

Chapter 2

Discovery and Preclinical Work

Daria Mochly-Rosen and Kevin Grimes

In any drug discovery and development effort, we must accomplish a number of critical steps to arrive at a compound that is safe and efficacious, and also exhibits the complex array of desired drug-like behaviors that warrants advancement to the clinic. These tasks include target identification and validation; screening for active compounds; chemical modification of candidate compounds to achieve optimized pharmacology; formulating the final drug product; and establishing safety in pre-clinical models. “Repurposing” drugs that have previously been approved (or shown to be safe in humans) for new clinical indications can provide a faster, less risky, and more cost-effective route for bringing a new therapy to patients. Such shortcuts in development can be particularly valuable to resource-constrained academicians. When performing drug discovery research, we must be particularly attentive to the robustness of our experiments, because inability to reproduce academic data continues to be a sticking point when projects are transferred to industry. Our experiments must be appropriately blinded, statistically powered, and meticulously documented so that our findings are worthy of the large investment required for their further translation into a drug. This chapter walks through the essential preclinical drug development steps that lead to a clinical drug candidate.

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2.1 Robustness of Preclinical Studies

Daria Mochly-Rosen

A number of recent commentaries challenge the robustness of academic preclinical studies. In one report, only 11% of published preclinical cancer studies from academic labs could be reproduced by Amgen scientists. This low rate was despite cooperation of the academic scientist who reported the original findings to reproduce the work at or with Amgen [1]. In another report, Bayer scientists found that ~75% of published academic studies brought in-house could not be reproduced, which resulted in termination of the effort to develop therapeutics based on these academic findings [2]. So what is going on?

The following discussion focuses on academic data related to animal studies. I will not repeat here the discussions of the importance of using the right animal models, how to confirm the findings using patient specimens, how to rely on proper understanding of pharmacokinetics and pharmacodynamics in using animal models, and how to use proper “endpoints” for the studies. All these issues are discussed in later sections of this chapter. Instead, I focus on factors that may contribute to irreproducible animal data published by academicians and some simple measures to mitigate these issues.

Box 2.1: What Surprised an Academician?

In 2004, when I temporarily moved from my academic lab to serve as the CSO of KAI Pharmaceuticals, I was hurt when our then CEO, who holds a B.A. in History, told me, “You will now learn that your academic work is not as robust as industry’s standard.” Like you, I take a great pride in our work in academia. I felt that conducting blinded studies, using several species, and reproducing the work in independent labs all combined to ensure high quality and valid data. That was not enough, I quickly learned. *—DM-R*

Box 2.2: Key Terms and Abbreviations

CSO: Chief Scientific Officer

Preclinical animal studies: animal studies done to validate a disease target and test the performance of a molecule prior to moving into human testing

***p*-value:** a statistical measure of the probability of obtaining a result at least as extreme as the one observed. If the *p*-value is less than the significance level (usually 0.05 or 0.01), one rejects the null hypothesis that there is no treatment effect

CDER: Center for Drug Evaluation and Research, within the Food and Drug Administration

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Box 2.2 (continued)

Endpoints: measurements (e.g., weight or tumor size) or observations (e.g., motor control or healthiness) used in a study to evaluate the effectiveness or safety of a treatment

Orphan indication: an FDA designation of a disease or condition that affects less than 200,000 people per year in the USA or for a treatment that is not expected to recoup its R&D costs due to pricing constraints

“me-toos”: drugs that are approved after other chemically similar compounds or molecules with the same mechanism of action are already on the market

2.1.1 Factors that Contribute to Irreproducible Data

2.1.1.1 Heterogeneous Experimental Conditions

Animal studies can be greatly affected by many factors. Yet, often we do not give proper attention to these potentially confounding factors and/or we do not record the conditions used in detail. For example, rodents are nocturnal animals. Data related to their immune response, eating, exercise, ability to learn tasks, etc. are greatly affected by the time of day when the experiment is conducted. The chow feed is another important variable that can affect animal-derived data; some feed is rich in soy and therefore contributes feminizing hormones to both males and females. Variation in the feed may affect response to drug uptake and metabolism, to the integrity of the immune response, etc. Other confounding factors relate to the housing conditions, including noise, strong smells, and crowding; and a good animal facility should minimize them. Latent or full-blown infection by viruses, bacteria, mites, and other parasites can also affect the results of the study (See Box 2.3). All these variables should be held to a minimum and detailed information should be recorded so that even if there is no room to provide it in full in the publication, we will be able to share the specific conditions used during our study when contacted by a commercial entity or another academic laboratory.

Box 2.3: Lack of Reproducibility May Relate to Previously Unsuspected Confounding Factors

Lack of reproducibility of preclinical reports does not mean that the data are fabricated or wrong. One of the better-documented cases of inability to reproduce data in mice relates to the induction of type I diabetes in NOD mice. Initial claims attributed increased diabetes incidence reported by some groups to the difference in housing the mice in germ-free conditions.

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Box 2.3 (continued)

However, more recent data showed that intestinal microbiota are the critical confounding factor; presence of *Bacillus cereus* in the gut delayed onset, and reduced incidence of type-1 diabetes [3].

2.1.1.2 Bias and Incomplete Reporting

It is critical that the investigators who assess the animal data will be blinded to the experimental conditions; unintended bias can greatly affect the analysis, especially when the endpoint determinants are subjective.

Another problem with bias results from dismissing and not reporting negative or inconsistent data. The investigator may have a reasonable rationale for wanting to exclude data related to certain animals; we should include the rationale in the method section and let the readers draw their own conclusions. All the data (positive and negative) should be reported, as they may often help identify important variables to consider in human studies. For example, the observation that gender and age can affect the therapeutic response to drugs in models of heart attack in animals was not reported for a long time. When these findings were finally reported, reviewers started requesting preclinical studies include animals from both genders.

Box 2.4: Recommendations to Improve Robustness of Preclinical Studies (Expanded from Ref. [4])

1. Keep detailed information about the experimental conditions.
2. Keep detailed information on the source of all the reagents and lot numbers used in the study.
3. Seek advice of statisticians during the study design to ensure that the study is powered to address the question at hand, and that the appropriate statistical tests are applied.
4. Include appropriate negative controls and—when possible—positive controls for the study.
5. Have each study reproduced by another investigator in the lab, and in an independent lab if feasible.
6. The investigators should be blinded to the identity of the control and treatment groups during data analysis.
7. Provide information on all the animals that were included in the study, those that were excluded from the study and the reasons for the exclusion.
8. Validate reagents for the intended application (e.g., selectivity of small molecule, appropriate antibody for immunohistochemistry).

All studies should include both positive and negative controls. For example, a group of animals treated with a drug that was approved for this indication will enable a side-by-side comparison of the benefit of our intervention, as well as confirm that the disease model is relevant. Academics sometimes assume that certain controls are wasteful—“We have done these controls before” is a reasoning we often use. However, the control experiments need to be done side-by-side with the treatment arm, as unexpected factors can contribute to the outcome. A recent investigator in SPARK told us that they omitted an oral gavage of their control subjects before the last blood draw, only to discover later that gavage alone increases neutrophil number in the blood—possibly due to animal stress. Needless to say, the entire study had to be repeated.

It is important that critical experiments are repeated by a different investigator in the same lab to ensure that the experimental protocol is detailed enough to be reproduced by an unbiased researcher. When I first reported on the benefit after heart attack of treating animals with an inhibitor we developed for delta protein kinase C, the benefit was so surprising that one skeptic refused to believe the results. It was good to be able to answer that three members of the lab reproduced the same data. It was even better to be able to report that two other labs reproduced our data, and it was really a coup when that skeptic obtained the same data in his own laboratory.

2.1.1.3 Insufficient Statistical Power of the Study or Inappropriate Statistical Analysis

To save on animal use, researchers in academia often use too few animals per treatment group. Unfortunately, a p -value smaller than 0.05, although significant, is not robust enough if the study was done with 5 or less animals per treatment group.

If you are like me, you contact a statistician only when you try to analyze the data. A recent commentary urges academicians to recognize the critical contribution of statisticians in preclinical research [5]. Statisticians should be engaged early during the study planning to ensure that the number of animals included is sufficient and that the study is powered to provide an unequivocal answer. This will not only ease the review process, but importantly will increase the rigor of the study. Let us not have our budget dictate the number of animals per group we use, or we risk sacrificing the robustness of our results!

Biostatisticians can also weigh in on the appropriateness of the statistical tests used to analyze the results. Often, there is more than one statistical test available to compare groups, but characteristics of the data (e.g., size, distribution, etc.) may make some tests inappropriate. For example, we should not use a t -test on nonparametric data.

2.1.2 Conclusion

Given the Amgen and Bayer reproducibility studies, should we even attempt to do preclinical work in academia? Let us not throw the baby out with the bath water. Academic research provides essential fuel for new drug development, in general, and for orphan indications in particular. In a recent analysis of 252 drugs approved by CDER between 1998 and 2007, only 47% were considered scientifically novel; and academic discoveries contributed to a third of those novel molecules [6]. In addition, of drugs approved for orphan indications during that period, almost 50% were based on academic discoveries. So academic research is an important engine for innovation in drug discovery. Nevertheless, as Begley and Ellis conclude, the bar for reproducibility in performing and presenting preclinical studies must be raised. More rigorous preclinical research in academia will reduce waste of research and money in industry, thus leading to a cheaper drug discovery effort and a benefit to patients.

Box 2.5: The Bottom Line

The bar for reproducibility in performing and presenting preclinical studies carried out by academic scientists must be raised, lest innovative academic work go unnoticed by industry partners.

2.2 Repurposing Drugs

Kevin Grimes

Drug repurposing (also called drug repositioning) refers to the practice of developing an existing drug for a new clinical indication. Typically, a drug selected for repurposing has been tested extensively in humans and has a known safety profile. The drug may have received regulatory approval for its original indication or may have stalled in development, perhaps for lack of efficacy or an unacceptable toxicity profile for a nonserious clinical indication.

Repurposing can be a faster, less risky, and more cost-effective route to benefit patients and is therefore particularly attractive for academics and other not-for-profit drug developers. Pharmaceutical companies, biotechnology companies, and health care investors are often less enthusiastic about supporting the development of a repurposed drug because the active compound is typically not patentable. Nonetheless, proprietary claims regarding formulation, dosing, or clinical indication may allow a period of exclusive marketing and lead to a profitable program. The repurposing of the teratogenic sedative thalidomide for the treatment of multiple myeloma is an example of the profitable exploitation of a drug whose patent had long ago expired.

While physicians often prescribe drugs for “off label” uses when caring for individual patients, a drug repurposing development program for a novel indication will require clinical human experimentation and, therefore, approval of your Institutional Review Board (IRB). Advancing a repurposed compound to clinical study may also require the filing of an Investigational New Drug application (IND) with the US Food and Drug Administration (FDA) or relevant national regulatory agency (if the clinical studies will be conducted outside of the USA).

Drug studies typically require a new IND if the research will be reported to the FDA in support of a marketing claim for the new indication, i.e., a new drug label, or if the research involves a “route of administration or dosage level or use in a patient population or other factor *that significantly increases the risks* (or decreases the acceptability of the risks) associated with the use of the drug product” [7]. When in doubt, check with your institution’s legal or compliance office or directly with the FDA.

