TEST SYSTEMS FOR EVALUATING THE ANTIVIRAL ACTIVITY OF NUCLEOSIDE ANALOGUES

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Several challenges confront the investigator involved in determining if a particular nucleoside analog has significant antiviral activity. These include, first, selecting an initial test system which will utilize the relatively small amounts of the new chemical usually made available to him. A second problem is to express the antiviral results obtained in a readily understandable, quantitative manner. Finally, and of major importance, is the challenge of selecting those test systems which will accurately predict the antiviral efficacy of a new substance to the human situation. Normally the answers to these challenges are not found in a single, all-encompassing test, but rather are resolved in stepwise fashion beginning with a simple in vitro screening test and then moving into appropriate predictive animal systems.

DESIGNING THE ANTIVIRAL EXPERIMENT

One should be aware that many factors must be considered if a truly acceptable antiviral chemotherapy experiment is to be achieved. Many of these are outlined in Table 1A.

When selecting the virus for the test, the kind of virus is important: Is it of public health importance? What type and strain is it? Has it been recently isolated, or is it a "standard prototype"? Is it natural to the host, or altered to a degree through cell or animal passage? These points will be discussed further in subsequent sections. The concentration of virus is critical. This is usually expressed in 50% infectious doses (ID_{50}) or 50% lethal doses (LD_{50}). In our experience, the ideal antiviral experi-

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ment utilizes a concentration of virus which approaches the maximal endpoint, i.e., in cell culture using 0 to 4+ cytopathic effect rating, we seek to achieve a 3 to 4+ endpoint, usually using a 10 to 32 cell culture ID_{50} virus dosage. In animals where death is used as an endpoint, the 90-95% lethal dose is ideal. Too much virus will overwhelm the antiviral effect of the test compound. In animal experiments the route of virus inoculation should be considered, with that route which produces an infection most similar to that seen in the human being sought. These aspects of infection will be further discussed in subsequent sections of this review.

Host considerations involve many factors. In cell systems, cells in which a discernible infection can be produced are paramount. Possible cell specificity should also be recognized, which requires the use of more than one cell type. The stage (resting or actively metabolizing) the cell is in when the test is run will seriously affect the eventual test results, with the actively metabolizing cells being much more sensitive to toxic effects of the drug. In animal studies, the age, sex, species, and strain of animal are all key. The older animals are usually more resistant to both virus and test compound than the younger hosts. In the mouse, particularly, the fighting tendencies of the male will often cloud accurate results. Species and strain of animal will affect its susceptibility to the virus and may influence the parameter to be chosen. All animals shipped from other areas should be quarantined prior to use. Certain animal species may be infected by, or contain antibodies to, natural virus infections such as murine hepatitis, epizootic diarrhea of infant mice, etc., suggesting the use of designated specific pathogen-free animals or germ-free animals in certain experiments.

Concerning the test compound itself, the factors listed in Table 1A are quite obvious. The use of multiple concentrations will provide data showing dose response and therapeutic index. The latter term is defined as the maximum tolerated dose divided by the minimum effective dose, which implies that in the design of the experiment, sufficient doses are planned to bridge that range. In cell systems, especially, one often seeks to determine the minimum effective concentration as an expression of drug efficacy. Many routes of administration of the test compound into the animal host are available for selection. Ideally, at least for initial studies, a route should be used which will allow adequate concentrations of compound to reach the infected tissues. Later studies may then be designed to determine if those routes most acceptable for human use, such as topical or oral, will allow the material to exert its antiviral effect. The vehicle used, especially in topical experiments, must allow the material to remain on the infected tissues, to penetrate interstitial spaces, and to release the material to allow its penetration into the cell. To our knowledge, no "universal vehicle" is available, since the test compounds will vary markedly in regard to solubility, etc.

The evaluation parameter will be discussed later. At this time, it is sufficient to stress the need for the parameters selected to be readily discernible, to be measurable, to relate, in the case of animal infections, to the human disease, and, hopefully, will be predictable to man.

The various time considerations listed in Table 1A can influence the sensitivity of the test. In cell culture studies, if cytopathic effect or other manifestations of viral infection are seen too early, it is usually an indication of too high a concentration of virus, which will tend to overwhelm the antiviral effects of the test compound. Too late an interval before such viral manifestations may be seen usually is indicative of insufficient virus and the cells may not be in satisfactory condition by the time the test is read. We generally use a 72 hour interval from time of adding virus to time of reading the test. The main point in this regard is to be consistent with whatever time is chosen. Toxicity observations in animals should be for a sufficiently long period of time to determine if delayed toxicity has occurred. We generally use a 30-day holding period. Treatment times may be chosen to allow the test to be as sensitive as possible, usually accomplished by beginning therapy prior to exposure to virus and continuing through the period when the infection is maximally manifested, or the time of therapy may be selected to only discern compounds acting as therapeutic agents. The latter therapy would be started after exposure to virus, usually at a time when the infection is beginning to be manifested in the host. Consideration should also be given in the in vivo experiment to how many treatments should be given per day, and to how long therapy should be continued. Knowledge of the pharmacokinetics of the test material would be of great benefit for determining the best treatment schedule.

