Preclinical Development of Antiviral Drugs

George M. Szczech

The early preclinical development of an antiviral agent is accomplished essentially in two stages. The first stage consists of gathering data to estimate the potential therapeutic index of the agent. This process includes testing for antiviral activity and for cytotoxicity in vitro and performing preliminary pharmacokinetic and toxicology studies in vivo. The second stage consists of carrying out more-extensive safety (toxicology) studies to determine any significant potential toxicities before the agent is used in humans. Chronic-toxicity studies, reproductive toxicity studies, and carcinogenesis bioassays are performed to support clinical trials of longer duration and, ultimately, approval of efficacious antiviral agents. It is essential to identify toxicities early in the developmental process for both safety and economic reasons. Progress in determining the mechanisms of specific toxicities will greatly aid in risk assessment and in our ability to predict and avoid these toxicities.

Early Evaluation of Antiviral Compounds

The preclinical development of an antiviral agent includes all the testing and evaluation of a compound, culminating in the filing of an investigational new drug (IND) application with the U.S. Food and Drug Administration and the initiation of clinical trials. An important component of this process is safety assessment, which is performed with use of a variety of test systems including laboratory animals. Chronic-toxicity studies and carcinogenesis bioassays are then performed to support clinical trials of longer duration and approval of candidate drugs that prove efficacious. Two stages are involved in assessing the safety of an antiviral during preclinical development. The first involves initial evaluation of a potential, or lead, compound to determine its basic chemical, physical, and pharmacological properties, and of most importance, to estimate its potential therapeutic index (potential efficacy vs. potential toxicity). If this evaluation yields promising results, preclinical development of the compound continues. The next step in development includes scale up of chemical synthesis and consists primarily of toxicology studies in laboratory animals and other test systems, which are performed before clinical trials are begun in humans.

In this article, I outline an approach to the early evaluation of antiviral compounds used at Wellcome Research Laboratories (Research Triangle Park, NC) and discuss the pitfalls encountered. I also comment briefly on mechanisms of toxicity and provide a perspective on how they can relate to some of the difficulties encountered in attempts to develop new antivirals.

Early Evaluation of Antiviral Compounds

Initial evaluation of antiviral lead compounds includes both in vitro and in vivo screening (table 1). For each assay or test, specific criteria must be met to proceed to the next stage of development.

As indicated in table 1, an important first step in evaluating a new compound is to estimate its therapeutic index. This is done by comparing the in vitro concentrations required to inhibit growth of the virus under investigation with in vivo concentrations at which cytotoxicity is encountered. Testing for antiviral activity includes determination of 50% inhibitory concentration (IC\textsubscript{50}) values for laboratory isolates, determination of activity against clinical isolates, and assessment of activity against drug-resistant isolates and surrogate viruses (e.g., mouse cytomegalovirus [CMV] and Rauscher leukemia virus) when appropriate. Another important step in assessing potential antiviral activity is to test the compound in an animal model of the viral infection, if one exists. However, such studies are usually not the first ones performed.

Testing for cytotoxicity includes in vitro studies with human T and B lymphoblastoid cells, MRC-5 cells, and normal human fibroblasts. In addition, the human bone marrow progenitor cell assay is quite helpful in providing an early prediction of hematologic toxicity [1, 2]. Although this is a difficult assay that requires ~2 weeks to perform, it does provide valuable information. The results of this assay (table 2) enabled prediction of important toxicity in the erythroid lineage for zidovudine, toxicity in the myeloid lineage for ganciclovir, and a large margin of safety for acyclovir; all of these findings have been reflected in clinical practice.

If in vitro testing shows that a compound has useful antiviral activity, discussions on obtaining a patent are initiated. A radio-labeled formulation of the compound is produced for use in metabolic studies and mechanism-of-action studies, and chemical synthesis is scaled up. Even this last step can be a critical determinant of the future of the compound. Not long ago, my colleagues and I worked with a nucleoside analog that has
The following examples from experience at Wellcome Research Laboratories illustrate many of the points made above. The first example shows that even when drug development efforts are concentrated in a defined area, one can encounter significant difficulties. After oral, intravenous, and topical formulations of acyclovir had been approved for clinical use, a preliminary study of... 28 days is preferable when the viral target—CMV or HIV, for example—requires treatment for more than a few days or weeks. These studies are carried out at a range of doses in small animals such as mice or rats and in larger animals such as monkeys or dogs. Endpoints evaluated include clinical signs; body weight; results of hematologic tests, serum biochemical tests, and histopathology; and pharmacokinetic parameters.

If the results of early pharmacokinetic and preliminary toxicology studies are favorable, it is important to conduct further studies of efficacy in animal models of infection, if they exist. Models that have been useful include mouse CMV; herpes simplex virus in rabbits, guinea pigs, and mice; mouse hepatitis B virus (HBV); the Rauscher leukemia virus; and the woodchuck HBV model.