Box 2.6: Key Terms and Abbreviations

Repurposing: finding a new indication, formulation or route of administration for an existing drug

Off-label: indications not listed on the drug label (and therefore not evaluated by the FDA)

IRB (Institutional Review Board): a committee formally designated by an institution to review, approve the initiation of, and conduct periodic reviews of biomedical research involving human subjects

IND: Investigational New Drug application; document filed with the FDA prior to initiating research on human subjects using any drug that has not been previously approved for the proposed clinical indication, dosing regimen, or patient population

FDA: Food and Drug Administration

NIH: National Institutes of Health

Drug Master File: a confidential document submitted to the FDA (or national regulatory agency) outlining specifications for the manufacturing, processing, packaging and storing of a therapeutic agent(s)

GLP: Good Laboratory Practice; extensive documentation of each procedural step to ensure high quality, reproducible studies

Pharmacokinetics: measurements of what the body does to a drug (absorption, distribution, metabolism and excretion)

2.2.1 Identifying Repurposing Opportunities

When we have discovered a novel, validated drug target, screening a library of previously approved drugs for activity against our target may lead to a drug repurposing opportunity. Researchers at the US National Institutes of Health

(NIH) have assembled a comprehensive list of drugs that have been previously approved by the FDA ($n = 2,356$) and by regulatory agencies worldwide ($n = 3,936$, inclusive of the FDA). In addition, they have compiled a library of 2,750 of these previously approved drugs and of 4,881 drugs that have undergone human testing, but have not been granted regulatory approval [8]. Researchers may apply to have the NIH test their targets against this library. Alternatively, many high-throughput screening (HTS) centers now also include a collection of previously approved drugs as a part of their chemical library.

A second path to repurposing is to apply a known modulator of a specific biologic target to a new disease. For example, eflornithine is an inhibitor of ornithine decarboxylase (ODC), a key enzyme in mammalian cells for converting ornithine to polyamines. The polyamines, in turn, are important in cell proliferation, differentiation and growth. Eflornithine stalled in development when it failed to show adequate efficacy as an antitumor agent, but has subsequently been successfully redirected as a treatment for African sleeping sickness, since ODC is also present in the causative parasite.

A third avenue for identifying repurposing opportunities is through astute clinical observation and exploitation of known or unanticipated side effects. For example, erythromycin is well known for causing gastrointestinal distress and diarrhea. This observation had led to its clinical use as a promotility agent in selected patients with a functional, non-obstructive ileus. Similarly, sildenafil originally entered clinical development as an anti-angina/antihypertensive agent. A serendipitous clinical observation led to its development as a treatment for erectile dysfunction—an extremely lucrative market opportunity.

The following sections will discuss the repurposing of drugs based upon the drug's regulatory status, patent status, and intended indication, dose, and route of indication. In general, the regulatory agencies will focus first and foremost on the safety of the proposed dosage and formulation in the new patient population. Of course, we must also show efficacy to gain regulatory marketing approval.

2.2.2 Previously Approved Drugs Using the Same (or Lower) Dose and Route of Administration

This category presents the fastest route to the clinic. If the drug is generically available and the intended patient population is not at increased safety risk, there are relatively few barriers to conducting a clinical study and publishing the results. Of course, we will require IRB approval prior to initiating the study. Once we publish our study results, physicians will be free to prescribe the drug off-label without a formal regulatory approval for the new indication. If there is reason to suspect increased risk or that the known drug risks are less acceptable for the intended indication and study population, we must file an IND.

If the drug is proprietary, we should consider approaching the company that markets the drug to solicit support for our study. Depending upon the size of the current market and the number of years remaining on the patents, the company may see our repurposing proposal as either an opportunity or a threat. Our proposed new market may represent an attractive pipeline extension. On the other hand, unanticipated negative adverse effects in the clinical study may threaten the existing franchise. If an IND is required, we must have the company's approval for the FDA to access their proprietary Drug Master File at the agency; thus, company consent is required. If an IND is not required, we may proceed with our study, even without the company's consent, assuming that we have obtained IRB approval and have adequate financial resources.

Working with the company can provide many advantages beyond financial support or free study drug. The company scientists will have an extensive working knowledge of the drug's metabolism, formulation, side effects, and potential drug–drug interactions. This information can be invaluable in the design and execution of the new clinical study.

2.2.3 New Route of Administration, Dosing, or Formulation

Regulatory agencies require that a drug be both safe and efficacious. When a drug is administered via a different route (e.g., via inhalation instead of intravenously), at higher dosages, or in a new formulation, the safety profile will be altered and human efficacy will be unproven. Therefore, an IND will be required.

Although prior human experience with the drug can be predictive and help guide preclinical studies, supplemental GLP safety studies will typically be required to determine that the route, dose, or formulation is safe to test in humans. At a minimum, preclinical studies should be conducted to assess safety and characterize pharmacokinetics for the new formulation and/or route of administration. Non-GLP preclinical efficacy studies can be useful in demonstrating biological effect and predicting the clinical dosing requirements. An open discussion with the regulatory agency early in the course of development can be invaluable in determining which preclinical studies will be required prior to entering clinical study.

2.2.4 Non-approved Drug with Human Trial Data

A number of drugs fail to advance beyond their initial phase 2 or 3 clinical study because of lack of efficacy for their intended clinical indication. These drugs are typically “shelved” by the sponsoring company, but can be very valuable if a new target or clinical indication can be identified. The timeline for developing a “shelved” drug for a new indication can be appreciably shortened and less costly because the company sponsor already has a complete preclinical package, human

safety data, and a Drug Master File with the FDA (or similar regulatory agency). Often, clinical grade drug product is also available if it still meets its quality specifications. Typically, we must work with the original company sponsor because the drug is under patent protection and/or all the previous data filed with the regulatory agency is proprietary and owned by the company. The US NIH has recently announced an industry/government collaboration program that provides access to academicians to test such compounds [9].

Box 2.7: The Bottom Line

Drug repositioning can be a faster, less risky, and less expensive route to develop a new therapy for a clinical indication. Repurposing is particularly attractive for academics and other not-for-profit drug developers who are seeking cures for patients, but have limited financial resources. Some repurposing programs can be quite successful commercially if they have intellectual property claims that block competitors or privileged regulatory status (e.g., orphan disease designation).

2.3 Developing Assays for High-Throughput Screening (HTS)

Bruce Koch

The aim of HTS of chemical libraries is to identify small molecules (chemical leads) that *hit* or affect a protein target or cellular phenotype. The screen typically identifies good starting chemical entities that will be improved upon (optimized) using medicinal chemistry. There are alternative approaches for identifying small libraries of chemical leads, such as searching the published literature (including patents) or screening substrate or transition state analogs. *In silico* and fragment-based screening are also options for screening large libraries of molecules, but these methods require prior target structure elucidation and high assay sensitivity, in the case of fragment-based screening. Here we focus on the development of assays for identifying and characterizing active compounds from large (>100,000 compounds) drug-like molecule libraries using HTS.

What is unique about HTS? It relies on robust, miniaturized, “mix and measure” assays. A robust assay is one with a high Z' -factor [10], good reproducibility between runs, and resistance to interference. With the large compound libraries typically screened via HTS, cost and logistics often dictate that only a single well per compound be run. Thus, even with a high Z' -factor, there are considerable opportunities for false positive (non-reproducible) and false negative (missed actives) results due to random variation. This should be considered when designing, optimizing, and characterizing the primary screening assay. Often, multiple iterations of assay design and testing are required to adapt a low-throughput (<50 samples) assay for optimal performance in HTS.

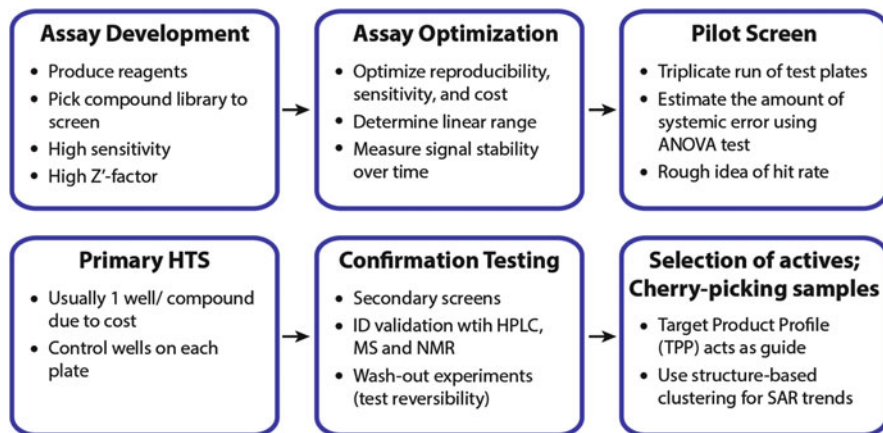


Fig. 2.1 General high-throughput screen workflow

The typical HTS workflow can be broken into the following steps (Fig. 2.1):

1. Procuring or scaling up production of the reagents (e.g., proteins or cells, substrates, solvents, reporters)
2. Developing the assay, including miniaturization
3. Assay optimization (e.g., Z' -factor, reproducibility, sensitivity)
4. Characterization of the optimized assay (e.g., sensitivity to time and temperature, linear range)
5. Pilot screen with triplicate runs of a small selection of the compound library
6. Primary HTS
7. Selection of actives and cherry-picking samples
8. Confirmation testing
9. Compound structure-based clustering
10. Confirmation of hits, evaluating the purity and identity of selected actives using LC-MS followed by NMR, and confirming activity in secondary assays

HTS assays are typically run in 2–30 μl in 384 or 1,536 well microtiter plates, although some assays resist miniaturization beyond 96 well plates. The choice of assay technology is often dependent upon the detection equipment available, cost of reagents (particularly for a screen of a large library of compounds), stability of the reagents, ease of use, and the potential for assay technology-dependent false positives.

Box 2.8: Key Terms and Abbreviations

Chemical hit: small molecule that affected the target or phenotype

HTS: high-throughput screening

Optimization: medicinal chemistry effort to improve the properties of a chemical lead

(continued)

Box 2.8 (continued)

Mix and measure assay: an assay that does not require washing away any of its components

Z'-factor: measure of assay signal relative to noise

Competitive inhibitor: molecule that binds to the target enzyme and excludes substrate binding (and vice versa)

Uncompetitive inhibitor: molecule that binds only to the target enzyme-substrate complex

Noncompetitive inhibitor: molecule that binds to the target enzyme independent of substrate binding

Edge-effect: situation in which outside wells of a multi-well plate have a bias toward different values than the rest of the plate

K_m (Michaelis constant): substrate concentration at which an enzymatic reaction rate is $\frac{1}{2}$ of the maximal reaction rate. K_m is a way to characterize the enzyme's affinity for the substrate.

Once an assay technology is chosen, assay design and optimization involves tradeoffs between assay sensitivity to compounds, Z'-factor, and cost. If cost is not a consideration, one can often add large amounts of detection reagent and get both an enhanced Z'-factor and an increased sensitivity to inhibition by compound. In practice, especially for an academic effort, cost is an important consideration. For enzyme assays, the choice of substrate concentration (relative to K_m) will affect the type of inhibitors or activators that are identified. Running the assay with the starting substrate concentration equal to K_m will give the best overall sensitivity to competitive, uncompetitive, and noncompetitive inhibitors [11]. Unlike most assays designed to study enzyme kinetics, HTS assays often allow substrate conversion to proceed to around 50%, since this produces much better signal/noise at a loss of only ~ 1.4 -fold in sensitivity to competitive compound inhibition [12]. Phenotypic screens use a biological response (e.g., cell death, protein translocation) to report compound activity. Because phenotypic responses reflect a complex biological cascade, they can be more accurate readouts of the therapeutic potential of a molecule. Confirmation via secondary assays, however, can be more difficult, as the compound target in a phenotypic screen may be unknown.