An acceptable antiviral chemotherapy experiment must have adequate controls. Toxicity controls ideally should be run at each treatment concentration. Such controls are sham infected with sterile virus vehicle and then exposed to the test compound. Virus controls are infected with virus but treated with drug vehicle only on the same schedule as in the regular treatment groups. Normal controls in cell culture are also known as cell controls, which are cells exposed to sterile virus and drug vehicles; such controls are used to determine if the various vehicles, the cells, and the container (plastic panel, glass tubes, etc.) in which the test is run are all in the appropriate condition for an acceptable test. In animal experiments, normal controls are particularly helpful if one has any question about the original condition of the animals and the environment in which the animals are being held. Inclusion of a known positive compound in a test run in parallel with the test substance will provide the investigator with information regarding whether the overall test was at the correct level of sensitivity,

Table 1A. Factors to be Considered in Designing Antiviral Chemotherapy Experiments.

1. Virus
   Kind, concentration, infection route
2. Host
   Cell, animal
3. Test Compound
   Quantity available, concentrations used, lability, solubility, route administered, vehicle
4. Evaluation parameter
   In: Cells, animals
5. Time considerations
   To: Examine parameter, observe toxicity, treat
6. Controls
   Toxicity, virus, known positive, normal
7. Environmental conditions
8. Statistics, reproducibility
9. Secondary infections

and, importantly, will provide a standard against which the activity of the test material can be compared. Literally any known active material can be used as the known positive control, as long as the investigator has had prior experience with that compound sufficient to know how it behaves in the test system used.

Substantial alteration of normal environmental conditions will seriously affect the results of a chemotherapy experiment. In cell culture, these especially include temperature, pH and the container in which the cells are established. In animal studies, changes in temperature, accidental deprivation of food or water, erratic light changes, abnormal odors and sound, and trauma of shipping will all influence an animal's response to virus and therapy.

Every test, especially in vivo studies, should contain adequate numbers of animals to allow acceptable statistical evaluation of the data. Should there be questions about the statistical design of a particular experiment, the chemotherapist would be wise to consult with a statistician. The results of any study should be readily reproducible, a fact determined only by the investigators repeating at least the essential portions of the experiment.

Finally, the spectre of the secondary infection must always be anticipated. In cell studies, the chemotherapist often combats this by inclusion of antibiotics, usually penicillin, streptomycin, and gentamycin, in the culture medium. In animal experiments, addition of tetracycline to the drinking water may be used.

Using the concepts outlined above, the remainder of this review will go into more specific areas of antiviral chemotherapy. This most appropriately may begin by briefly considering some known active compounds. It is fortunate that we are at a time in which a number of substances active in antiviral tests have now been studied in the clinic against human viral disease. We thus know, to a degree, at least, of the predictive nature of some of the more common antiviral test systems. A list of the more significant clinically tested antiviral drugs (including some nonnucleosides) is shown in Table 1, together with the viral disease against which each was tested. Shown are compounds with which considerable investigation has been published on their effect in experimental antiviral test systems. As the various antiviral test systems are discussed in this review, reference will be made to the activity of some of these clinically active drugs.

There are a great number and variety of viruses causing significant infections in man, and thus the task of selecting the most appropriate virus for antiviral testing can be rather difficult. For simplification in this review, the field has been rather arbitrarily narrowed to those viruses that are responsible for infections of major public health importance and that are presently not readily controllable by the use of vaccines. These are listed in Table 2. Of these viruses, the last three listed (Arenaviruses, Alphiviruses, Flaviviruses, rabies) occur infrequently and vaccines are available, although outbreaks still are reported, indicating the potential need of chemotherapeutic measures. The severity of each of these latter diseases justifies their inclusion in the list. Test systems for the Arenaviruses are limited to these few laboratories having facilities sufficient to contain the viruses.

A general review of all important chemotherapy test systems has been previously published[1]. In the present review, since it is oriented particularly towards antiviral systems for evaluating nucleoside analogues, we shall concentrate primarily on those

Table 1. Representative Clinically Active Antiviral Substances[a]

Drug	Chemical Name	Viral Disease Studied[b]
Methisazone	1-Methylisatin-3-thiosemicarbazone	Smallpox, severe vaccinia
Iododeoxyuridine	5-Iodo-2'-deoxyuridine	Herpes eye infections, cutaneous infections, and encephalitis Cytomegalic infections Smallpox, severe vaccinia
Trifluorothymidine	5-Trifluoromethyl-2'-deoxyuridine	Herpes eye infections Adeno eye infections
Cytarabine	1-β-D-Arabinofuranosylcytosine	Herpes eye infections, cutaneous infections Cytomegalic infections
Vidarabine	9-β-D-Arabinofuranosyladenine	Herpes eye, cutaneous and neonatal infections Cytomegalic infections Smallpox Hepatitis B
Amantadine HCl	1-Adamantanamine HCl	Influenza A, B
Rimantadine HCl	α-Methyl-1-adamantanamine HCl	Influenza A, B
Ribavirin	1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide	Hepatitis A, B Influenza A, B Herpes cutaneous, mucocutaneous infections Measles

[a]Not included are substances thought to be active by immunomodulation (interferon inducers, cellular immunity enhancers, etc.) or substances active as virus inactivators.

[b]All clinical viral diseases studied, regardless of final activity seen in the study.