Once in vitro and in vivo studies have identified a potentially useful antiviral agent, more-extensive toxicology studies are undertaken in preparation for clinical trials to further evaluate the safety of the compound. For many reasons, this is a critical stage in the preclinical development of a compound. Amounts of the compound needed increase markedly. It is at this point that the tests and study designs relevant to support the safe introduction of the compound into clinical trials are determined. Early detection of drug interactions, toxicities, unacceptable pharmacodynamic activity, or other problems that may affect clinical use of the antiviral conserves expensive drug development resources in addition to satisfying safety considerations.

### Pitfalls Encountered in Attempts to Develop New Antivirals

The following examples from experience at Wellcome Research Laboratories illustrate many of the points made above. The first example shows that even when drug development efforts are concentrated in a defined area, one can encounter significant difficulties. After oral, intravenous, and topical formulations of acyclovir had been approved for clinical use, an

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**Table 1.** Steps in evaluating candidate antiviral compounds.

1. In vitro assays
   - Antiviral testing (10–20 mg)
   - Cytotoxicity screens (10–20 mg)
2. Initiate patent discussions
3. Order radiolabeled compound
4. Scale up chemical synthesis
5. Pharmacokinetics
   - Metabolism
   - Mechanism of action
6. Preliminary toxicology
   - Rat (>50 g) or mouse (>7.5 g) for 28 days
   - Monkey (100–250 g) or dog (300–750 g) for 28 days
7. Animal model efficacy
   - Mouse, guinea pig, rabbit HSV
   - Mouse CMV
   - Mouse HBV
   - Rauscher leukemia model
   - Woodchuck HBV

**NOTE.** Numbers in parentheses are approximate amounts of test compound required. CMV = cytomegalovirus; HBV = hepatitis B virus; HSV = herpes simplex virus.

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strong in vitro activity against strains of human CMV. However, we were unable to synthesize it in quantities sufficient for supporting development, even after we had invested ~12 chemist-years; therefore, the project was stopped.

The next steps (5–7) shown in table 1 relate to in vivo studies. Early pharmacokinetic studies are carried out to gain additional information about the compound in laboratory animals. These studies guide the selection of species and study designs for further toxicologic investigation. Pharmacokinetic studies are usually performed in rats, mice, monkeys, and, when appropriate, dogs and woodchucks.

The evaluation of toxicity in dose-range-finding studies is a significant step. It is the first wholly in vivo experience that can be used to confirm the therapeutic index estimated by earlier comparison of results from in vitro studies. Dosage regimens of 7 and 14 days are often used in range-finding studies; however, a preliminary study of ~28 days is preferable when the viral target—CMV or HIV, for example—requires treatment for more than a few days or weeks. These studies are carried out at a range of doses in small animals such as mice or rats and in larger animals such as monkeys or dogs. Endpoints evaluated include clinical signs; body weight; results of hematologic tests, serum biochemical tests, and histopathology; and pharmacokinetic parameters.

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**Table 2.** In vitro human bone marrow progenitor toxicity assay.

<table>
<thead>
<tr>
<th>Antiviral agent</th>
<th>No. of experiments</th>
<th>CFU-GM (μM)*</th>
<th>BFU-E (μM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zidovudine†</td>
<td>55</td>
<td>10 ± 3</td>
<td>0.3 ± 0.06</td>
</tr>
<tr>
<td>Ganciclovir‡</td>
<td>12</td>
<td>19 ± 3</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>Acyclovir§</td>
<td>4</td>
<td>150 ± 13</td>
<td>330 ± 57</td>
</tr>
</tbody>
</table>

**NOTE.** Methods are from [1]. IC<sub>50</sub> = 50% inhibitory concentration; BFU-E = burst-forming unit-erythroid lineage; CFU-GM = colony-forming unit-granulocyte/macrophage lineage.

* Estimates are subject to twofold variation.
† Data are from [3].
‡ Data are from [4].
§ Data are from [2].
Table 3. Plasma levels of 134U73 and acyclovir 1 hour after 134U73 dosing in rats.

<table>
<thead>
<tr>
<th>Dose of 134U73 (mg/(kg·d))</th>
<th>Plasma level (μM) on indicated day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>19</td>
</tr>
<tr>
<td>400</td>
<td>77</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>56</td>
</tr>
</tbody>
</table>

*NOTE.* Doses of 25 mg/(kg·d), 50 mg/(kg·d), and 100 mg/(kg·d) were selected for the carcinogenesis bioassay. Data are from [10].

Table 4. Plasma levels of 515U74 and acyclovir in dogs in a 1-year study.

