and chronic leukemias or as anti-inflammatory effects for lymphomas and hypernephromas. (3) Estrogens for patients with estrogen positive breast cancer and diesthylstilbesterol for prostate cancer. (4) Progestins are used in advanced, well-differentiated renal cell, endometrial, and breast carcinomas. The precise mechanism of action of these steroids in their use treating cancer lacks molecular detail and their use is generally supportive rather than front line care.

There are over 4000 natural and synthetic molecules with structures related to retinoic acid (vitamin A). Retinoids used as anti-cancer therapies influence cellular differentiation, have anti-proliferative properties, and are pro-apoptotic. All-trans

retinoic acid (tretinoin) is FDA-approved for the treatment of acute promyelocytic leukemia. 9-cis retinoic acid (alitretinoin) is FDA-approved for topical treatment of Kaposi's sarcoma lesions (Baumann et al. 2005) and shows promise in prevention of mammary and prostate cancers (Christov et al. 2002; Wu et al. 2000). 13-cis retinoic acid (isotretinoin) is used in treatment of thyroid cancer and maintenance therapy in patients treated for neuroblastoma (Matthay et al. 2009). These nuclear receptor ligands may alter tumor resilience as an un-explored mechanism of action.

It is time to incorporate assays to explore transcriptome plasticity in the discovery and development of new therapeutics. Chemical libraries rich in nuclear receptor ligands are available to laboratories as a foundation for alternate splicing screening. Bioinformatic techniques have been developed so that even a novice can identify alternate splicing events. What remains is the refined hypothesis linking a specific disease to transcriptome plasticity features. The reward is likely to drive precision medicine and resulting highly effective therapies for refractory diseases.

The Placebo Effect A curiosity encountered in human drug evaluation is the apparent therapeutic benefit of inert agents in individuals that have not received the drug. A placebo group is highly recommended in "well-controlled" blinded or double-blinded drug trial study designs so that neither the patient nor the caregiver knows whether the test article is drug or inert agent. Placebo treated patients have responded with a broad spectrum of changes including heart rate, blood pressure, depression, anxiety, fatigue, and improvement in asthma symptoms. Scientists argue about the placebo mechanism. Some believe the placebo to be artifacts of data collection procedures while others pose a patient's expectations of benefit leads to altered levels of hormones or other endogenous substances capable of objective physiological responses. Both points of view are probably true.

An early lesson in pharmacology taught a drug could not produce an effect the cell cannot produce. While this statement predates gene therapy, the fundamental premise remains true. Placebo effects have the potential to mimic drug effects using endogenous mediators. I find the explanation that as a patient believes they feel different leads to actually feeling different lacks molecular precision and is untestable. Earlier discussion described robust brain transcriptome plasticity in response to events. High placebo effects may utilize dopamine and mu-opioid activity capable of analgesic, anti-Parkinson's disease, and anti-depressant responses. These placebo effects may not require transcriptome plasticity but the drivers of neuronal resilience are endogenous molecules including steroids and hormones. Strategies to enhance transcriptome plasticity to refine placebo responses would represent an avenue to precision medicine.

Aroma Therapy Humans can detect thousands of different compounds by smell, most are within the volatile organic compound (VOC) class. Many of these VOCs have a biological origin from lipid biosynthesis that is necessary for cell membranes. An important group of lipids begins with the synthesis of a 5-carbon molecule called isoprene. Two isoprenes can combine to form a 10-carbon geranyl which can be oxidized to form geraniol, the primary component of rose oil, palmarosa oil,

and citronella oil. These small compounds are found in geranium, lemon and many other essential oils. Geraniol can contribute to synthesis of flavors such as peach, raspberry, grapefruit, red apple, plum, lime, orange, lemon, watermelon, pineapple, and blueberry. Compounds with chains of 5 to 10 carbons are generally VOCs that have easily detected odors but also represent building blocks to larger VOCs including terpenes, fatty acids, and cholesterol. A variety of these molecules are ligands for different nuclear receptors capable of facilitating RNA transcription.

Capsaicin is a curious VOC composed of a single 6-carbon aromatic ring with three substituent groups attached. It is the essence of hot in chili peppers. I investigated the metabolism of capsaicin finding it to form a reactive metabolite capable of reacting with the cytochrome P450 2E1 (CYP2E1) enzyme for which it is a substrate. Our studies confirmed capsaicin inhibition of CYP2E1 led to reduced mutagenesis from nitrosamine compounds found in processed meats that are also activated by CYP2E1 (Gannett et al. 1990). The publication of these data led to considerable attention from gardeners, hot sauce manufacturers, fans of Mexican food, and the military. Capsaicin proved to be exceptionally potent as we could sense the compound room across the room when someone opened the reagent bottle of capsaicin. Capsaicin is chemically similar to vanillin and sesimol, also pungent molecules with very different fragrances confirms the diversity in how humans experience highly related chemicals.