Table 2. Important Human Viruses Especially in Need of Chemotherapeutic Measures

Virus	Nucleic Acid Type	Family[a]	Type of Infection
Rhino	RNA	Picornaviridae	Respiratory
Coxsackie	RNA	Picornaviridae	Respiratory, CNS, cardiovascular
Corona	RNA	Coronaviridae	Respiratory
Influenza	RNA	Orthomyxoviridae	Respiratory
Parainfluenza	RNA	Paramyxoviridae	Respiratory
Respiratory syncytial	RNA	Paramyxoviridae	Respiratory
Adeno	DNA	Adenoviridae	Respiratory, ophthalmic
Rota	RNA	Reoviridae	Gastrointestinal
Herpes	DNA	Herpetoviridae	Cutaneous, mucocutaneous, CNS, ophthalmic
Cytomegalo	DNA	Herpetoviridae	Generalized neonatal, respiratory,
Hepatitis	?	Unclassified	Hepatic
Arenaviruses (e.g., lassa, hemorrhagic fever)	RNA	Arenaviridae	CNS, generalized
Alphi, Flaviviruses (e.g., dengue, etc)	RNA	Togaviridae	CNS
Rabies	RNA	Rhabdoviridae	CNS

[a]Classification according to Fenner[21].

systems we have used and are using in our laboratory for testing such compounds. It is felt such an approach is pertinent, since two of the substances we have studied extensively[2-11], 9-β-D-arabinofuranosyladenine (vidarabine) and 1-β-D-ribofuranosyl-1,2,4,-triazole-3-carboxamide (ribavirin) have been used against clinical viral disease with varying degrees of success.

IN VITRO ANTIVIRAL SYSTEMS

The in vitro test is usually the "primary" screening tool to discern if a new substance has significant antiviral activity. In the test, living metabolizing cells susceptible to the targeted virus are cultured in artificial medium, usually being allowed to divide until a confluent monolayer is established. When the virus is introduced into the medium, it enters the cell and within a period of time varying from a few hours to days, the cells are altered. Such cellular alterations (cytopathic effect, CPE) due to viral infection can be expressed visibly, as seen by holes or plaques appearing in the cell layer, by individual cell destruction viewed under the microscope, by color change due to pH alteration of the medium caused by altered metabolism of the cell, or occasionally by inhibition of the uptake of labeled metabolites such as thymidine or uridine into virus nucleic acid. With some viruses, the cell infection is so subtle that methods such as the use of immunofluorescence techniques[12] to discern infected cells are used. We almost exclusively use CPE inhibition in 96-well disposable plastic micro tissue culture plates in our primary in vitro assays according to a previously published procedure[13]. Such a system allows for seven concentrations of 3 to 4 compounds to be evaluated on each plate, with approximately 1 mg of test compound required for each virus tested. The plate includes all necessary controls (cell controls, virus controls, toxicity controls) and enables us to obtain reasonabl quantitative data in each test.

Expression of antiviral activity in such studies can be as the minimum inhibitory concentration (MIC), which is the least amount of test compound which will still cause viral inhibition, or numerically as a virus rating (VR), in which the degree of inhibition of viral CPE and the toxicity of the compound for the cell monolayer are considered[1]. A typical antiviral experiment, using vidarabine tested against 10 and 100 cell culture 50% infectious doses ($CCID_{50}$) of KB cells is seen in Table 3, with calculations of VR and MIC indicated. Scoring of CPE was from 0 (no CPE) to 4 (100% cell destruction). The VR and MIC varied according to virus concentration used, illustrating the need for relatively constant virus concentrations to be used, and for a known positive drug to be run in parallel in each experiment. In our experience, a VR of >0.5 is usually indicative of antiviral activity, but this minimum value may vary with the virus used.

Table 3. Typical Type 1 Herpesvirus-Inhibitory Effect of Vidarabine Using the Micro Tissue Culture Plate System

Vidarabine Concentration µg/ml	Cyto-toxicity	CPE Score: 10 $CCID_{50}$ of Virus		CPE Score: 100 $CCID_{50}$ of Virus	
1000	Toxic	T	T	T	T
320	P[a]	0	0	0	0
100	Sl. P[a]	0	0	1	0
32	0	0	0	1	1
10	0	1	1	2	2
3.2	0	3	2	4	4
1.0	0	4	4	4	4
0 (Cell Control)	0				
0 (Virus Control)		4	3	4	4

$$VR = \frac{\Sigma(\text{Total Virus Control Score} - \text{Treated Score of Each Drug Conc.}^{a})}{10 \times (\text{Number of Cups Used per Drug Concentration})}$$

$$VR\ (10\ CCID_{50}) = \frac{(3.5 + 3.5 + 7 + 5 + 2)}{10 \times 2} = \frac{21}{20} = 1.1; \quad MIC = 3.2\ \mu g/ml$$

$$VR\ (100\ CCID_{50}) = \frac{(4 + 3.5 + 6 + 4)}{10 \times 2} = \frac{17.5}{20} = 0.9; \quad MIC = 10\ \mu g/ml$$

[a]Differences in virus control and treated scores divided by 2 at partially toxic (P) drug levels.

For example, activity against type 1 herpes virus is often seen at VR's >1.0, but activity against rhino, adeno, parainfluenza and influenza viruses is not usually quite as pronounced. We occasionally also determine intra- and extracellular virus titers at each drug concentration[9,13] but inhibiton of virus titer usually correlates closely with CPE inhibition[9].