<table>
<thead>
<tr>
<th>Dose of 515U74 (mg/(kg·d))</th>
<th>Plasma level (μM) of indicated agent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acyclovir</td>
</tr>
<tr>
<td>20 mg/(kg·d)</td>
<td>20</td>
</tr>
<tr>
<td>40 mg/(kg·d)</td>
<td>35</td>
</tr>
<tr>
<td>80 mg/(kg·d)</td>
<td>50</td>
</tr>
</tbody>
</table>

*The prodrug 515U74 was given in two equal doses separated by ~6 hours.*
levels of ara-M was begun. Twenty-six 6-substituted arabinosides [19] and 50 prodrugs of ara-M (twenty-five 2' esters and twenty-five 5' esters) were studied [20, 21] for the ability to provide greater systemic availability of ara-M. The latter compounds were evaluated in rats to determine urinary recovery of ara-M, but the results were not sufficient to identify a specific potential antiviral. Additional studies of aqueous solubility, stability of the produrg, and urinary recovery in rats showed that 170U88, given orally, delivered four times as much ara-M to plasma as did ara-M itself and that it also delivered more ara-M to plasma than did the other esters. Two percent of ara-M was recovered in the urine of rats given ara-M, as compared with 25% in the urine of rats given 170U88. These data were promising enough to advance 170U88 to project status, with the near-term objective of filing an IND and beginning clinical trials.

However, 90-day toxicology studies with 170U88 showed irreversible neurotoxicity in both rats and monkeys (table 6). The CNS as well as the peripheral nervous system were affected in both species [22]. Histopathologic examination showed patterns of large vacuoles in the cerebellums of rats and degenerative alterations in the sciatic nerves, primarily involving long axons, of monkeys. Signs of this toxicity appeared after 30 days of dosing and did not reverse during the postdose period. Development of 170U88 was discontinued when this important and irreversible toxicity was discovered. Another investigative compound, 642U88, produced similar neurotoxicity in rats. The candidate antivirals 170U88 and 642U88 both are nucleoside analogs that are metabolized to hypoxanthine arabinoside, which is then converted to adenosine arabinoside (ara-A, or vidarabine). We postulated that both of these compounds may have produced greater exposures to ara-A, thereby enhancing the known toxicities of ara-A.

The final example illustrates how preclinical studies can provide insight into alternatives that help avoid pitfalls. As clinically important nephrotoxicity was encountered with (S)-

**Table 5. Oral bioavailability of valacyclovir and conversion to acyclovir in laboratory animals.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Percent of drug bioavailable*</th>
<th>Acyclovir/valacyclovir levels in plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>82</td>
<td>28:1†</td>
</tr>
<tr>
<td>Rat</td>
<td>68</td>
<td>54:1†</td>
</tr>
<tr>
<td>Rabbit</td>
<td>84</td>
<td>17:1†</td>
</tr>
<tr>
<td>Monkey</td>
<td>59</td>
<td>42:1†</td>
</tr>
</tbody>
</table>

* Oral bioavailability of acyclovir in mice, rats, and monkeys is 10%–15%.
† Ratio for mean plasma levels after lifetime dosing at 120 mg/(kg·d).
‡ Ratio for mean plasma levels in one-year study at a dose of 120 mg/(kg·d).
§ Ratio for mean plasma levels in three-month study at a dose of 300 mg/(kg·d).
** Ellipses indicate that no valacyclovir was detected in plasma (0.08 μg/mL = limit of sensitivity of assay) in three-month study at a dose of 600 mg/(kg·d).
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** Ellipses indicate that no valacyclovir was detected in plasma (0.08 μg/mL = limit of sensitivity of assay) in three-month study at a dose of 600 mg/(kg·d).

The data in table 5 demonstrate that this has been shown in laboratory animals, and these data also show that the conversion of valacyclovir to acyclovir is excellent, even at large doses. No valacyclovir was detected in the plasma of most of the animals when it was obtained 30 or more minutes after they were dosed.

In preclinical safety testing, valacyclovir was found to have the same favorable profile as acyclovir [13]. The only significant systemic toxicity seen in laboratory animals was obstructive nephropathy, similar to that seen in animals given large parenteral doses of acyclovir [14]. Reproductive and genetic toxicity studies with valacyclovir showed only those toxicities that had been previously observed with acyclovir [15, 16], and these occurred at the same exposures or concentrations, in the case of in vitro studies. In addition, carcinogenesis bioassays performed with valacyclovir were negative in rats and in mice, as they were when performed with acyclovir [9]. The clinical safety of valacyclovir [13] is consistent with that of acyclovir [5]. Thus, valacyclovir is an antiviral that met the objective of designing a molecule that enhanced systemic delivery of acyclovir while maintaining its demonstrated safety.