Floral scent plays a central role in pollinator attraction and specificity. Angiosperms emit a diverse array of floral scent molecules. Male bees produce perfume compounds in scent glands during courtship to convey fitness and identity. Orchids are known to release at least 83 volatile compounds of which 64 are known molecules; 31 aromatics, 31 terpenes (15 monoterpenes and 16 sesquiterpenes), and 1 nitrogenous compound. Many compounds are common to different orchids (e.g. eugenol, 1,8-cineole, terpinen-4-ol, β-ocimene, and β-pinene), while others are rare and specific to individual orchid species (e.g. dimethoxybenzene, methyl benzoate, ipsdineol, trans-geranyl acetone, isoeugenol, and cis-methyl p-methoxycinnamate) (Hetherington-Rauth and Ramirez 2016). These fragrant molecules are capable of communicating with insects as an attractant to facilitate pollination.

Aromatherapy (AT) has been use to manage depression, anxiety, muscle tension, sleep disturbance, nausea and pain. More than 40 plant derivatives have been identified for aromatherapy but lavender, eucalyptus, rosemary, chamomile, and peppermint are the most frequent therapeutics. A meta-analysis of twelve studies in which AT was employed to treat pain revealed a significant positive effect compared to placebo ($p < 0.0001$). The most effective applications of AT involved treatment of postoperative pain and obstetrical and gynecological pain. AT can successfully treat pain particularly when combined with conventional treatments (Lakhan et al. 2016).

AT is a popular complementary alternative approach to medicine for the treatment of depression. The current reluctance to accept aromatherapy practices by established medical practices invoked a meta-analysis of twelve randomized clinical trials of aromatherapy intervention in patients with depression. Seven studies showed improvement in depressive symptoms suggesting aromatherapy potential as

an effective option for relief of depressive symptoms (Hetherington-Rauth and Ramirez 2016). The observation is surprising given the lack of specific chemical definition. A highly speculative hypothesis posits a resilience relationship between fragrant molecules employed in AT and binding to transcriptional regulators capable of recruiting pre-mRNA splicing regulators.

Environmental Resilience Everything in the environment is changing over space and time obscuring the concept of steady-state conditions. Since our genomes adjust over evolutionary time, the changing transcriptome adjusts to rapid changes. This resilience makes life robust and not fragile.

Pheromones are secreted chemicals that can trigger social responses within a species. Adult humans release steroid pheromones such as androstenol and androstenone. Androstenol is a female pheromone capable of synchronizing menstrual cycles among women (Stern and McClintock 1998). Androstenone is thought to be released by males to attract females (Grammer et al. 2005). These molecules are ligands that bind to multiple receptors including the nuclear receptors capable that can influence alternate exon use. The concept introduces social resilience to the repertoire of possible roles of transcriptome plasticity.

Secreted chemicals that can trigger interspecies social responses include allomones and kairmones. While human examples are not described, the concept can be described for karimone in which a terpene, mycrene, is released by the Ponderosa pine when damaged attracts the Western pine beetle to the tree to the detriment of the tree. The berothid lacewing larvae release an allomone to subdue aggressive termites so they can feed on them. Mice infected with *Toxoplasma gondii*, a neurotropic protozoan parasite, lose their fear of cat odors. The cats are infected after they consume the infected mice enhancing the life cycle of the parasite (Ingram et al. 2013). These interspecies chemical communication schemes reveal an integrated ecological web that expands the role of chemical detection and response. While highly speculative, transcriptome plasticity offers a greater refinement in possible responses to a chemically complex environment.

Conclusions

Our exposure to light, radiation, chemicals, and infections demand a rapid, measured, and regulated response with the capacity for incredible diversity. The temporal demands fall outside the genome sequence changes associated with evolution. The expression of RNA serves to supplant evolution in this period in a manner where survival is a matter of resilience. The preceding discussion elaborates a sequence of events in which a receptor senses the environment in a way that imposes specific conformation changes (shapes) from a range of possibilities, each capable of recruiting specific co-regulatory molecules from a pool of competing possibilities. These environment specific and nearly unique complexes combine with RNA polymerase to transcribe a defined number of pre-mRNA. The proposed next step

involves this transcriptional complex in selection of which sequence features will define exon boundaries for further processing to create multiple variant forms of mRNA.

Splice-variant mRNAs are formed in addition to prototype mRNAs. A collection of quality control filters such as nonsense mediated decay, nuclear export, and translation exclude mRNAs to limit the possible phenotypes. The postulate is that certain splice-variants will function to improve resilience, survival in the face of a changing environment. However, experimental evidence linking splice variants to environmental resilience remains scant. Further, all of the mechanisms for regulating the inventory of splice variants for each gene are not known. The purpose of this book is to propose a logical sequence of molecular events that may mediate resilience.

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