The ability of an in vitro antiviral system to predict efficacy in vivo is difficult to assess, since many factors may enter in to prevent such correlation (Table 4). Metabolizing enzymes in the animal not found in vitro may either break down the compound to an inactive form, or may activate an otherwise inactive material. In connection with this are other aspects of pharmacokinetics: Absorption and organ distribution of the test compound in the animal host, which are of considerable importance if the compound is to

Table 4. Factors Preventing In Vivo Correlation with In Vitro Antiviral Tests

1. Metabolizing enzymes in the animal host.
2. In vivo absorption of the compound.
3. Organ distribution of the compound.
4. Insoluble test compound.
5. Immunity factors in the animal host.
6. Virus concentration.
7. In vitro incubation time.
8. In vitro contaminating mycoplasma.
9. Cell specificity.

exert a significant antiviral effect in the host. Thus, the material must be capable of being absorbed into the animal's system after administration orally or by injection, and it must then achieve active concentrations in the virus-infected organs. The insolubility of test compounds in cell culture media will usually thwart the demonstration of in vitro antiviral effect. In vivo, the insoluble material can often be eventually absorbed. The in vitro system does not ordinarily have the variety of immune systems provided by the animal host, such immune systems usually playing a major role in the recovery of the host from viral disease. In our experience, the majority of the antiviral compounds need the assistance of such host immunity in order to render a maximal antiviral effect, especially if those antivirals are virus-inhibitory in their action. The in vitro test conditions, such as virus concentration and time of incubation, are key factors. As can be seen in Table 3, a one-log increase in virus concentration considerably reduced the antiviral activity. Greater increases will usually obscure any significant activity from being demonstrated. Similarly, viral CPE which can be read prior to 72 hours may indicate a viral concentration which is too high for accurate test sensitivity. The viral concentration effect can also be carried over to the in vivo test system. Contaminating mycoplasma in the cell culture system may enzymatically alter the compound[14]. Finally, cell specificity can apparently be a problem in evaluating a new test compound. We have found that with some compounds, antiviral effect can only be seen in certain cell lines[9], for as yet not fully understood reasons.

IN VIVO ANTIVIRAL CHEMOTHERAPY SYSTEMS

The in vivo systems to be discussed in this review will be confined to those major viral diseases shown in Table 1, and again will be primarily those we have found useful in our antiviral evaluative programs. Each viral disease will be discussed separately.

Rhinoviruses

Rhinoviruses appear to be responsible for the majority of the viral induced acute upper respiratory diseases, and hence have been a target for many chemotherapy programs[15]. The viruses offer a serious challenge, however, because of their lack of pathogenicity for laboratory animals. Thus the chemotherapist is faced with choosing a nonrelated virus for in vivo study, an elaborate indirect system, or the use of expensive, difficult-to-handle nonhuman primates for an adequate preclinical evaluation of effective antiviral agents. Another problem is the lack of clinically effective antirhinovirus drugs, so we are uncertain of the predictive value of any of the above choices.

A general scheme for evaluating a potential antirhinovirus compound, once activity is seen in vitro, would be to initially repeat the in vitro test using other types of rhinovirus as well as other RNA viruses causing acute respiratory disease, such as corona, influenza, parainfluenza and respiratory syncytial viruses. This would achieve the purpose of, first, determining if the compound has a sufficiently broad spectrum of activity to warrent further testing, and second, if activity is seen against either influenza or parainfluenza viruses, an acceptable in vivo system can then be used employing one of these latter agents. Assuming sufficient efficacy is seen against a spectrum of rhinoviruses but not against the influenza or parainfluenza viruses, then one could run an indirect antiviral study, in which the test compound is administered to mice or rats, preferably orally, and then serum levels of the drug, taken at varying times after treatment, are ascertained by assay in vitro using a sensitive rhinovirus strain. Such a test would indicate that the compound had been absorbed and was circulating in an active form. Even more applicable would be the demonstration of active drug levels in the target respiratory tissues.

The only animals susceptible to rhinoviruses are nonhuman primates, particularly the chimpanzee and gibbon ape, with the infection exhibited as shedding of the virus in the pharynx and by seroconversion[16,17]. Equine rhinoviruses induce similar infections in vervet monkeys[18]. The animals are given a general anesthetic, and the virus instilled intranasally. Virus isolation from nasopharyngeal swabs, and removal of blood are accomplished after brief anesthesia

with an anesthetic such as nitrous oxide[19]. Treatment is usually done by ingestion to alleviate undue trauma to the animal. Such a test system is difficult to establish and requires especially trained personnel and special animal facilities. We have limited our rhinovirus chemotherapy studies to the in vitro and indirect in vivo studies described above.

Coxsackie Viruses

This group of viruses is included as a "secondary" target for antiviral chemotherapy, since they alone are perhaps of not of sufficient public health importance to warrent a full chemotherapeutic program. They can induce upper respiratory disease in man and may be a cause of herpangia, aseptic meningitis, and myocarditis in infants.

A variety of experimental systems are available for studying the efficacy of anticoxsackie virus drugs in animals. These are summarized in Table 5. To date, no clinically effective drugs for treatment of coxsackie virus diseases are available, so the reliability of these models for predicting efficacy to human disease remains to be established.

