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Review

In vitro and in vivo assay systems for study of influenza virus inhibitors

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Abstract

Evaluation of potential influenza virus inhibitors may utilize multiple steps. First would be to determine if the viral target (e.g. influenza virus neuraminidase) being focused upon will be inhibited in the appropriate assay. Standard in vitro antiviral assays, used next in antiviral evaluations, may utilize inhibition of viral plaques, viral cytopathic effect (CPE), and viral hemagglutinin or other protein, with inhibition of viral yield used in follow-up evaluations. The CPE can be determined visually and by dye uptake. Animal models used for study of potential influenza virus inhibitors include the ferret, the laboratory mouse, and the chicken, with a variety of parameters used to indicate the severity of the infection and its inhibition by therapy. Multiple parameters are recommended in any in vivo antiviral evaluation. The ferret and the mouse infection models have been useful in studying the development of drug resistance and the relative virulence of drug-resistant viruses. The influenza mouse model has also been of value for the evaluation of immunomodulating effects of test compounds and for the study of the utility of antiviral drugs for use against influenza virus infections in the immunocompromised host. In considering the use of any animal model, species differences in drug pharmacology and metabolism must be taken into account. This review has described the systems which have been used most frequently by antiviral investigators, using, as examples, recent studies with the clinically approved influenza virus neuraminidase inhibitors oseltamivir and zanamivir.

Keywords: Influenza; Zanamivir; Oseltamivir

1. Introduction

Control of influenza continues to be a major public health concern and much effort has been extended towards the discovery and development of antiviral drugs for treatment of this significant viral disease. The selection of both in vitro and in

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vivo antiviral test systems that will provide selective, sensitive, and reproducible results is, therefore, a priority in this discovery and development process.

This review will focus on the systems utilized for the study of potential influenza virus inhibitors using primarily the experiences with the recently developed drugs oseltamivir and zanamivir for illustrative purposes.

2. In vitro assay systems

An appropriate first step in evaluation of potential influenza virus inhibitors will be to determine if the viral target being focused upon will be inhibited in an appropriate assay. This concept is especially illustrated using the influenza virus neuraminidase (NA) inhibitors, since two of these materials, zanamivir and oseltamivir, have been used successfully in the clinic (Hayden et al., 1996a,b, 1997, 1999a,b; Mist Study Group, 1998; Monto et al., 1999a,b,c). In the studies run with GS4071 (oseltamivir carboxylate), the parent compound of oseltamivir (Mendel et al., 1998), the viral NA enzyme was pre-incubated with varying concentrations of the test compound or with diluent only; fluorogenic substrate (2-[4-methylumbelliferyl]-α-D-*N*-acetylneuraminic acid) was then added, followed by incubation at 37°C and the reaction stopped with NaOH in ethanol. Fluorescence was then quantified by a fluorimeter. A 50% inhibitory concentration, relative to the activity in the reaction mixture containing virus but no inhibitor, was determined.

It has been found that some clinical influenza virus isolates have insufficient NA to be readily detectable in fluorogenic assays. To compensate for this, a rapid chemiluminescence assay with improved sensitivity has recently been reported that utilizes a 1,2-dioxetane derivative of sialic acid as the substrate (Buxton et al., 2000). Using this assay with a number of influenza viruses, zanamivir exhibited potent inhibition of NA that compared well with the results seen using the fluorogenic method.

Ideally, in such assays, the enzyme from a spectrum of influenza viruses representing those implicated in current epidemics should be used to illustrate the breadth of potential influenza virus inhibition. In the above-cited experiments with GS4071, several viruses representing influenza A (H1N1), A (H3N2) and B were used. The viruses selected were both relatively new clinical isolates as well as recognized laboratory strains. Controls in such studies should include NA from other sources to show that the enzyme inhibition was selective for influenza viruses only. In the GS4071 study, these sources included human liver, two bacteria (*Clostridium perfringens* and *Vibrio cholerae*), and two unrelated viruses (Newcastle disease, parainfluenza 3).

In vitro antiviral assays using cell-based systems commonly use inhibition of viral plaque formation, viral cytopathic effect (CPE), or viral hemagglutinin (HA) or other viral protein, with inhibition of virus yield used in follow-up evaluations. The cells primarily used are Madin Darby canine kidney (MDCK), although other cells (e.g. primary chick embryo, chick kidney, calf kidney, Vero, mink lung, and human respiratory epithelial cells) may be used. Trypsin and ethylene diamine tetraacetate are added to the medium to enhance the viral CPE (Appleyard and Maber, 1974; Tobita and Kilbourne, 1974). Influenza virus, when added to cell cultures, may efficiently replicate, producing new infectious virus, or the virus may not undergo a complete replicative cycle with non-infectious (incomplete) virus released; with some viral isolates no cell infection may occur (Stuart-Harris and Schild, 1976). This ability to replicate in cells is quite dependent upon the virus, with new clinical isolates often requiring passage in the amniotic cavity of eggs and multiple cell passage before efficient replication will occur.