Box 2.9: Z'-Factor Defined

The Z'-factor reports the statistical effect size of the difference between an assay's signal (positive control) and noise (negative control). Good HTS assays have a Z'-factor between 0.5 and 1.

$$Z' \text{-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

Where data follows a normal distribution and:

(continued)

Box 2.9 (continued)

σ_p : standard deviation of positive control replicates
 σ_n : standard deviation of negative control replicates
 μ_p : mean of the positive control replicates
 μ_n : mean of the negative control replicates

Once the assay has been developed, it often will require optimization to obtain an adequately high Z' -factor and robustness. This is particularly true if the assay suffers from “edge-effects,” a situation where the outside wells (in a control plate) have a bias toward different values than the rest of the plate. This can be caused by differences in temperature (plates warm up from the outside), evaporation, and in the case of plated cell-based assays, differential cell growth. It can take considerable experimental effort to identify the cause(s) of the artifact and redesign the assay to minimize its effects. As an example, for a thermal gradient edge effect, a long incubation with a lower enzyme concentration might replace a short incubation to allow time for thermal equilibration before assay readout.

Box 2.10: What Surprised an Academician?

Nearly all high-throughput screens identify reproducible (i.e., not produced by variance) false positives. This is why it is so important to have secondary assays with different reporters to confirm hits.

During assay optimization, the assay conditions should be characterized with regard to linearity with the concentration of the target protein (e.g., binding and enzyme assays), linearity with time, stability of the reagents on the assay equipment (necessary because of the time required for assay runs), solvent (typically DMSO), sensitivity, and pharmacology (if suitable standards are available). If it doesn't interfere with the assay, it is advisable to use fairly high concentrations (5–10% v/v) of DMSO, since this tends to increase the solubility of many compounds. However, cell-based assays are typically relatively sensitive to DMSO, with the limit often being at 0.5–2% v/v.

After assay optimization, the assay protocol is “frozen” and a pilot screen is run to rigorously test whether the assay is ready for HTS. Three identical sets of compound plates (typically several thousand unique compounds) are run through the assay, one set (in randomized plate order) per run. The data are analyzed using analysis of variance to determine the sizes of the systematic errors due to plate order, plate row, plate column, etc. Ideally the variance is almost all “random,” with only very small contributions from systematic errors.

All HTS assay designs result in the identification of reproducible (i.e., not produced by variance) false positives. These can result from compound interference with the assay readout or from undesirable modes of interaction with the target.

Examples include reactivity of the test compound leading to covalent modification of the target, or compounds that inhibit the detection of a reporter gene directly; a common concern in a luciferase-based assay. Thus, it is essential to develop additional independent assays to validate the *hits* (active compounds) from the primary screen or, if that is not possible, to eliminate potential mechanisms producing false positives.

These validation assays should seek to answer the following questions:

1. Does the compound interact directly and reversibly with the molecular target, and with reasonable stoichiometry?
2. Does the reported structure of the active compound match what is in the well? Is it reasonably (>90%) pure? If the compound is <99% pure, is the activity quantitatively the same after purification or resynthesis?
3. Does the compound interfere directly with the reporter readout used?
4. Is compound activity quantitatively reproducible using a different assay technology (e.g., cell-based versus *in vitro*)?
5. Is the activity reversible after washout? (The relationship between potency and expected off-rate should be considered.)
6. Is there evidence of a structure–activity relationship (SAR) for the active compounds? Are there related inactive compounds in the library?
7. Is the compound just generally reactive under the assay conditions? This can be assessed by comparing compound activity before and after incubation with potential target moieties (e.g., 5 mM lysine dissolved in assay buffer).

Following these steps should result in a well-characterized primary screening assay and a set of secondary assays suitable for a HTS campaign in academia or one of the NIH Molecular Libraries Probe Production Centers Network.

Box 2.11: Recommendations

For a biochemical HTS assay, substrate concentration should be equal to K_m to help identify competitive, uncompetitive, and noncompetitive inhibitors. Different conditions may be required to identify activators (depending on the sensitivity of the assay). Usually 50% of the substrate should be converted to product for optimal signal/noise.

For cell-based assays, the percentage of organic solvents should be minimized and solvent-alone should be run as a control during assay development. Live cell imaging can be particularly challenging for large libraries unless the microscope is also in a temperature, % CO₂, and humidity-controlled environment.

Box 2.12: Key Web Sites

NIH Molecular Libraries Program

<http://commonfund.nih.gov/molecularlibraries/overview.aspx>

Lilly/NCGC Assay Guidance Manual

http://www.ncgc.nih.gov/guidance/manual_toc.html

Society for Laboratory Automation and Screening

<http://www.slas.org/>

Journal of Biomolecular Screening

<http://jbx.sagepub.com/>

2.4 Medicinal Chemistry and Lead Optimization

Daniel A. Erlanson

Lead optimization means taking a small molecule with promising properties and transforming this “hit” into a drug. It is like molecular sculpture, but instead of developing an aesthetically pleasing statue (which sometimes occurs), the aim is to construct a safe and effective molecule for treating a specific disease. And instead of chisels and plaster, practitioners—medicinal chemists—apply the tools of chemical synthesis.

The previous section covered HTS which, if successful, has generated a hit, a small molecule that has some activity for the target or phenotype of interest. Of course, this hit is likely a long way from being a drug. Improving affinity is often the first task of lead optimization. A drug should be as potent as possible to reduce the cost of production, to minimize the size of the pill or injection needed, and to reduce the potential for off-target effects. Most drugs have IC_{50} or EC_{50} values (half maximal inhibitory concentration or half maximal effective concentration) around 10 nM or so, with considerable variation to either side. Hits from HTS are sometimes nanomolar potency, but more often low micromolar, which means that binding affinity may need to be improved by several orders of magnitude.

2.4.1 *Lead Optimization Considerations*

2.4.1.1 Improved Affinity

Knowing how the molecule binds can generate ideas on how to improve potency. For example, there may be a pocket on the protein near the small molecule, and adding a chemical group (or moiety) to reach this pocket may pick up additional interactions and thus additional binding energy. Alternatively, a structure may reveal an unfavorable contact: perhaps a hydrophobic (water-hating) portion of the ligand is exposed to solvent, or a hydrophilic (water-loving) portion is buried in

a greasy hydrophobic part of the protein; the medicinal chemist would make analogs of the molecule without the unfavorable contact and test the activities of the new molecules. Ideally this will lead to better potency, but often changes are less dramatic than expected, and additional molecules will need to be made. This iterative process is called structure-based drug design. In the best cases, it is possible to obtain structural information of how the small molecule binds to the target using experimental techniques such as X-ray crystallography or NMR spectrometry. Failing this, computational modeling can give some idea of the binding mode if the structure of the target is known or is believed to be similar to another characterized target.

It is also possible to do lead optimization in the absence of structure by making somewhat random changes to the molecule and seeing what effects these have on activity. Over the course of several iterations, structure-activity relationships (SARs) emerge. SAR can provide a wealth of knowledge that a medicinal chemist can use to understand the binding mode. Although experimental structural information has become a key tool in medicinal chemistry, it is worth remembering that X-ray crystallography was not sufficiently rapid and general for routine use until the 1980s and 1990s, and even today medicinal chemistry is applied to many targets for which direct structural information is not available, such as most membrane proteins.

Box 2.13: Key Terms and Abbreviations

HTS: high-throughput screening

IC₅₀: half maximal inhibitory concentration

EC₅₀: half maximal effective concentration

Chemical moiety: a functional group or portion of a molecule

SAR: structure–activity relationships

Lipophilicity: the tendency of a molecule to partition between oil and water

PK: pharmacokinetics

ADME: absorption, distribution, metabolism and excretion

PD: pharmacodynamics

hERG channel: human Ether-à-go-go-Related Gene channel, a potassium ion channel that is important to normal electrical activity of the heart. Inhibition of this channel can lead to sometimes fatal cardiac arrhythmias

CYP: cytochrome P450; a large and diverse group of enzymes that play a major role in drug metabolism

2.4.1.2 Improved Selectivity

Selectivity is another critical factor in lead optimization. Researchers generally want their drug lead to be active against the target of interest but not active against other proteins. Selectivity is most readily assessed by simply measuring activity of the molecule against other proteins, especially closely related ones, but this can be a

daunting task. For example, there are about 500 protein kinases in the human genome, so measuring activity against all or even most of them can get pricey. Fortunately, enough companies have been working in the kinase field that there are now commercial offerings to confirm selectivity against a large number of kinases in a short period of time. However, such selectivity testing for newer classes of targets and enzymes is often not available. Note that selectivity testing within a related family of enzymes or receptors does not rule out the possibility that your compound will bind to a protein outside that family. Before compounds advance into the clinic they are tested against a panel of up to several hundred targets that could cause problems (see below). However, not everything can be tested *in vitro*, and off-target effects often manifest as side effects and toxicity during *in vivo* studies.

2.4.1.3 Improved Physicochemical Properties

Throughout the course of lead optimization, it is important to keep an eye on the physicochemical properties of the molecule such as solubility and lipophilicity (the way it partitions between water and oil or membranes). Solubility, in particular, can be a tricky balancing act because improving potency often involves increasing the size and lipophilicity of a molecule, leading to decreased solubility. Chemists sometimes refer to particularly insoluble compounds as “brick dust.”

2.4.1.4 Improved Biological Potency

Initial screens are often conducted using pure isolated proteins under highly artificial conditions. Therefore, it is essential that potency be determined in more biologically relevant systems such as whole cell assays; all too often compounds that show activity against the isolated protein will show less or no activity in cells. Sometimes this is due to factors that a medicinal chemist may be able to fix rationally. For example, compounds that are negatively charged can have difficulty crossing cell membranes to interact with targets inside the cell. In other cases, it is unclear why there is a disconnect; in these cases it may be necessary to make more dramatic changes to the lead series, or switch to another series entirely.

2.4.1.5 Improved Pharmacological Properties

Potency and selectivity are important, but other parameters also require optimization. In fact, a rookie mistake is to focus exclusively on potency. Many things can happen to a drug on its way to its target. This is especially true for oral drugs: the body treats anything coming in through the mouth as food and tries to digest it or, failing that, to excrete it. The study of what happens to a drug *in vivo* is called pharmacokinetics (PK), which is covered in more detail in Sect. 2.8. A critical

aspect of lead optimization is to measure and improve the ADME (absorption, distribution, metabolism and excretion) properties of a molecule, keeping it in the body for long enough and at sufficient levels to do its job without causing problems. Many of the individual proteins that affect a drug's path into and through the body are known, and experiments with isolated enzymes, plasma, or liver extracts can be helpful, but ultimately animal studies are essential to understand a molecule's PK.

Because so many different factors are at play in pharmacokinetics, medicinal chemists often turn to empirically derived rules to try to tune the properties of their molecules. The most famous of these is Chris Lipinski's Rule of 5, a set of guidelines concerning molecular weight, lipophilicity, and other properties that predict the likelihood a drug candidate will be orally bioavailable [13]. When performing SAR to optimize PK, often a specific moiety may be prone to metabolism, and by altering this bit of the small molecule the overall stability can be improved. Keep in mind that such rules are not hard cut-offs, but directional guidelines to improve the probability of success.

2.4.1.6 Target Validation

Pharmacokinetics is sometimes characterized as “what your body does to a drug.” Conversely, pharmacodynamics (PD) can be thought of as “what a drug does to your body.” On a fundamental level, the drug needs to be active against the target of interest.