Coronaviruses

Like the coxsackie viruses, the coronaviruses would be more likely included as a secondary target for chemotherapy, since the total human disease induced by them is considered relatively low. They are definitely associated with acute upper respiratory illness, and hopefully would be one of several upper respiratory viruses which could be attacked by a chemotherapeutic agent. The only upper respiratory animal model for this virus is the infectious bronchitis of chicks induced by the avian infectious bronchitis virus[20], which is included in the same viral class and has similar properties[21]. This avian infection is appealing because it is a naturally occurring infection and the symptoms seen are similar in some respects to the human illness.

Influenza Viruses

The influenza viruses are considered a high priority target for chemotherapy programs, since the disease persists as a major incapacitating, even life taking, threat throughout the world, and as yet is not adequately controlled by vaccines. Because of the importance of influenza as a chemotherapeutic target, a multitude of in vivo experimental systems has been studied, and several drugs have reached widespread clinical trial, showing the systems commonly used

Table 5. Coxsackie Viruses: Available In Vivo Model Systems

Virus Type	Animal	Evaluation Parameter
A	Suckling mouse	Flaccid paralysis; muscle inflammation, degeneration; myocarditis
A	Suckling hamster	Paralysis
A	Suckling ferret	Paralysis
A7	Monkey, cotton rat, suckling mouse	Paralysis
B	Suckling mouse	CNS-associated mortality; myocarditis
B4	Cynomolgus monkey	Myocarditis
B	Weaned mice	Pancreatic necrosis

can be predictive, although several compounds active in the lower animal models did not prove unequivocally efficacious in man and no one system can yet be considered acceptable by all investigators.

The available in vivo model systems for the influenza viruses are summarized in Table 6. Most commonly used, and the model we prefer, is the laboratory mouse, with increases in mean survival time or decrease in lung consolidation and in virus titer in the lungs being common measures of antiviral efficacy. Less used parameters are inhibition of rales and changes in weight and water intake. The virus is usually administered in droplets intranasally to the lightly anesthetized animal, or in an aerosal to unanesthetized mice. The use of mice does pose problems. Newly isolated virus strains are usually not too infectious for mice until adapted to the animal by multiple lung passages; use of barely weaned mice which are more susceptible to the virus may obscure demonstrable antiviral activity because such animals usually also do not tolerate drug treatment well.

The ferret is an animal often used for influenza chemotherapy experiments in those countries where they are available. They are readily infected with influenza virus, exhibiting many typical signs of influenzal disease, including nasal discharge, loss of appetite, congested eyes and ruffled fur[22]. Virus can be recovered from the respiratory tract. Ferrets which are anesthetized and then heavily infected with influenza virus will develop a usually lethal pneumonia. Examination of comparative experiments run with the same

Table 6. Influenza Viruses: Available In Vivo Model Systems

Virus Type	Animal	Evaluation Parameter
A, B, C, Swine	Mouse	Pneumonia-associated mortality; lung consolidation; virus content of lungs; rales; weight change; water intake
A, B, C, Swine	Ferret	Pneumonia-associated mortality; nasal discharge; virus content of nasal washings, turbinates, trachea and lung; antibody; general symptoms of influenza
A, B	Hamster	Pneumonia-associated mortality; lung consolidation; virus content of lungs, nasal washings
Swine, A	Swine	Pneumonia; virus content of nasal washings
A, B	Squirrel monkey	Sneezing and coughing; nasal discharge; fever; virus content of nasal washings

compounds in ferrets and in mice does not reveal one animal system to be significantly more sensitive than the other.

The only other animal used to any extent for influenza virus chemotherapy experiments is the squirrel monkey[23], which develop symptoms of the disease closely resembling those seen in the human.

Parainfluenza Viruses

The parainfluenza viruses occur principally in infants and young children, causing effects ranging from inapparent infection to those of life-threatening intensity in the lower respiratory tract. Diseases particularly seen include the croup syndrome, rhinitis, pharyngitis, bronchitis and bronchiolitis. The viruses could therefore be considered as primary targets for antiviral chemotherapy, although because of the many viruses responsible for respiratory disease and the lack of rapid exact diagnosis of such viral infection, an antiviral drug should ideally be active against those other respiratory disease viruses as well.

Available in vivo model systems for the parainfluenza viruses are shown in Table 7. The chemotherapy systems available vary according to virus type, with the type 1 virus causing the most easily discernible infection. The type 3 virus model in hamsters particularly mimics the upper respiratory infection in humans; in our studies with ribavirin[24], it was found that parenteral therapy did not significantly influence the infection, but a nasal spray application had a marked effect on the various disease parameters. We concluded that parenteral therapy did not allow sufficient drug to reach the infected tissues.

Respiratory Syncytial Virus

The respiratory syncytial virus is classified in the Paramyxoviridae family with the parainfluenza viruses, although in a separate genus. The virus should be considered with the parainfluenza viruses when studying antiviral drugs with broad spectrum potential. The virus has been considered the single most important viral respiratory pathogen of infancy[24].