When performing in vitro screenings on a number of test compounds, the 96-well microplate is quite useful (Sidwell and Huffman, 1971). Our assays utilize visual determination of viral CPE inhibition, with the data confirmed by neutral red (NR) dye uptake (Huffman et al., 1997; Sidwell et al., 1998). Others have used MTT, XTT, or crystal violet staining of cell monolayers (Gabrielsen et al., 1992; Ozes et al., 1992; Klein et al., 1996; Shigeta et al., 1996) Various one-half $\log_{10}$ con-

centrations of test compound were used in each test; in initial screening the compound is added approximately 5 min prior to virus exposure and remains on the cells until the test is read. Since the antiviral activity of most antiviral compounds is dependent upon the viral multiplicity of infection (m.o.i.), an inoculum is selected that will induce near-maximal (4+) CPE in 72–96 h. We utilize approximately 50 cell culture 50% infectious doses ($CCID_{50}$) per microplate well, which equates to an m.o.i. of 0.001 infectious particles per cell. Cytotoxicity controls in uninfected cells are included with each concentration of test compound. Other controls include normal controls (uninfected cells with test medium only) and virus controls (cells with virus and drug diluent). A known positive control drug is recommended to be run in parallel in every antiviral screening test. Antiviral activity is expressed as the 50% effective (virus-inhibitory) concentration (EC_{50}), determined by regression analysis of the CPE inhibition data. Cytotoxicity is expressed as the 50% cytotoxic concentration (CC_{50}) determined by plotting percentage of cytotoxicity versus test compound concentration. A selective index (SI) is determined as CC_{50}/EC_{50}.

Experiments run comparing plaque, CPE, and HA inhibition assay results have not been run, so direct comparisons of these assays cannot be made. However, to illustrate how the visual and NR uptake data compare, the effects of the oseltamivir carboxylate (GS4071), and zanamivir on a number of influenza virus strains are summarized in Table 1. None of the compounds exhibited cytotoxicity at the highest concentration employed, 1000 μM, hence the SIs are all expressed as '>' the calculated value. In general, it can be seen that both compounds have similar antiviral efficacies, and the visual EC_{50} values compared well with those determined by NR uptake. It was interesting that the A/NWS/33 (H1N1) virus appeared quite resistant to these compounds; as will be seen later, however, mice infected with the identical virus pool responded very favorably to treatment. Sequencing of the NA and HA genes of this virus has not been done. A similar contrast between in vitro and in vivo efficacy against the same virus has also been reported by others using zanamivir versus influenza A/Stockholm/24/90 (H1N1) virus (Von Itzstein et al., 1993; Woods et al., 1993) suggesting the in vitro data are not always predictable to what may occur in the animal.

Compounds considered active by CPE inhibition determined visually and by dye-uptake should be further tested to determine influence on virus yield. This test is run in a similar manner to the initial test, with the plate then frozen and thawed and eluates from each set of wells assayed for virus titer. This viral titration can be run by adding varying dilutions of each sample to a monolayer of cells in 96-well microplates, with viral CPE after a standard incubation period used as endpoint. Alternatively, the virus may be quantified by plaque formation or hemagglutinin (HA) assay, although this latter method is less acceptable because the virus particles will differ by strain in their ability to cause HA (Schulze, 1975). In addition, the amount of HA will vary depending upon the species of erythrocytes, temperature, pH, and ionic components of the reaction (Schulze, 1975). The embryonated egg has been used widely for end-point infectivity titrations of influenza virus (Henle, 1953). The presence of HA activity in the egg fluids is used as evidence of infection, although recent influenza A (H1N1) and A (H3N2) clinical isolates have lost an ability to agglutinate chicken red blood cells (Rimmelzwaan et al., 1998). In addition, virus grown in the presence of NA inhibitors causes intensive self-aggregation of progeny virions, which interferes with erythrocyte agglutination. The allantois-on-shell technique (Fazekas de St. Groth and White, 1958) has had limited use.