Unfortunately, it is possible to inhibit or activate a biological target and yet have no effect on the disease of interest—this is particularly true for newer targets. Validated targets are targets for which modulation of their activity alters a disease state, and the best way to validate a target is through the use of a small molecule (or peptide or protein). A tool compound can be used for target validation; this is a molecule that has sufficient activity and ADME properties to answer basic biological questions about the target, but may not be suitable as a drug, perhaps because it is toxic or has other deleterious properties.

Box 2.14: What Surprised an Academician?

We started KAI with three drug candidates for three different clinical indications. When asked by the VC to rename them (to differentiate them from those used in my academic laboratory), I thought it was silly that they did not accept the names KAI 001, KAI 002 and KAI 003. In my naiveté, I was sure that we would not need to make more than 999 compounds after all the preliminary work in my university lab. I also did not realize that a company should not reveal to others how many compounds were made (e.g., if few were made, the IP might not be that strong). So instead of giving sequential numbers, our VC dubbed KAI-9803 based on my answers to “what year did you design that peptide?” and “where did it fall in the sequence of peptides you designed that year?”.

—DM-R

2.4.1.7 Reduced Toxicity and Drug–Drug Interactions

There is a growing consensus that virtually all drugs have off-target effects, and it is important to understand these and determine whether they will cause adverse events. Toxicology is concerned with specific toxic effects, for example liver damage. A number of molecular substructures are known to have caused toxicity in the past, and medicinal chemists try to avoid having these moieties in their lead molecules. Ultimately though, it is impossible to predict whether a given molecule will be nontoxic without doing *in vivo* experiments.

Moreover, toxicity is not the only problem; there are many other “anti-targets” that a drug lead should avoid hitting. One of the most important is a cardiac ion channel protein called hERG, which when inhibited can cause severe and sometimes fatal heart problems. This has led to the withdrawal of several marketed drugs, and medicinal chemists today almost universally assess the hERG activity of their leads. The SAR of hERG binding is partially understood, and often medicinal chemists can engineer promising leads to maintain potency against the target protein and also avoid hitting hERG.

Similarly, many of the enzymes involved in metabolizing drugs (particularly a large class of enzymes called CYPs) can also be inhibited by small molecules, which can lead to drug–drug interactions if the enzymes in question are necessary for metabolizing other drugs. During the course of lead discovery it is important to measure CYP inhibition and, ideally, to make changes to the molecule to reduce or eliminate it.

Pharmacokinetics and pharmacology are both utterly dependent on animal models, but it is important to always remember that mice are not furry little people: drugs metabolized rapidly in mice may be stable in humans and vice versa. Because of such differences, obtaining animal data in at least two different species is usually necessary before moving a drug into the clinic.

2.4.2 Other Issues

A recent trend in medicinal chemistry is fragment-based drug discovery. Instead of starting with low micromolar IC_{50} lead-sized or drug-sized molecules, this approach starts with smaller “fragments” with molecular weights one-quarter to one-half the size of typical drugs and potencies in the mid to high micromolar range. Because there are fewer small fragments than larger molecules (just as there are fewer two letter words than four letter words), it is possible to more efficiently screen chemical diversity. Moreover, smaller, simpler molecules are less likely to have extraneous bits that do not help the overall potency but cause problems with PK or PD. Of course, identifying and optimizing lower affinity molecules are challenges in their own right.

Box 2.15: The Bottom Line

Multi-parameter molecule optimization in the absence of complete data is what makes medicinal chemistry as much an art as a science. The fact that an acceptable solution may not exist can make it a particularly frustrating art.

Ultimately lead optimization requires the medicinal chemist to improve numerous parameters simultaneously: potency, selectivity, solubility, PK and PD. Unfortunately improving one may exacerbate another. Medicinal chemistry requires picking the best possibilities to explore, even though it is impossible to gather all data for every compound.

In fact, there is no guarantee that it is even *possible* to produce a molecule that satisfies all the necessary parameters; targets for which this is the case are called “undruggable.” This multi-parameter optimization in the absence of complete data is what makes medicinal chemistry as much an art as a science, and the fact that a solution may not exist sometimes makes it a particularly frustrating art. The next time you take a drug, it is worth reflecting on the effort, skill, and serendipity that went into discovering that little molecular sculpture.

Box 2.16: Resources

1. *Journal of Medicinal Chemistry* (<http://pubs.acs.org/journal/jmcmar>)

This is probably the premier journal for medicinal chemistry but has onerous requirements for compound characterization.

2. *Bioorganic and Medicinal Chemistry Letters* (<http://www.sciencedirect.com/science/journal/0960894X>).

Because medicinal chemistry papers are often not submitted for publication until years after the work has been completed, some compounds may be missing key data, and so many researchers, particularly in industry, publish in this journal. It has a lower bar to publication, but some excellent work appears here too.

3. *In the Pipeline* (<http://www.corante.com/pipeline/>)

This is probably the best chemistry-related blog out there. The author, Derek Lowe, is an experienced medicinal chemist who writes prolifically about a range of topics, and his posts attract dozens of comments.

4. *Practical Fragments* (<http://practicalfragments.blogspot.com/>)

For all things having to do with fragment-based drug discovery and early stage lead optimization, my blog is a good resource.

2.5 Vaccine Development

Harry Greenberg

Few if any biomedical interventions have been as successful at preventing morbidity and mortality as vaccines. The eradication of smallpox, the near eradication of paralytic polio and the potential reduction of the global burden of hepatocellular and cervical cancer are just a few of the many benefits that have been rendered by vaccines in the last 50 years. Along with their great impact, in many ways vaccines are one of the most egalitarian of all health interventions, since their benefits generally are well suited for delivery to both wealthy and poor countries alike. Therefore, vaccines have the ability to rapidly and efficiently alter the face of global health and well being.

Vaccines are molecular moieties (or antigens) that are administered to people via a number of routes, such as parenterally (e.g., intramuscular, subcutaneous, intradermal) or via a mucosal surface (e.g., orally or intranasally). In general, they are administered on only one or a few occasions because they are designed to work indirectly by eliciting a long lasting immune response in the host. They can be formulated of simple proteins or peptides, polysaccharides, nucleic acids, or complex mixtures of these constituents. In addition, vaccines can be created using complex infectious agents that are attenuated in some fashion and whose replication is restricted. These infectious agents can, on occasion, also be used to carry and express exogenous proteins.

To date, the most successful vaccines have been live attenuated infectious agents, inactivated infectious agents, or complex components of infectious agents or polysaccharides conjugated to protein carriers. Vaccines are employed to induce a host immune response that is either protective or therapeutic. Thus far, vaccines have been more effective as preventative, to avoid contracting the disease, than as therapeutic, after you have the disease, interventions. The general or even specific applicability of the “therapeutic vaccination” concept remains to be determined in humans.

Vaccination has been most successfully employed to prevent a wide variety of infectious diseases caused by many different viruses and bacteria. Vaccination against parasitic diseases has been much less successful. “Vaccination” has also been used with more limited success for treatment of allergy. In addition, a variety of experimental vaccines for the treatment of substance addiction, for birth control, and for treatment of autoimmune diseases have been studied but have not yet been widely successful. The remainder of this brief summary will therefore focus specifically on preventative vaccines against infectious diseases.

Box 2.17: Key Terms and Abbreviations

Antigen: entity that activates an immune response

Parenteral: routes for drug absorption outside the gastrointestinal tract

HIV: human immunodeficiency virus

CMV: cytomegalovirus

RSV: respiratory syncytial virus

HCV: hepatitis C virus

HA: hemagglutinin antigen

Adjuvant: compound that increases the host immune response to an antigen

2.5.1 Vaccine Efficacy

The past 50 years have witnessed the development of many highly successful new vaccines. The remaining important infectious disease targets, such as HIV, tuberculosis, malaria, CMV and RSV have remained much more difficult to prevent. Vaccine development has the highest likelihood of success when the natural infection induces a strong and enduring immunity to subsequent infection or illness. This was, for example, the case for smallpox, measles and hepatitis A and B. In other cases where reinfection can occur (usually at a mucosal surface) but secondary infection is not as often associated with severe sequelae, vaccination approaches have also been successful. This is the case with rotavirus and influenza vaccines.

When one or a few natural infections do not lead to the development of significant immunity—as is the case for HIV, HCV, gonorrhea, rhinovirus infection and malaria, for example—then it is likely that the pathway to an effective vaccine will be far more difficult. In these cases, it is likely that identification of novel immunization strategies will be required in order to develop a successful vaccine.

Two key elements in vaccine development are the availability of a predictive functional assay to measure vaccine response and a relevant animal model in which to test various immunization strategies. Animal models that replicate actual wild type infections of the microbial pathogen in the human host are most likely to be relevant. The duration, specificity and strength of the host response, as measured by a validated functional assay, are key determinants of the efficacy of the vaccine.

Box 2.18: What Surprised an Academician?

Unless the targeted disease is quite prevalent, a large number of patients must be included in vaccine trials to demonstrate efficacy—even for a highly effective vaccine. This can greatly add to development costs and duration.

2.5.2 How Vaccines Generally Work

Vaccines are designed to induce the host to mount an immune response that prevents or eliminates infection by the targeted pathogen. The induction of host immunity involves a variety of factors including many aspects of the innate immune system, the site of immune induction, the nature of the antigen, and the quantity and duration of antigen exposure. Each of these aspects needs to be carefully considered to maximize the chances of eliciting an acquired antigen-specific immune response that has functional therapeutic activity.

Whereas both T and B cell responses are often induced by vaccination, as a generality, most existing successful vaccines “work” at the effector level on the basis of the B cell and antibody responses induced. Many methods have been and are being examined to enhance the immune response to vaccines, including using an adjuvant to boost the innate immune response, using protein carriers to induce immune memory to polysaccharide antigens, and using replicating vaccines to produce more antigens with greater diversity at the site of infection. As mentioned above, when natural infection induces protective immunity, it has been relatively straightforward to design a vaccine that mimics the effective component(s) of that infection. When natural infection is not a very effective inducer of protective immunity, vaccine development has been much more difficult.

2.5.3 Some New Technologies in Vaccine Development

This short review cannot cover all the new technologies that are currently being explored to develop novel or improved vaccines. A few examples are provided to invite the reader to examine the field more extensively. Many pathogens avoid host immunity by altering or expanding their antigenic diversity. Examples include such diverse organisms as influenza, HIV and pneumococcus. Recent advances in immunology have demonstrated the existence of “common” or “shared” antigens on several pathogens, such as the finding that the influenza HA stalk is a target of a protective antibody. Such targets could provide an “Achilles’ Heel” to which the host can target its immune response and thereby circumvent the problem of pathogen antigenic diversity. Currently many investigators are working to design new vaccines directed at such shared antigens of influenza, HIV and pneumococcus, for example.

As an alternate approach, directed regulation of the innate immune response holds the promise of greatly enhancing the level and duration of acquired immunity following vaccination. Many investigators are now exploring the safety and efficacy of new adjuvants that directly target specific signaling molecules, thereby enhancing the innate immune response.

Finally, immunization using nucleic acids (either DNA or RNA) encoding antigenic proteins holds promise to greatly simplify vaccine manufacturing, while

substantially reducing cost and enhancing safety. To date, such strategies have been highly promising in small animal models but less so in people. Continued innovation in this area, if successful, could greatly facilitate vaccine development.