Table 7. Parainfluenza Viruses: Available In Vivo Model Systems

Virus	Animal	Evaluation Parameter
Type 1	Mouse	Bronchopneumonia-associated mortality; virus content of lungs, nasal washings.
Type 1	Ferret	Virus content of lung, nasal washings; antibody; general symptoms influenza.
Type 1	Rat	Virus content of lung, nasal washings; antibody.
Type 2	Hamster	Virus content of nasal washings; antibody.
Type 2	Vervet monkey, baboon	Virus content of nasal washings; antibody.
Type 3	Hamster	Virus content of lung, nasal washings; lung consolidation; antibody.
Type 3	Vervet monkey, baboon	Virus content of nasal washings; antibody.
Type 4	Guinea pig	Antibody.

No fully acceptable animal model has yet been developed for the respiratory syncytial virus. The agent will infect mice, hansters, and ferrets, but clinical disease is not produced[25]. Therefore, only the less direct chemotherapy approaches, such as inhibition of antibody, have been used.

Adenoviruses

There are at least 60 related but immunologically distinct viruses in the Adenoviridae family. Approximately 30 have been associated with human acute respiratory disease, pharyngitis and pharyngoconjunctival fever, pneumonia of infants, keratitis and conjunctivitis[26]. The adenovirus has been used in antiviral programs by many laboratories, although to date no clinically effective drug inhibitory to them has been developed.

Few acceptable animal models mimicing the human respiratory diseases caused by adenoviruses have been developed. The human viruses have limited pathogenicity in animals, with disease detected only by isolation of virus from tissues or by specific antibody formation. Such a system has had some limited use in antiviral studies[27]. Pigs defined as "pathogen-free" develop mild bronchopneumonia accompanied by virus recovery from the lungs and histopathological changes in lung epithelial tissue when they are exposed intranasally to types 1,2,5, and 6 adenovirus[28]. The only other animal infection induced by adenovirus which relates to the human respiratory disease is seen in the vervet monkey[29]. When these animals are infected intranasally with SV-17 (simian) adenovirus, reddening and swelling of the pharyngeal mucous membranes and tonsils is seen and virus can be recovered from the nose and throat. These latter pig and monkey systems, while expensive and relatively cumbersome, offer the most realistic evaluation of a potential adenovirus inhibitory drug.

Rotavirus

The rotavirus, formerly referred to as a reo-like gastroenteritis virus, is the major cause of diarrhea in infants and children. Adult populations also occasionally manifest the disease[30]. It has been estimated by the World Health Organization that in 1975 there were about 500 million episodies of diarrhea in children, resulting in up to 18 million deaths. In the study, rotaviruses were considered the cause of 50-60% of the diarrhea cases in children aged 6-24 months[31]. No vaccine is available for the disease, and no antiviral drugs have yet been developed; this virus would therefore appear to be a prime target for antiviral measures.

The human virus has not yet been successfully grown in cell cultures, although the closely related bovine, simian, and porcine rotaviruses replicate well in several cell systems, with viral CPE and immunofluorescing cells used as indicators of infection[32-35]. Infant mice infected orally with the murine rotavirus develop an acute, often fatal gastroenteritis closely resembling that seen in the human. The disease is manifested in the form of acute diarrhea, weight loss or failure to gain weight, scoreable changes in the gross appearance of the intestine, and recoverable virus in the diarrheal fluid and in the villi epithelium of the intestine, the latter best demonstrated by specific immunofluorescent staining[36]. The system has been used only in a single antiviral investigation to our knowledge[37].

Herpesviruses

The herpesviruses induce a wide variety of disease manifestations which are high priority goals for chemotherapy programs. The diseases include infections of the skin and mucous membranes, viscera, nervous system and the eye. The viruses are also implicated in other diseases such as lymphoma, cervical and possibly other forms of cancer, infectious mononucleosis, and arthritis. Several antiviral drugs have found use against clinical forms of the more recognized herpesvirus infections, so the predictability of some of the animal herpesviruses chemotherapy systems has been determined. The available animal models are summarized in Table 8.

Two animal systems have particularly been used with success to mimic the infection caused by the herpesviruses in the human eye for chemotherapy studies. These are herpetic keratitis in the rabbit eye and keratitis-encephalitis in the hamster. The rabbit model has been particularly useful, for it can be standardized, provides a variety of criteria for studying a drug's effects, and seems to predict well to the human situation. Both types 1 and 2 herpesvirus can be used in the model. The cornea (usually topically anesthetized) is traumatized by either rubbing with dry cotton swab or by scarifying with a needle, and the virus is placed on the surface of the eye. The infection is usually initially manifested as small punctate lesions accompanied by conjunctival erythema. The infection then progresses to dendritic lesions and, finally, to severe geographic ulceration of the cornea. The Draize (immunologic) signs of the infection progress to extensive swelling of the conjunctiva, nictitating membrane, and eyelid, and to considerable discharge. Iritis is also apparent later in the infection. If a neurotropic strain of virus is used, the infection will eventually progress to the brain and fatal encephalitis ensues. Test compound is applied topically to the eye in saline, ophthalmic ointment, or 1.4% polyvinyl alcohol. The later two vehicles are