The next example of a pitfall illustrates the desirability of discovering problems early in the drug development process. In this case, toxicity that was clearly unacceptable occurred before there was any human exposure and was sufficient to result in redirection of resources to more promising candidate antivirals. The attempt to develop 170U88 as a specific treatment for varicella-zoster virus (VZV) infections was supported by the observation that eight strains of VZV were susceptible to 6-methoxypurine arabinoside (ara-M), having IC₅₀ values of 0.5–3 mM [17]. It was also known that ara-M is converted to ara-ATP selectively in VZV-infected cells [17, 18]. Thus additional extensive work with 76 purine nucleoside analogs [19–21] that was aimed at selectively delivering therapeutic...
Mechanisms of Toxicity

It is obvious that the identification of significant toxicities at the preclinical stage would be greatly aided if a given toxicity could be predicted on the basis of a compound’s molecular structure and/or activity. Unfortunately, it is unlikely that such correlations will be reliable until the mechanisms behind specific toxicities are understood more fully. Current research in areas including DNA synthesis in mitochondria, intracellular enzymes, phosphorylation, and membrane transport is providing important keys to the understanding of these mechanisms. Toxicologists can add to these efforts by becoming involved at the earliest stages of drug development and by continuing to develop screening tests. Such tests can provide clues about potential common denominators (e.g., the potential for a given nucleoside to affect lactic acid metabolism) or the pathogenesis of adverse effects that will ultimately allow us to fully understand mechanisms of toxicity, to improve benefit-risk assess-
ments and, ideally, to design antivirals that have more predictable characteristics.

Although neither the pathogenesis nor the molecular mechanism is completely understood for any of the toxicities associated with nucleoside analogs, significant progress has been made. The clinical toxicities encountered with 2'-fluoro-2'-deoxy-5-iodoarabinoside U (FIAU) and ddC (zalcitabine) are good examples. The correlative work reported by Lipman and colleagues [24], who sought additional information on the neurotoxicity produced by ddC, is illustrative. Even after adjusting for differences in the oral bioavailability of ddC, there are marked differences in the sensitivity of various species to its neurotoxicity. Unfortunately, humans are the species most sensitive to this adverse effect [24]. While ddC at doses of 2,000 mg/(kg·d) in mice and at doses of 4,000 mg/(kg·d) in rats produced no neurotoxicity after 4 and 52 weeks, respectively, doses of 35 mg/(kg·d) in rabbits produced neurotoxic effects on peripheral myelin and on distal axons after 16 weeks. Distal axon toxicity was observed in cynomolgus monkeys after 18 weeks when ddC was administered at a dose of 5 mg/(kg·d), and this effect was observed in humans after 10 weeks when only 0.1 mg/(kg·d) was administered. Similar findings with regard to hematologic toxicity have been attributed to ddC [24]; however, it is important to note that it is not hematologic toxicity but neurotoxicity that is the dose-limiting adverse effect associated with the use of ddC in clinical practice.

Lipman and co-workers [24] postulated that nucleoside phosphorylation and intracellular levels of phosphorylated metabolites may play an important part in the mechanism behind species-dependent sensitivity to ddC-related neurotoxicity. They examined the intracellular pool size of nucleotides (ddCTP, ddCDP, and ddCMP) in both fibroblasts and in peripheral lymphocytes from five species at 1 hour, 3 hours, 6 hours, and 24 hours following exposure to ddC. An elution profile revealed that ddC, ddCMP, ddCDP, and ddCTP each eluted at 6 minutes, 11 minutes, 14 minutes, and 15 minutes, respectively. These authors found that intracellular concentrations of nucleotides in rabbit and human fibroblasts were 142 and 653 times higher, respectively, than they were in mouse fibroblasts. Similarly, they found that intracellular concentrations of nucleotides in rabbit and monkey lymphocytes were 220 and 3,640 times higher, respectively, than they were in mouse lymphocytes. In addition, the relative concentration of ddCTP at 24 hours was highest in human fibroblasts (i.e., 3.1 times that in rabbits and 7.5 times that in mice) and in monkey peripheral lymphocytes (11 times that in rabbits, 35 times that in mice, and 41.1 times that in rats). The concentration of ddCTP peaked at 6 hours in all of the species except monkeys and humans; in the latter two species, it continued to rise for 24 hours.

Lipman et al. [24] pointed out that these findings do not completely explain the neurotoxicity associated with ddC. Recently, Werth and colleagues [25] speculated that ddC-induced neurotoxicity may result from altered calcium buffering in dorsal root ganglion neurons, a phenomenon they observed in rat
neurons studied in an in vitro system. However, this example illustrates the value of correlating research findings in isolated systems with clinical observations when attempting to elucidate mechanisms of toxicity. Coordinated multidisciplinary research will continue and will accelerate progress in development of new antivirals.

Acknowledgments

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