2.5.4 Special Considerations Concerning Safety and Cost

There are a variety of factors that distinguish vaccine development from virtually all other areas of therapeutics development. Of course, like all other medical interventions, vaccines must be shown to be efficacious. However, unlike most other interventions, vaccines are generally given to healthy individuals with the intent of preventing a possible illness in the future rather than treating a current problem. Because of this fact, the level of tolerance for risk associated with vaccination is very dependent on the level of perceived danger from the infection being prevented. For example, when polio epidemics were common, the public clamored for a preventative intervention. However, since polio has disappeared from the Western hemisphere, even one case of immunization-induced polio per million vaccinations represents an unacceptable risk in the USA and Europe.

This common and pervasive concern with vaccine risk is often intensified because vaccines are most frequently given to young healthy children, who can be considered most vulnerable to untoward risk. In addition, the benefits of vaccination are most easily measured at the societal rather than the individual level because the odds that any given individual will be infected are frequently quite low. This dichotomy further complicates acceptance of vaccines by the public. Because of these factors, vaccine development often requires investment in very large and extensive safety testing before registration, as well as substantial post-licensing follow-up that is both expensive and complex.

Because vaccines are given to healthy individuals, because they are generally given only a few times during the life of an individual, and because of the prolonged regulatory pathway due to safety concerns as discussed above, they have frequently been perceived as providing a poor return on investment by drug developers. This is, of course, a shame, given their immense societal impact over the years.

Box 2.19: The Bottom Line

Although only one or a few doses of a vaccine are administered, vaccines are generally administered to healthy people, most often children, who are at low risk of acquiring the disease. As a result, the safety hurdle is very high, further adding to the time and cost of vaccine development.

Finally, many of the most important remaining challenges in the area of vaccine development (HIV, tuberculosis, malaria) are diseases that generally afflict the poor, disadvantaged and less developed regions of world. This fact has likely

inhibited the rate of progress for these much needed interventions. Despite these issues, recent advances in immunology, material sciences and systems biology provide exciting opportunities for the vaccine innovators of the future. During the coming decade, we are likely to see vaccination for several of these challenging diseases reduced to practice.

2.6 When to Begin Animal Studies

Daria Mochly-Rosen

We have identified a new chemical entity or a known drug that affects our validated target/pathway and have shown its efficacy in a cell-based assay. What is the next step?

Experts are divided on whether it is advisable to begin animal studies right away or whether it is better to first identify the optimal compound. By generating and testing analogs of the original “hit,” it may be possible to improve potency or specificity for the target. *In vitro* studies to obtain an optimal formulation for a drug or simply better solubility can also improve the chance for success once animal studies begin. And there are other considerations, such as *in vitro* assessment of drug toxicity and metabolism, including liver enzyme assays, hERG channel effects, etc. In other words, we can easily spend a year and thousands of dollars in studies aimed at improving our initial hit.

In vitro and cell-based assays are usually cheaper and faster to run than animal studies, but they are not always predictive of the *in vivo* behavior of the molecule—which is ultimately most important for determining if our hit will make a good drug. So, how are drug development programs to decide, with their limited funds, between screening lots of analogs *in vitro* versus testing only a handful of molecules in animals? As an academician that has followed over 70 programs in SPARK, my answer to this question is simple.

2.6.1 Take a Short Cut

We should start animal studies as soon as we can. It is true that many improvements to our compound can be made, but a short *in vivo* study can be extremely valuable in helping to optimize the compound and induce greater interest from partners and investors. A great deal can be learned from an imperfect drug. We might even be lucky and find that our compound shows a therapeutic benefit and drug-like properties!

We must also recognize that failure to demonstrate efficacy at this stage is not a reason to discontinue our project. These are exploratory studies and much can still

be done to improve the compound's selectivity, potency, solubility, bioavailability, safety, metabolism, route of administration and final formulation.

Box 2.20: Key Terms and Abbreviations

Alzet[®] pumps: miniature osmotic infusion pumps for the continuous dosing of a drug to a laboratory animal

hERG channel: human Ether-à-go-go-Related Gene channel is a potassium ion channel that is important to normal electrical activity of the heart. Inhibition of this channel can lead to sometimes fatal cardiac arrhythmias

ip: intraperitoneal; within the abdominal cavity

sc: subcutaneous; beneath the skin

SAR: structure–activity relationship

2.6.2 What Animal Model to Use?

It is best to read the literature and use an animal model that is accepted in the field for the given indication. It is inadvisable to develop a new model for this first *in vivo* trial. Better yet, we can find a collaborator that is using this animal model and have them do the study for us. It is rare that such a study will generate new intellectual property—and the collaborator can provide an independent and unbiased assessment of our compound.

2.6.3 How to Deliver the Drug?

Even if we believe that oral administration is the ideal route for our clinical indication, it is ill advised to attempt to do the first efficacy study in animals using oral gavage. Instead consider intraperitoneal (ip) injection. If the drug is not very soluble, we can deliver the drug with ethanol, DMSO or polyethylene glycol; animals will tolerate quite a high dose of these solvents. If there is concern that the drug dose will be too low using ip injection, we can consider using a subcutaneous (sc) Alzet[®] osmotic pump. The company's Web site details a number of sizes and recommended solvents as well as training on how to implant them—all very easy.

Box 2.21: What Surprised an Academician?

When selecting a delivery formulation for these initial animal studies, simpler is always better. We once used over the counter beauty lotion for an initial topical delivery study because it had the desired aqueous formulation properties. –DM-R

2.6.4 Start with a Small Safety Study

To make sure that the drug dose is not fatal, we can inject a couple of healthy animals and observe them for a few hours for obvious signs of toxicity. A veterinary nurse can help with monitoring for adverse events. Once we know that the dose selected is not acutely toxic, we can jump into efficacy and longer safety studies in the chosen animal model of disease.

2.6.5 Learn as much as You Can from the First In Vivo Study

Animals are precious and should be used sparingly. Therefore, we should plan experiments carefully to include proper controls. If there is a drug that is known to be efficacious in the model, we should treat three to four animals with that drug to serve as a positive control. We can include a vehicle control if we are worried about effects of the vehicle. Otherwise, for this first study, just compare drug-treated to non-treated animals. When euthanizing the animals, we should collect as many organs and bio-fluids as possible for analysis. A pathologist can advise us on how to preserve the tissues and store samples for later analysis. We should attempt to collect as much data as possible relevant to our disease and to compound safety. The bottom line: we need to maximize the information obtained from this first set of studies.

2.6.6 If the Short-Cut Failed

We are not done! Remember that we have committed to take the long route even if the shortcut failed. We can go back and perform further SAR studies with analogs of our hit and additional studies on drug solubility and *in vitro* toxicity. We can now focus on correcting the problems identified based upon the first *in vivo* experiment.

2.6.7 If the Short-Cut Succeeded

Congratulations! The work has just begun. But now we have more compelling data that the project is worth pursuing. Make sure to consult Sect. 2.1 on robust preclinical work and Sect. 2.7 on *in vivo* pharmacology to plan your next steps.

Box 2.22: The Bottom Line

An early small *in vivo* study can be extremely helpful in demonstrating both efficacy and preliminary toxicity of our drug. Results can also inform further rounds of optimization of the compound. During initial animal studies, the drug should generally be administered using a parenteral route (ip or sc via osmotic infusion pump).

2.7 *In Vivo* Pharmacology: Multiple Roles in Drug Discovery

Simeon I. Taylor

Classical drug discovery relied primarily upon testing compounds for activity in established animal models. When following this paradigm, it was not necessary to ask questions such as why one conducted *in vivo* pharmacology experiments or whether there was value in studying animal models of disease. Rather, screening in various animal models was often the first step in the drug discovery process. The use of animal models played an essential and central role in the classical drug discovery process. In the past, the molecular target was frequently unknown at the time a drug was approved for use in patients. Indeed, as illustrated by the example of sulfonylurea drugs, the molecular target (e.g., the sulfonylurea receptor) was identified several decades after the drugs were in widespread use to treat type 2 diabetes mellitus.

How times have changed! Modern drug discovery most often relies on a radically different research paradigm. Target-based drug discovery has become so entrenched that some scientists actually question whether *in vivo* experiments in animal models have any value in the modern approach to drug discovery. This section illustrates the many ways in which *in vivo* pharmacology studies in experimental animals contribute to drug discovery.

2.7.1 *Target Identification and Validation*

How are drug targets identified in the first place? While there is no simple answer to this question, proposals for new targets are often based upon genetic experiments. Genetic diseases (either in humans or in experimental animals such as mice) can generate hypotheses suggesting potential drug targets. In some cases, a gene mutation (most often a loss-of-function mutation) causes disease. For example, homozygous loss-of-function mutations in the genes encoding either leptin (*ob/ob*) or the leptin receptor (*db/db*) cause obesity in mice. Based upon the identification in 1994 of a loss-of-function mutation in the leptin gene as a cause of obesity in mice,

a biotechnology company paid a large sum of money to license the relevant intellectual property from an academic institution. In other words, a biotechnology company viewed this genetic evidence as compelling validation that leptin represented a therapeutic protein to treat human obesity.

Ultimately, the clinical studies in humans were disappointing. Although leptin is efficacious in rare human diseases associated with low leptin levels (e.g., mutations in the leptin gene or lipoatrophic diabetes), it did not deliver the desired efficacy in patients with the common forms of obesity. In short, the predictive value of leptin-deficient animal models was limited to predicting the response of leptin-deficient humans to pharmacologic therapy with leptin. However, most obese patients turn out to be leptin-resistant rather than leptin-deficient. Accordingly, human responsiveness to antiobesity treatments was better predicted by a leptin resistant model (i.e., the *db/db* mouse with mutations in the leptin receptor gene).

Box 2.23: Why Do Some Scientists Question the Value of Studies in Animal Models?

There are many examples where data obtained from experiments in animal models fail to predict the outcome of clinical studies. It would be fallacious, however, to infer that animal studies in general are entirely without value. Animal models are idealized versions of disease where all subjects are the same age (usually young), eat the same food, and have the same routines. Human subjects are much more varied, and so will have a more variable response to treatment. This is why it is very important to know the limitations of your chosen animal model when extrapolating to expected effect in humans.

What lessons can be drawn? There are many animal models. It is essential to exercise scientific judgment before extrapolating from an animal model to human disease. For example, multiple animal models of a particular disease may yield discordant predictions. Whereas the *ob/ob* mouse model suggested that leptin would be a highly efficacious treatment for obesity, the *db/db* mouse model predicted the exact opposite. It is often necessary to carefully compare results from animal models to clinical specimens or observations to assess the predictive value of a particular animal model for a particular human disease.

There are at least three other limitations which make it difficult to extrapolate from genetic models such as knock-out mice:

- Because mutations are present at the earliest times in development, there can be important developmental effects which might not be relevant to pharmacology in adult animals. For example, if a mutation in a particular gene impacts development of an organ, this would have a profound effect upon physiology. Pharmacological inhibition of the function of the same gene product in an adult animal would not necessarily lead to the same physiological deficit.

- Many loss-of-function mutations cause disease. Accordingly, to treat the disease it may be necessary to find a drug to activate the function of the gene product. However, as illustrated by the example discussed above, leptin was not an efficacious treatment of obesity despite the fact that leptin deficiency causes obesity. In contrast, there are examples where loss-of-function mutations have been shown to promote health. For example, loss-of-function mutations in Pcsk9 lead to decreased LDL levels, thereby decreasing the risk of cardiovascular events. Subsequent data demonstrated that loss-of-function mutations in the Pcsk9 gene reliably predicted the pharmacology of Pcsk9-neutralizing antibodies.
- It seems likely that loss-of-function mutations may accurately predict the pharmacology of inhibitors or antagonists. For a variety of reasons, agonists and activators may not always exert pharmacological effects which are the opposite of the phenotype of loss-of-function mutations.