When assaying the effect of NA inhibitors on viral yield, we have found the frozen/thawed eluates require low-speed centrifugation ($3200 \times g$ for 5 min) to separate extracellular from cell-associated virus. The effects of zanamivir and GS4071 on cell-associated and extracellular virus yields are shown in Fig. 1. It can be seen that these compounds markedly affect the extracellular virus yields, but had a lesser effect on the cell-associated virus, a result to be expected considering the mechanism of action of these compounds. In the absence of NA, virus particles remain clumped at the cell surface instead of being released into the medium (Liu et al., 1995).

Additional information on in vitro influenza virus antiviral testing of NA inhibitors can be found in a recent review by Tisdale (2000).

3. The ferret model

The ferret has been a preferred animal model for some influenza researchers because they exhibit many of the typical signs of influenza infection, such as nasal discharge, loss of appetite, congested eyes, otologic manifestations, and, importantly, fever. Virus in high titer can be recov-

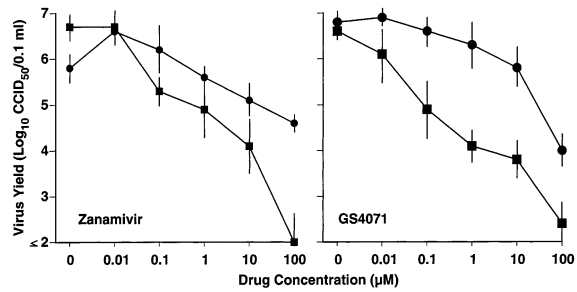

Fig. 1. Effects of zanamivir and GS4071 on cell-associated (●) and extracellular (■) influenza A/Texas/36/91 (H1N1) virus yields from Madin Darby Canine Kidney cells.

Table 1
Comparison of the in vitro influenza virus-inhibitory effects of GS4071 and zanamivir using visual and neutral red uptake determination of cytopathic effect

Virus	GS4071				Zanamivir			
	Visual EC_{50}[a] (μM)	SI[b]	NR[c] EC_{50} (μM)	SI	Visual EC_{50} (μM)	SI	NR EC_{50} (μM)	SI
A/Bayern/07/95 (H1N1)	3.0	>330	2.7	>370	3.6	>275	3.4	>290
A/PR/8/34 (H1N1)	0.35	>2850	0.22	>4540	0.30	>3330	0.42	>2380
A/NWS/33 (H1N1)	57	>17	>100	–	60	>16	>100	–
A/Texas/36/91 (H1N1)	0.16	>6250	0.17	>5880	0.14	>7140	0.22	>4540
A/Beijing/52/92 (H3N2)	0.13	>7690	0.23	>4340	0.30	>3330	0.65	>1530
A/Johannesburg/33/94 (H3N2)	0.18	>5550	0.50	>2000	0.12	>8330	0.62	>1610
A/Shangdong/09/93 (H3N2)	0.04	>25 000	0.32	>3120	0.07	>14 280	0.5	>2000
A/Victoria/3/75 (H3N2)	0.06	>16 660	0.06	>16 660	0.10	>10 000	0.41	>2440
A/Duck/MN/1525/81 (H5N1)	0.19	>5263	0.22	>4540	0.18	>25 000	0.20	>5000
A/Gull/PA/4175/83 (H5N1)	0.27	>3700	0.26	>3840	0.22	>4540	0.22	>4540
B/Beijing/184/93	70	>100	>100	–	60	>100	>100	–
B/Harbin/07/94	0.23	>4350	0.36	>2770	0.16	>6250	0.21	>4760
B/Hong Kong/5/72	2.0	>500	2.5	>400	0.8	>1250	1.0	>1000
B/Panama/45/90	0.7	>1430	1.5	>600	0.9	>1100	1.3	>770

[a] Fifty percent effective (virus-inhibitory) concentration.
[b] Selective index (EC_{50}/CC_{50} (CC_{50} >1000 μM)).
[c] Neutral red.

ered from the respiratory tract (Potter et al., 1976; Smith and Sweet, 1988). However, the animals appear more responsive to infections by influenza A than influenza B viruses (Pinto et al., 1969).