Table 8. Herpesviruses: Available In Vivo Models

Herpesvirus	Animal	Evaluation Parameter
Types 1 & 2	Rabbit (eye)	Keratitis; virus recovery from surface of eye; CNS-associated mortality
Types 1 & 2	Hamster (eye)	Keratitis; CNS-associated mortality; virus content in brain
Type 1 with hyaluronidase	Rabbit (back)	Lesion size
Types 1 & 2	Mouse (tail	Lesion size; virus content of tail
Type 1	Hairless mouse, newborn rat (back)	Lesion size; CNS-associated mortality
Type 1	Guinea pig (back)	Lesion size
Types 1 & 2	Suckling and adult mouse (nasal)	CNS-associated mortality
Types 1 & 2	Mouse, rat (brain)	CNS-associated mortality
Type 2	Mouse, hamster, guinea pig (genital)	Vaginitis; virus recovery from genital area; CNS-associated mortality
Type 2	Cebus monkey	Vaginitis

more satisfactory for such infections because they hold the test substance on the eye for longer periods of time and may allow more penetration into the eye. Efficacy is determined by reduction of scores applied to each visual criterion of the infection, as well as by reduction of recoverable virus from the corneal epithelium and possibly reduction in CNS-associated death of the rabbits, if a neurotropic virus is used. We use a weighted scoring procedure applied to both infectivity and Draize parameters, giving more emphasis to the inhibition of infection than to immunosuppression which may occur[7,8,39].

The hamster infection[40] is induced by ether-anesthetizing the young adult animal and inoculating the eye by corneal scarification, using a neurotropic herpesvirus. The animals develop corneal dendritic patterns and then within 4 days exhibit signs of CNS involvement, with death occurring by the 8th day of the infection. Test compounds are usually applied topically. A drug such as vidarabine is markedly effective against this infection.

As seen in Table 8, a variety of herpesvirus induced skin infections have been studied. The two models we prefer are the tail lesion infection in mice[42] and the cutaneous lesions on the backs of guinea pigs[43]. Both models do not require special procedures to induce the infection, and the infection tends to remain at the site of initial virus exposure, not progressing to the CNS as with the other models shown. Varicella virus, the causal agent of zoster and chicken pox in humans, does not induce infections in laboratory animals, but the type 1 herpesvirus-induced skin infections have so far been reasonably accurate predictors of activity against this virus infection as well.

The encephalitis models using the newborn or adult mouse are the most commonly used systems for determining efficacy against encephalitis. Vidarabine, active in these models, has proven also effective in man for treatment of herpesvirus-induced encephalitis. We have often used intracerebral injection of test drugs administered a few hours after similar injection of virus in mice for evaluating potential antiviral compounds[44]. The procedure minimizes technician's time and the amounts of new test compound needed. The results obtained were usually similar to those derived from herpesvirus encephalitis studies using multiple administration of the same compounds by other routes.

A major concern of public health authorities today is the increasing incidence of herpesvirus type 2-induced genitalis in humans. The infection remains with the individual, reoccurring periodically, with the potential to infect others by genital contact, as well as infants born to infected mothers. The infection in the woman is thought to be a possible cause of cervical cancer. No drug is yet known which will effectively treat this infection to the point of eliminating it from the individual. Several animal models, summarized in Table 8, have been used in chemotherapy studies. The two models most widely accepted are the mouse and the guinea pig infections. In each model, the vaginal area of the animal is exposed to high concentrations of the virus for at least one minute. To facilitate better infection "takes", the area may be swabbed, first, with a dilute solution of sodium hydroxide and, second, with a dry swab prior to exposure to virus[45]. Dry swabbing alone is relatively effective. The animals are treated either topically, parenterally, or by a combination of topical + parenteral treatment. Vehicle again is important. In our experience saline and water are not efficaceous; 1.4% polyvinyl alcohol, specially prepared gels, ointments and dimethysulfoxide have all been relatively satisfactory[45]. Antiviral activity is usually evaluated by the combined parameters of lesion inhibition, reduction of recoverable virus titer from genital swabs, and lessening of mortality in the animal.

Yet to be fully studied for evaluating the ultimate efficacy of antiherpesvirus compounds is a reproducable latent infection in a laboratory animal.

Cytomegalovirus

This virus, a member of the Herpetoviridae family along with the herpesviruses, has been of concern to clinicians because it induces cytomegalic inclusion disease in infants, and it has become a serious secondary infection arising in immunosuppressed patients. Chemotherapy appears to be the best approach for the control of the infection. Although several drugs are known to markedly inhibit herpesvirus infections, none yet have unequivocably been of use for cytomegalovirus disease. One reason for this failure is the need of most drugs for the "back up" of the host's immune system, which, in most of the human cases treated to date, has been seriously suppressed.

The murine cytomegalovirus is relatively infectious for mice, large doses administered intraperitoneally being lethal[46]. Smaller virus concentrations tend to induce chronic infections, presumably similar to that seen in man, in mice, guinea pigs, hamsters and rats. In each animal species the virus strain specific for that animal is used. Such chronic infections provide an excellent model for studying the effect of an antiviral substance on eliminating the latent virus from the tissues of the animals[47]. Pregnant mice, when infected with the murine virus, exhibit a considerable diminution in number of viable offspring[48], another potential model for antiviral drug evaluation. Because of the importance of the disease in the immunosuppressed individual, thought should be given to using infections in immunosuppressed animals as an ultimate test for cytomegalovirus active compounds.

Hepatitis Viruses

Human viral hepatitis is generally regarded as being caused by at least three viruses, classified as type A (infectious), type B (serum), and type C (non A non B) hepatitis. The diseases, particularly types A and B, are of considerable public health importance and ideal targets for chemotherapeutic drugs. Ribavirin has been used with some degree of success in treating type A hepatitis particularly[49]; vidarabine has had some limited use against chronic type B hepatitis[50].