Box 2.24: Key Terms and Abbreviations

Pharmacodynamic efficacy: the ability of a compound to affect the *in vivo* activity of a target

Disease efficacy: the ability of a compound to improve the effects of disease

Off-rate: the rate of compound release after binding to the target; irreversible binders have a zero off-rate

Drug exposure: also called the AUC (Area under the curve); the integral under a plot of plasma drug concentration versus time

Pro-drug: a compound which requires metabolism after administration in order to show therapeutic activity

PK: pharmacokinetics; measurements of the absorption, distribution, metabolism and excretion of a molecule after administration

PD: pharmacodynamics; measurements of drug action in the body (e.g., target inactivation, receptor off-rate, etc.)

NOAEL: no observed adverse effect level

2.7.2 Assessing Efficacy During Lead Optimization

As a prelude to discussing the role of animal experiments in the lead optimization process, it is important to distinguish between two concepts:

- *Pharmacodynamic efficacy.* This refers to the ability of a compound to engage the molecular target *in vivo*, and also to modulate *in vivo* biology. Among other things, this requires that the compound be delivered in appropriate concentrations to the biological compartment where the target resides. It also requires that the pharmacokinetics will provide sufficient exposure of the drug to the target. There are at least two complementary approaches to assessing pharmacodynamic efficacy. (a) In some cases, it is possible to assess target occupancy

(e.g., by assessing the ability of a drug to inhibit binding of PET ligands to the drug target). (b) It is often useful to assess the function of the target (e.g., by assessing the ability of a protein kinase inhibitor to decrease the phosphorylation state of a specific kinase substrate).

- *Disease efficacy*. This refers to the ability of a compound to ameliorate the manifestations of a disease. Needless to say, evidence of disease efficacy in an animal model is frequently interpreted as suggesting that the drug will also be efficacious in human disease. This expectation is not always borne out. Nevertheless, this is not a reason to entirely abandon the use of animal models simply because they are imperfect predictors of human pharmacology. Situations in which compounds show strong pharmacodynamic efficacy but lack disease efficacy can also occur, and suggest target validation was in an oversimplified model of disease.

Whether or not they turn out to predict disease efficacy in humans, animal models provide essential information for the pharmaceutical R&D process. For example, animal models can provide important insights for lead optimization:

1. Which parameters of *in vitro* pharmacology best predict disease efficacy? In many cases, the *in vitro* potency (e.g., the thermodynamic affinity with which the compound binds to its target) will be the best predictor. However, in some cases, the kinetic off-rate may be more relevant. For example, when neurotransmitters are released at synapses, this leads to very high local concentrations that persist for short durations. If a competitive antagonist has a rapid off-rate, this will allow the high concentrations of neurotransmitter to compete effectively with the drug. In contrast, if the drug has a slow off-rate, the drug will remain bound to the target during the brief time the neurotransmitter achieves its peak level. In this nonequilibrium condition, a drug with a slow off-rate will out-perform a drug with a fast off-rate even if both drugs have the identical *in vitro* potencies during equilibrium binding conditions.
2. Which parameter(s) of drug exposure best predict disease efficacy *in vivo*? In some cases, peak drug levels drive disease efficacy—e.g., for transcriptional activators that promote expression of long-lived proteins. In other cases, drug exposure (the integral of drug concentration over time) drives disease efficacy—e.g., if it is necessary to sustain inhibition of a target for 24 h a day.
3. Does the drug reach the appropriate compartment to drive disease efficacy? Sometimes, a drug can accumulate in an organ because it is tightly bound to an irrelevant protein. To derive the desired pharmacology, it is necessary to achieve sufficient levels of free drug to drive the required occupancy of the correct molecular target.
4. Do metabolites show pharmacological activity? In some cases, compounds will undergo metabolic transformation into active species. In some cases, the administered compound (i.e., the “prodrug”) may be inactive and undergoes metabolic transformation into an active species. For example, prednisone is

inactive, but must be converted into the active compound, prednisolone, by 11β -hydroxysteroid dehydrogenase. In other examples, active metabolites are to blame for a compound's undesired side effects; in which case medicinal chemistry efforts will modify the lead compound to reduce that mode of metabolism. *In vivo* pharmacology experiments are essential to identify and quantitate the levels of drug metabolites and also to assess their contribution to overall pharmacology.

5. What is the projected human dose? As part of the feasibility assessment, it is necessary to estimate the expected dose required for efficacy in humans. There are at least two factors which enter into the dose projection: first, quantitation of the exposure required for efficacy in at least one animal model; and, second, prediction of the expected pharmacokinetic (PK) profile in humans. The projection of human PK is generally based upon measurement of PK in multiple species (e.g., mouse, rat, dog, and nonhuman primate).
6. How safe are the compounds and what is the therapeutic index? Safety assessment is generally conducted in two nonclinical species (one rodent and one non-rodent) prior to initiating human studies. The "no observed adverse effect level" (NOAEL) is defined as the highest exposure that can be achieved without causing adverse effects in the test species. The therapeutic index is defined as the ratio of the NOAEL exposure: efficacious exposure. To calculate the therapeutic index, it is essential to define the exposure required for efficacy in at least one animal model. This is one of the most important reasons why it is essential to have conducted efficacy studies prior to advancing a compound into development.

2.7.3 Identifying Clinical Biomarkers

Relatively long periods of treatment are often required to assess efficacy in human disease. Prior to embarking upon such studies, it is essential to define the relevant dose range to study. Toward that end, it is very useful to assess the effect of the drug on translational clinical biomarkers. For example, in the development of sodium-dependent glucose transporter-2 (SGLT2) inhibitors as antidiabetic drugs, it was possible to assess the drug's pharmacodynamics (PD) efficacy by measuring excretion of glucose in the urine. There are at least two questions which must be addressed in order to interpret clinical biomarker data:

1. Does the biomarker predict disease efficacy? In the case of SGLT2 inhibitors, loss of glucose in the urine is a direct consequence of inhibiting the transporter that mediates reabsorption of glucose from the glomerular filtrate. In addition, loss of glucose in the urine is the key mechanism that drives the decrease in plasma glucose levels. This line of reasoning provides a compelling rationale to believe that glucosuria is a valid biomarker to predict glycemic efficacy in patients with type 2 diabetes.

2. What degree of change in the biomarker is required to drive disease efficacy? By studying the biomarker in animal models of disease, it is possible to obtain experimental data to calibrate the biomarker relative to assessments of disease efficacy. There is no guarantee that the calibration derived from animal models can be extrapolated quantitatively to human disease, but it does provide a reasonable starting point. In the absence of such data from animal models, clinical investigators have no alternative but to guess at how to calibrate the biomarker.

2.7.4 Conclusion

In vivo pharmacology studies in animal models make critical contributions to many aspects of pharmaceutical R&D—including target identification, target validation, lead optimization, safety assessment, and translational biomarker identification, validation, and calibration. Unfortunately, for a variety of reasons, nonclinical studies are only imperfect predictors of clinical pharmacology. Nevertheless, perfection is seldom achieved in human endeavors. While researchers must take this limitation into account, it would be a mistake to let the perfect be the enemy of the good.

2.8 Pharmacokinetics and ADME Properties

Werner Rubas and Emily Egeler

Initial screening efforts and secondary assays to identify compounds with desired efficacy and specificity for the intended target focus on issues of pharmacodynamics (PD), which in layman's terms can be defined as "actions of a molecule (drug) on the body." For a drug to be successful, however, the active molecule must be able to reach the intended target at high enough concentrations and for a long enough time to exert its therapeutic effect. The body must also be able to remove the active molecule without significant buildup of toxic species, or the drug will fail in clinical trials. These considerations are evaluated in pharmacokinetic (PK) studies; summed up as "actions of the body on a molecule."

Pharmacokinetic studies measure the absorption, distribution, metabolism, and excretion of an administered molecule—often abbreviated as ADME characteristics.

Box 2.25: Key Terms and Abbreviations

PD: pharmacodynamics; measurements of drug action in the body (e.g., target inactivation, receptor off-rate, etc.)

PK: pharmacokinetics; measurements of the absorption, distribution, metabolism and excretion of a molecule after administration

ADME: Absorption, Distribution, Metabolism, and Excretion

CYP: Cytochrome P450, a class of enzymes important in drug metabolism

Polymorphism: genetic variation in enzymes that affects their activity and leads to differences in drug metabolism rates

iv: intravenous

po: oral

SDPK: Single dose pharmacokinetic

SAD: single ascending dose

2.8.1 Key ADME Parameters

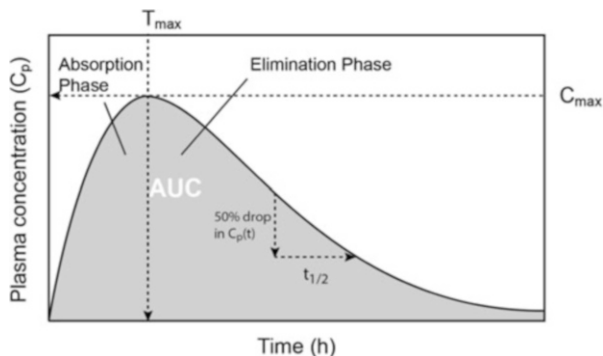
ADME characteristics depend on both intrinsic properties of the molecule such as pK_a , size, and lipophilicity; and extrinsic properties such as formulation or route of administration. Excellent resources exist for detailed description of the influence of each pharmacokinetic factor discussed briefly below [14].

Important ADME characteristics include those listed below and pictured in Fig. 2.2:

- **Bioavailability (F)**—The percentage of an administered dose that reaches the systemic circulation. Molecules administered intravenously have 100% bioavailability, whereas molecules delivered topically or orally with a high first-pass effect would have a lower bioavailability.
- **Volume of distribution (V_d)**—The apparent volume required to dissolve the administered dose at the drug concentration measured in the plasma. For a drug retained exclusively in the vascular compartment, the volume of distribution is equal to the plasma volume (0.04 L/kg body weight). For a drug that is extensively bound in peripheral tissues, the V_d can greatly exceed the total body volume.
- **Clearance (CL)**—A fraction of blood or plasma volume completely purified of drug per unit time. Total CL depends on elimination rate constant ($t_{1/2}$) and V_d . Clearance at specific organs, such as liver, kidneys, skin, lungs, etc., is dependent on the blood flow through the organ; so disease states can alter drug clearance. Intrinsic clearance (CL_{int}) refers to the measured *in vitro* clearance.
- **Half-life ($t_{1/2}$)**—The time required for the drug concentration to fall by 50% of an earlier measurement. Terminal half-life is calculated from the clearance and volume of distribution.

$$t_{1/2} = ((\ln 2) \times V_d)/CL$$

Fig. 2.2 Plasma concentration curve with PK metrics



- Area under the curve (AUC)—The integral under a plot of plasma drug concentration versus time. The AUC reflects the “total exposure” from a single dose of drug. The dose normalized ratio of $AUC_{\text{oral}}/AUC_{\text{intravenous}}$ yields bioavailability.
- First pass effect—The extent of metabolism that occurs before an orally administered drug enters the systemic circulation.

2.8.2 Drug Metabolism and Drug–Drug Interactions

The simplest form of elimination is direct excretion of an unchanged drug molecule into the urine, bile, or occasionally tears, sweat or air. More commonly, molecules undergo biotransformation, a process of metabolism that involves building or breaking chemical bonds within the molecule to improve the body’s ability to excrete it. Biotransformation is grouped into Phase I and Phase II reactions; Phase I enzymes catalyze oxidations, reductions and/or hydrolysis to introduce or unmask functional groups in the molecule. Phase II enzymes conjugate endogenous small polar molecules to the unmasked functional groups to inactivate the drug and improve its water solubility for elimination. A drug may be subject to Phase I metabolism, Phase II, or both. Sometimes, knowledge of a drug’s metabolism is exploited by chemists to devise a prodrug, a molecule whose metabolism creates the true therapeutically active compound, to improve ADME properties.

The cytochrome P450 (CYP) family of enzymes is composed of a number of related isozymes and is responsible for a major portion of drug Phase I metabolism. CYP enzymes are primarily located in the liver, but also occur in a number of other tissues. CYP isozymes differ in their abundance and importance to metabolism across different tissues. For instance, the CYP3A4 isoform is very abundant in the liver and intestinal epithelium and contributes to the biotransformation of almost one half of drugs, whereas CYP2D6 is one of the least abundant isozymes and yet is involved in the metabolism of a quarter of all drugs [15].

Identifying which CYP isozymes are responsible for metabolism of the lead compound, called reaction phenotyping, is important for two reasons. First, a

number of genetic polymorphisms have been identified for CYP isozymes. Polymorphisms result from inherited differences in enzyme expression or mutations that alter enzyme activity. These differences create variation in the rates of drug metabolism within a patient population. Dosing regimens may need to be adjusted to properly treat slow or ultra-fast metabolizers.

The second reason for reaction phenotyping is that many drugs display off-target activity on CYP isozymes, acting as inhibitors, inducers, or both. Co-administered molecules may show altered metabolism to that of a single drug. These drug–drug interactions must be carefully screened for, as they can either negatively (creating side-effects) or positively (improving ADME properties) impact the metabolites produced.

2.8.3 *In Vitro Experiments*

Initial studies of ADME characteristics are likely to be *in vitro* due to the high cost of animal studies. Although algorithms exist to extrapolate *in vitro* data to living systems, preliminary *in vivo* studies should be performed to confirm that *in vitro* data are indeed predictive. If the results are in concurrence, a strategy of *in vitro* screening with limited *in vivo* testing can be adopted. This approach allows more rapid and cost-effective identification of compound liabilities and better selection of a formulation before moving into animal models.

A number of different test systems are available to measure the *in vitro* or intrinsic clearance (CL_{int}) and are listed in Table 2.1. CYP reaction phenotyping is typically done with panels of purified enzymes and their cofactors. Systems derived from human material are preferred for identifying drug metabolites, but other animal models are important for initial studies of drug safety. *In vitro* experiments are useful for reaction phenotyping, screening for drug–drug interactions, measuring intrinsic clearance, and identifying metabolites.

In addition, there are a number of *in vitro* models (Caco-2, MDCK, mucosal tissues and skin) to predict absorption via different routes of administration.

2.8.4 *In Vivo Experiments*

The goal of *in vivo* PK experiments is to calculate bioavailability, AUC, volume of distribution and half-life while validating the clearance and metabolite identity data collected from *in vitro* studies. The FDA requires safety studies in at least two mammalian species, including one non-rodent species. These animal studies in concert with pharmacokinetic and pharmacodynamic studies will help predict the dosing range and regimen for desired therapeutic effect and expected safe dose in humans before starting phase 1 clinical trials. Because upper dosing levels are usually set at the appearance of adverse side effects (or in the case of oncology

Table 2.1 *In vitro* test systems for intrinsic clearance

Test system	Specific models
Cell extracts	<ul style="list-style-type: none">• S9 fraction (Phase I and II)• Microsomes (Phase I only)
Cell culture	<ul style="list-style-type: none">• Hepatocytes (fresh or cryopreserved)• HepG2 cells transfected with CYP isozymes
Whole tissue	<ul style="list-style-type: none">• Liver slices

drugs, severe adverse effects), *in vivo* pharmacokinetics studies go hand-in-hand with toxicology studies. For this reason, some people refer to *in vivo* testing as ADMET studies.

Initial *in vivo* PK studies should be done in rodents, preferably rats, in parallel with the *in vitro* testing. Dosing routes should include intravenous (IV) and the intended clinical route of administration, often oral (po). To gather as much PK data as possible, both urine and blood samples should be collected; with other fluids such as cerebrospinal fluid, perspiration, or breath collected as applicable. The second species for *in vivo* testing, often dogs or monkeys, should be chosen based on program-specific issues such as metabolite profile and pharmacology.

The first test is often a single-dose pharmacokinetic (SDPK) study to follow the ADME properties of a single bolus of administered drug. Samples are collected at many time points to create a plasma concentration curve similar to that shown in Fig. 2.2. Once the compound's ADME characteristics look promising, animal PK studies move into single ascending dose (SAD) experiments to establish the maximum acutely tolerated dose. Further studies with radiolabeled drug are used to confirm the identity of major metabolites and look at drug deposition in different tissues.

2.8.5 *The Bottom Line*

Pharmacokinetic studies tell researchers how the lead compound is absorbed, distributed, metabolized and excreted from the body. *In vitro* PK testing is used to identify initial metabolism rates and routes, in addition to identifying potential drug–drug interactions. *In vivo* PK testing is essential for establishing a pharmacokinetic/pharmacodynamic relationship and the maximum tolerated dose and therapeutic window in animals for a lead compound, which becomes the basis for planning safe and effective doses moving into human trials. Because different crystal forms, salts and formulations of the same compound can have different ADME characteristics, it is very important to show favorable PK properties before scaling up GMP production for clinical trials to avoid costly reformulation delays. Proper PK studies can help drug developers maximize their therapeutic window between minimum efficacious dose and maximum tolerated dose.

2.9 Route of Administration and Drug Formulation

Terrence F. Blaschke

The route of administration and the formulation of a drug are often intertwined by virtue of the chemistry and the desired onset and duration of action of the drug. The route of administration of a drug can be broadly separated into three categories: (1) Enteral, (2) Parenteral, and (3) Topical. In each of those categories, there are a number of subcategories, as follows:

1. Enteral Administration

- (a) Oral
- (b) Buccal or sublingual
- (c) Rectal

2. Parenteral Administration

- (a) Intravenous
 - Slow Bolus
 - Slow infusion, then stop
 - Continuous infusion (long-term)
- (b) Subcutaneous
 - Bolus
 - Continuous infusion (long-term, e.g., insulin)
 - Depot
- (c) Intramuscular
 - Bolus
 - Depot

3. Topical application

- (a) Transdermal (intended for systemic effects)
- (b) Epidermal/dermal (intended for local effects at site of administration)
- (c) Vaginal (usually intended for local effects)
- (d) Intranasal
- (e) Pulmonary inhalation (intended for local or systemic effects)

Each of these routes of administration requires a different type of formulation. Many companies are developing drug delivery technologies involving oral, nasal, inhalation, transdermal, and parenteral delivery platforms.

Box 2.26: Key Terms and Abbreviations

Enteral: routes for drug absorption through the gastrointestinal tract

Parenteral: routes for drug absorption outside the gastrointestinal tract

Buccal: in the mouth

Sublingual: under the tongue

Intranasal: in the nose

Bolus: a single large dose of drug

Depot: store of drug deposited in the body that is slowly released over time

Bioavailability: the fraction (or percent) of the dose of chemically unchanged drug found in the blood based on the route of administration

SR: slow release

XR: extended release

API: Active Pharmaceutical Ingredient

Bio-betters: new formulations of biologic therapeutics to improve dosing schedule or route of administration

Therapeutic index: the ratio of the toxic dose to the effective dose; a larger therapeutic index suggests a larger safety window

2.9.1 Oral Route

The most common, desirable, and usually the least expensive route of administration is the oral route; especially if the drug is intended for multiple doses or chronic administration. However, for many drugs, the oral route may not be feasible or practical, as the drug may show poor oral bioavailability and not reach the systemic circulation after oral dosing. For the oral route, there are many forms (tablet, capsule, liquid, suspension, etc.) chosen and manufactured on the basis of the bioavailability of the drug.

Another important characteristic of an oral formulation is its rate of absorption. In some settings, rapid absorption is desirable, to achieve a rapid onset of action (e.g., drugs given for pain or for sleep). Tablets may be formulated as “quick dissolve” versions. In other settings rapid absorption is problematic, as the high peak concentrations associated with rapid absorption may result in unwanted side effects, sometimes serious or life-threatening. There are many examples of this in the cardiovascular field.

There are a number of special formulations used for oral administration intended to prolong the duration of action and/or avoid high peak concentrations. These are often called “slow release” (SR) or “extended release” (XR) formulations, to distinguish them from immediate release formulations. Such formulations may allow a drug to be administered at longer dosing intervals that improve patient adherence to the medication (e.g., once instead of twice daily, or twice instead of three times daily). Other special oral formulations include enteric-coated formulations that protect the drug from the acidic environment of the stomach and dissolve

in the intestines, or fixed-dose combinations containing two or more active pharmaceutical ingredients (APIs) that are used for conditions benefiting from combined drug therapy (e.g., hypertension, diabetes and HIV).

Box 2.27: What Frustrated an Academician?

Not all drugs reach their target when delivered in a simple formulation. Proper formulation and route of delivery is also critical when using new pharmacological agents for basic research, whether in culture or *in vivo*. It is important to include studies on drug stability and distribution for each formulation of a new pharmacological agent.

2.9.2 Parenteral Route (Injectables)

For drugs that cannot reach the systemic circulation after enteral or transdermal administration, or for drugs for which a very rapid onset of action is needed, parenteral dosage forms are required. Parenteral routes also avoid the first-pass metabolism in the liver experienced by orally administered drugs. For direct intravenous administration, the drug must be solubilized in a liquid suitable for direct injection into a vein, or—much less commonly—into an artery. Speed of injection (bolus, slow infusion or constant infusion) is dependent on the indication. For anesthetics and sedative/hypnotics used in procedures, and for some cardiac arrhythmias, slow bolus injections are often used. However, for many other agents that are not orally available (e.g., many anticancer agents and the rapidly increasing number of biologics on or close to the market) a slow infusion is preferable to avoid toxicity associated with high peak concentrations and rapid distribution into tissues where unwanted effects can occur (e.g., the central nervous system, heart or other vital organs). With the advent of reliable, miniaturized infusion pumps, there is increasing interest in research evaluating whether the therapeutic index could be improved by longer-term infusions. The subcutaneous infusion of insulin is an example of this approach to therapy of diabetes. Examples in other chronic diseases will no doubt follow.

2.9.3 Epidermal or Transdermal Route

Epidermal or transdermal formulations are generally patches or gels. If systemic absorption is the goal of transdermal delivery, there are many characteristics of the drug that may limit this route. In particular, drugs must be of high potency, be able to penetrate the epidermis, and benefit from a fairly constant concentration in the blood. Alternatively, transdermal or epidermal routes may be selected to deliver a high local concentration of drug and avoid systemic exposure. There is increasing

interest in this route of administration. Patches are easy to use (improving patient adherence), provide continuous dosing of a steady drug concentration, and avoid first-pass metabolism. A number of companies are developing new technologies to improve transdermal absorption. A few examples of very successful transdermal systemic delivery systems include the opiate pain reliever fentanyl, contraceptive patches, and clonidine for hypertension. Examples of successful drugs used for local effects include topical steroids, antibiotics and local anesthetics.