The type B hepatitis virus will induce an infection in chimpanzees similar to chronic type B hepatitis in man[51]; such an infection has been used to a limited extent in chemotherapy evaluations, with negative results relating well to similarly negative results seen in

clinical studies with the same drug[52]. The expense involved, coupled with the difficulties in using such animals, lead us to seek more practical chemotherapy systems. A variety of viruses, most probably unrelated to the human hepatitis agents, are known to induce hepatitis in various lower animals[53]. These include the murine hepatitis virus, adenovirus type 5, murine cytomegalovirus, latent rat virus, equine abortion virus, infectious canine hepatitis virus, Rift valley fever virus, yellow fever virus, marmoset hepatitis virus, duck hepatitis virus, and turkey hepatitis virus. We have preferred the murine hepatitis virus, an RNA virus which induces a rapidly fulminating hepatitis in mice, and the equine abortion virus, a DNA containing agent causing a similar disease in hamsters, in our studies. Each system appears to yield relatively reproducible data, to provide a number of parameters for drug evaluation, and at least one antiviral drug has been found active in each[54-56]. Both infections are characterized by severe damage seen visibly in necropsied animals and also discerned by increased glutamic oxaloacetic transaminase, glutamic pyruvic transaminase and bilirubin levels in the serum. The animals usually die of the infection within 5 to 10 days after intraperitoneal injection of the virus.

VIRUS SPECIFIC ENZYMES

A concept gaining increasing support among chemotherapists is to approach the design and evaluation of antiviral substances by considering their potential to selectively inhibit enzymes known to be associated with viruses[57-61]. Several compounds appear to have this capability. Among these are vidarabine, which, as its triphosphorylated metabolite, inhibits herpesvirus-specific DNA polymerase[62], and the ribavirin triphosphate metabolite, which inhibits influenza virus RNA polymerase[63]. Table 9 lists most of the known virus-associated enzymes which could be used as biochemical targets in developing new antiviral compounds. Such enzyme inhibition studies would probably best be achieved as a follow up to active in vitro tests, using wherever possible similar cell-associated enzymes to determine if the enzyme inhibition seen is selective for the viral enzymes.

RELATING IN VIVO TEST RESULTS TO CLINICAL STUDIES

Despite the improving kinds of animal models being developed for antiviral chemotherapy study, there is still that uncertainty one feels as a candidate substance proceeds from highly successful animal experiments into clinical trial. Will efficacy be seen? If not, why? In summarizing this review, it is appropriate to briefly consider some of the factors which affect an accurate prediction from animal to man (Table 10). There may exist considerable

Table 9. Virus-Associated Enzymes Available for Antiviral Followup Studies

Enzyme Activity	Representative Viruses
Neuraminidase	Influenza, parainfluenza
Protein kinase	Vaccinia, herpes, oncoviruses
Nucleases	Adeno, vaccinia, oncoviruses
Nucleotide phosphohydrolases	Vaccinia
DNA-dependent RNA polymerase	Vaccinia
RNA-dependent RNA polymerase	Influenza, parainfluenza, vesicular stomatitis, rota
RNA-dependent DNA polymerase (Reverse transcriptase)	Oncoviruses

differences between the test animal and man in the ability to absorb the drug, to metabolize it, and to distribute it in sufficient quantity to affected tissues. Marked variation in tolerance to the drug may occur, which, if the tolerance is lower in man, may seriously affect the clinical therapeutic index. The actual viral infection in man is often difficult to mimic in the laboratory, resulting often in extraordinary efforts to establish a recognizable laboratory infection, especially if a human virus isolate is used. Thus, unnatural routes of virus exposure and the use of very large amounts of viral inoculum may be required. Often greatly magnified signs of the disease are needed to accurately measure the animal infection; this again is unnatural and does not relate well to the clinical disease. The viral chemotherapist will tend to use certain treatment schedules and routes of drug administration in the laboratory test, all which may not necessarily be too applicable to the human situation. The in vivo system usually lacks supportive therapy, such as the use of analgesics, antihistamines, etc., and the proper nursing care that is provided to human patients to allow better rest and comfort. Such aids may significantly assist the body in its recuperative efforts, and their omission in vivo will further tax the already seriously compromised animal. Finally, secondary infections in either the animal or man may also influence responses to the infection or to the drug. Such infections may even affect absorption and metabolism of the drug.

Despite such problems, we gain some consolation in the fact that such experimental antiviral chemotherapy systems as described here can and do work. Antiviral substances, significantly most being nucleosides, have traversed the difficult road from initial

Table 10. Factors Which May Affect Prediction of In Vivo Antiviral Data to Clinical Studies

1. Absorption of the drug
2. Metabolism of the drug
3. Tissue distribution of the drug
4. Toxicity reaction to the drug
5. Route of virus administration
6. Viral inoculum
7. Host for the virus
8. Infection parameters
9. Drug administration schedule
10. Route of drug administration
11. Supportive therapy
12. Nursing care

synthesis through in vitro and in vivo evaluation to eventual successful clinical trials. The challenge to the viral chemotherapist is to consider all those factors affecting such experimental studies in order that the results achieved are useful and predictive.

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