2.9.4 Biologics Require New Delivery and Formulation Methods

The rapid increase in the number of biologics already on the market or in the pipeline has resulted in a dramatic increase in the development of new technologies to improve their delivery and efficacy/toxicity. A 2010 survey, conducted by Global Industry Analysts, forecasts that protein drug sales will be worth more than \$158B by 2015 and expects therapeutic antibodies to emerge as the market leaders. Of new first-in-class agents approved between 1999 and 2008 and having novel molecular mechanisms of action, 50/75 (67%) were small molecules and 25/75 (33%) were biologics. Many, such as rituximab (Rituxan[®]), bevacizumab (Avastin[®]), epoetin alfa (Epoen[®]), and etanercept (Enbrel[®]) are multibillion dollar markets, and several are coming off patent in the next few years. This has resulted in an emerging market to devise parenteral formulations to produce so-called “bio-betters” that require less frequent administration and have an improved therapeutic index. A recent survey found that there are more than 20 independent drug delivery companies doing research on controlled release depot injection formulations, along with most of the major pharmaceutical companies that have internal programs. The formulation technologies that are being explored in these efforts to deliver biologics include microspheres, liposomes, microparticles, gels, and liquid depots (see examples listed in Box 2.30). Currently there are 13 depot products on the market, and the market size for such products is estimated to be >\$2 billion dollars.

Box 2.28: What Surprised an Academician?

A formulation consultant suggested that we formulate our intracoronary drug at pH 3, as the drug was more stable in acidic conditions. Supporting his arguments, he cited a few drugs on the market. Luckily, our clinical director knew that the drugs mentioned produced phlebitis and helped me, the basic researcher, to push back on that formulation recommendation. Consultants are not always right and, if something does not seem right, we should do our own diligence. *—DM-R*

Box 2.29: The Bottom Line

There is a process of trial and error leading to the identification of optimal formulation. Understanding the clinical setting and drug dosing for the patients is critical for proper formulation development. Compromise may be required to fit the pharmacodynamics and chemical properties of the API.

Box 2.30: Suggested Resources

Reviews:

- Liechty WB, Kryscio DR, Slaughter BV and Peppas NA (2010) Polymers for Drug Delivery Systems. Annual Review of Chemical and Biomolecular Engineering. 1:149–173. (Broad and comprehensive review of this topic. Contains 149 references and other related resources)
- Wang AZ, Langer R, Farokhzad OS (2012) Nanoparticle Delivery of Cancer Drugs. Annual Review of Medicine 63: 185.
- Timko BP, Whitehead K, Gao W, Kohane DS, Farokhzad OC, Anderson D, Langer R (2011) Advances in Drug Delivery. Annual Review of Materials Research 41: 1. (This review discusses critical aspects in the area of drug delivery. Specifically, it focuses on delivery of siRNA, remote-controlled delivery, noninvasive delivery, and nanotechnology in drug delivery.)

Book:

- Rowland M, Tozer TN (2011) Clinical Pharmacokinetics and Pharmacodynamics: Concepts and Applications, 4th Edition. Wolters Kluwer/Lippincott Williams and Wilkins, ISBN 978-0-7817-5009-7. (These authors and this book are recognized worldwide as the authorities in teaching the basic principles of pharmacokinetics and pharmacodynamics. Each chapter contains Study Problems (with answers!) and by purchasing the text can be accessed anywhere that you have an internet connection. Pharmacokinetics and pharmacodynamics simulations are also available on the Web site.)

Web sites:

- NIH Clinical Center “Principles of Clinical Pharmacology”
- <http://www.cc.nih.gov/training/training/principles.html>
- (This course is taught by faculty members from the National Institutes of Health (NIH) and guest faculty from the Food and Drug Administration (FDA), the pharmaceutical industry, and several academic institutions from across the USA. Course materials are available online via the above URL.)
- American College of Clinical Pharmacology, Educational Offerings
- <http://www.accpl.org/videos.shtml>

(continued)

Box 2.30 (continued)

- (This Web site has a free course on pharmacogenomics, covering 13 different modules, each having *overview* and *depth* sections. There is also a Web-based course on pharmacometrics.)

2.10 Preclinical Safety Studies

Michael Taylor and Kevin Grimes

“*Primum non nocere*,” translates from Latin to “First, do no harm.” This fundamental ethical principle in the practice of medicine is equally applicable when exposing individuals to investigational drugs. Virtually all substances can be toxic to human beings if the dose is high enough. Even drinking excessive quantities of water or breathing 100% oxygen for prolonged periods can result in severe organ damage or death. Therefore, when administering a novel compound to human subjects, we have both an ethical and legal duty to ensure that the risk has been minimized as much as possible.

Safety is difficult to prove without extensive human exposure. Lack of safety, on the other hand, can be proven. We perform preclinical safety studies to better characterize the likely effects and the risk/benefit ratio of administering a novel compound to humans. While experiments using cell lines and animal models will not mirror with certainty what will happen in human subjects, the results can be extremely helpful in predicting dose-limiting side effects and appropriate dose ranges.

The US Food and Drug Administration (FDA) and the International Committee on Harmonization (ICH) have developed guidance documents that outline a series of *in vitro* and *in vivo* experiments that should be conducted prior to each phase of clinical development for a new molecular entity (NME). These studies help predict the drug’s on-target and off-target toxicities, reversibility of these toxicities, limits on the dose and duration of treatment, early predictors or signals of impending serious toxicity, and safety margin between the doses where efficacy and dose-limiting toxicity occur. Additional studies are performed to further characterize the drug’s pharmacologic effects on major organ systems, pharmacokinetics, metabolism, and likely interactions with food or other drugs. Preclinical safety studies that will be submitted to regulatory agencies to support subsequent clinical testing must be performed according to Good Laboratory Practice (GLP). GLP studies require extensive documentation of each study procedure and are quite costly.

Box 2.31: Key Terms and Abbreviations

FDA: US Food and Drug Administration

ICH: International Committee on Harmonization; joint effort of European, Japanese, and US regulatory authorities and pharmaceutical industries to provide uniform standards and guidance regarding drug development

NME: New Molecular Entity; a new drug submitted to the FDA Center for Drug Evaluation and Research (CDER)

GLP: Good Laboratory Practice; extensive documentation of each procedural step to ensure high quality, reproducible studies

C_{max}: peak plasma level of a drug that is achieved after dosing

AUC: Area under the curve; plasma concentration of a drug integrated over time after dosing

Excipients: inactive materials (e.g., fillers, binders, coatings) included in the drug product formulation

API: Active Pharmaceutical Ingredient

Although there is always opportunity for discussion and negotiation, the FDA (and other regulatory agencies) typically requires a specific battery of nonclinical safety studies to be completed before advancing to phase 1 human studies. In general, the duration of drug exposure in animal studies should equal or exceed that of subsequent clinical studies. Therefore, additional general animal toxicology studies of longer durations are often performed to support increasing duration of clinical dosing prior to phase 2 and phase 3 studies. Specific studies of relatively long duration assessing reproductive toxicity and carcinogenicity are generally required before exposing large numbers of patients to study drugs in phase 3 studies.

The guidance documents include discussions of various types of studies to assess specific toxicities including safety pharmacology of the cardiovascular, pulmonary, and neurologic systems; genotoxicity; reproductive toxicity; and carcinogenicity. In addition, they outline preclinical safety requirements for specific disease indications (e.g., oncology).

In addition to identifying possible toxicities, nonclinical safety studies are also important for identifying potential biomarkers for monitoring untoward effects, establishing the first dose to be administered to humans, and establishing the upper limits of dosing (exposure) in humans. This latter purpose is particularly important when severe or non-monitorable toxicities are encountered.

Guidance regarding the development of approved drugs for new indications, by comparison, is limited. Specifically, there is a guidance that speaks to the kind of animal studies that are required for reformulated old drugs (also termed repurposing or repositioning). There is also an FDA expectation that an old drug being developed for a new indication meet current regulatory standards.

Before conducting animal studies, it is important to define how the drug will be given to patients: formulation, route of administration, and frequency of dosing. Generally speaking, animal testing should make use of the same formulation and route of dosing to be used clinically. Both the excipients (inactive ingredients of the final formulation) and active pharmaceutical ingredient (API) need to be considered and evaluated. It is important to appreciate that excipients are scrutinized during the approval process similarly to the drug under development.

When determining which excipients to include in the final formulation, the FDA inactive ingredients listing can be useful. A novel excipient or novel use, outside the limits of its current use (e.g., route, dose), will normally require additional evaluation. The use of some excipients is limited by toxicity (e.g., dimethylacetamide, cyclodextrin) and therefore it is necessary to carefully consider the excipient dose and the patient population for which the product is intended. A good strategy for excipient evaluation is to use the clinical formulation without API as the vehicle formulation (control group) in animal studies. It is also advisable to include an additional negative control group, to confirm lack of effects by the excipient.

The selection of the API lot for animal testing is also important. The tested material should be representative of the material intended for clinical use, such that the impurity profile should be both qualitatively and quantitatively similar to the clinical material. There are several guidances that discuss the acceptable limits of API impurities and the necessary steps for impurity qualification when such limits are surpassed. A good practice, particularly for the IND-enabling studies, is to use the same lot of API for nonclinical safety studies that is to be used in the clinic.

Box 2.32: What Surprised an Academician?

The drug tested in GLP toxicity studies should not be too pure. If the clinical lot has higher levels of impurities than the toxicology lot, which can occur from manufacture scale up, further GLP toxicology studies will be required to characterize the potential toxic effects of the new or increased impurities. This can significantly impact development timelines and budgets. So it took me some time to understand, when told by the VP of Drug Development, that my pride in purifying our non-GLP material to 99.5% purity before using it in pig efficacy studies was misguided and potentially a very costly mistake.
—DM-R

Appropriate dose selection is important to the conduct of useful and therefore successful animal studies. In part, success should be considered based on efficient use of animals. Although the use of two species of animal models is central to drug development and evaluation, there is an ever-increasing awareness and responsibility to follow humane practices and to thoroughly justify the need for animal use and numbers.

The fundamental premise of dose selection for animal studies is that the animal doses and exposures (C_{\max} , AUC) should exceed those proposed for humans.

Ideally, the high dose for animal studies is best selected by clear evidence of toxicity, such as decreased body weight gain, changes in clinical condition, or abnormalities in clinical pathology parameters. The low dose should be a small multiple (2–3×) of the projected clinical dose (exposure) and the mid dose should be set between the high and low doses. It is important to separate doses such that the exposures between groups do not overlap. For many orally delivered small molecules or parenterally delivered macromolecules, doses can be adequately spread using half log or log intervals. Since there is less pharmacokinetic variability for intravenous administration, the dose intervals can be smaller.

Because both dose and time influence toxicity, it is difficult to predict doses that will be tolerated for chronic administration. Therefore, it is best to plan studies of increasing duration sequentially. Selection of doses for the first studies can be challenging and one should draw on all available information. Whereas rodents are usually the species chosen for the early efficacy studies, there is typically no information available for dosing in the non-rodent model. If no or limited data are available, short duration non-GLP pilot studies (1–3 days) with minimal numbers of animals should be performed to assist in selecting the appropriate dose range. For compounds with limited evidence of toxicity, the high dose can be set based upon consideration of the animal exposure relative to humans and practical limits such as dose volume or API solubility.

This discussion provides an introduction to the types and extent of preclinical safety studies required to support drug development. Please also consult the previous sections on formulation and drug metabolism, as these are also important considerations for successful safety evaluation.

Box 2.33: The Bottom Line

When administering a novel compound to human subjects, we have both an ethical and legal duty to ensure that the risk has been minimized as much as possible. Preclinical safety studies help to minimize risk to human subjects by identifying potential toxicities, appropriate dosing ranges, and early signals of toxicity.

Box 2.34: Resources

Specific FDA guidance on Nonclinical Safety Studies:

<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM073246.pdf>

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