It is interesting that in some of the earliest antiviral studies reported using ferrets, amantadine therapy appeared to enhance the infection as seen by mortality and increased lung damage (Cochran et al., 1965). This observation was confirmed by Squires (1970) using daily rectal temperatures, which were overall higher in the drug-treated group compared to controls. Amantadine has exhibited significant anti-influenzal disease activity in mice and in the clinic (Hoffman, 1973), suggesting the data obtained in ferrets may not always be predictive of clinical efficacy. As indicated in Section 6 of this study, viral resistance to amantadine develops quickly in vivo; it is possible that the lack of efficacy seen in the ferret is a manifestation of this resistance. Ribavirin, which has exhibited a variable activity against influenza in human patients (review, Sidwell, 1996), has been shown to reduce the virus-induced febrile response, nasal wash protein, nasal antibody titer (Schofield et al., 1975) and the nasal wash virus titer (Potter et al., 1976; Ryan et al., 1995; Fenton et al., 1999) in ferrets. Zanamivir and oseltamivir have similarly been reported to be highly effective in reducing nasal wash influenza virus titers in ferrets (Ryan et al., 1995; Mendel et al., 1998). Oseltamivir was also shown to significantly reduce the febrile response and inflammatory cell count in nasal washes of infected ferrets (Mendel et al., 1998).

The ferret model has been used extensively to study the infectivity and virulence of influenza virus mutants resistant to the neuraminidase inhibitors (Blick et al., 1998; Gubareva et al., 1998; Barnett et al., 2000). Blick et al. (1998), utilizing a zanamivir-resistant influenza virus having an NA and HA mutation, reported the virus to exhibit no detectable resistance to zanamivir in the ferret model but it showed a small decrease in sensitivity to the drug in mice, indicating some differences in the two animal models in their role in studying drug-resistant influenza viruses.

4. The mouse model

The laboratory mouse can be experimentally infected with both influenza A and B viruses, although some adaptation to the animal must be done by multiple passage of the virus through their lungs. Such mouse-adapted viruses have developed an ability to infect alveolar cells (Mulder and Hers, 1972) and are reportedly associated with changes in the antigenicity of the surface HA (Gitelman et al., 1983). Recent clinical isolates which are not mouse-adapted may induce a toxic pneumonitis in the absence of significant viral replication (Sugg, 1949, 1950; Ginsberg, 1954; Barker and Hoyle, 1972). We have used such a viral infection induced by non-mouse-adapted A/Beijing/32/92 (H3N2) virus in mice to evaluate the effects of treatment with amantadine and with ribavirin. Amantadine exhibited a significant inhibitory effect on the lung consolidation and on decline in arterial oxygen saturation (SaO_2), but ribavirin was considered ineffective (Sidwell et al., 1995). Since ribavirin is inhibitory to in vitro infections and to infections induced in mice by mouse-adapted influenza viruses (Sidwell, 1996), these data suggest caution in interpreting the results of animal experiments using non-mouse-adapted influenza viruses, although the changes occurring in the mouse-adapted virus may also have the potential of yielding misleading antiviral results.

A number of parameters may be used to monitor influenza virus infections in mice. These are summarized in Table 2. Some of the disease manifestations are dependent upon the infectivity and challenge dose of the virus. When the murine infection is not a clinically manifested illness, the effects of antiviral therapy can be monitored using parameters such as quantitation of recoverable virus from the lung, increase in lung weight, and increase in α_1-acid glycoprotein (α_1-AG), all of which increase in the non-lethally infected mice.

As the influenza virus neuraminidase inhibitors have been studied, particular emphasis has been made on the route of administration of the test compound. Zanamivir, with its low oral adsorption, has exhibited the greatest efficacy when mice or ferrets were treated intranasally (Ryan et al.,

1994, 1995; Sidwell et al., 1998). It is important to note that when intranasal therapy is administered, the viral challenge should be reduced because the repeated administration of fluids into the lung exacerbates the infection (Takano et al., 1963). This effect may be circumvented if the compound is administered by small-particle aerosol in the manner described by Wyde et al. (1986). The oral gavage route of treatment has also been used extensively; using this route, oseltamivir has exhibited strong inhibitory effects against influenza A and B in mice (Mendel et al., 1998; Sidwell et al., 1998). Surprisingly, zanamivir was also efficacious when administered by this route, although its antiviral effect was less than that of oseltamivir (Sidwell et al., 1998).

Table 2
Parameters used in mouse influenza virus antiviral experiments

Parameter	Reference[a]
Pneumonia-associated death	Davies et al. (1964)
Mean time to death	Davies et al. (1964)
Arterial oxygen saturation	Sidwell et al. (1992)
Serum α_1-acid glycoprotein increase	Wong et al. (2000)
Pulmonary gas exchange	Tschorn et al. (1978)
Rales	Kaji and Tani (1967)
Lung score	Sidwell et al. (1968)
Lung weight	Sidwell et al. (1994)
Ratio of lung weight to body weight	Schulman (1968)
Lung water content	Arensman et al. (1977)
Change in host weight	Hoffman (1973)
Change in water intake	Hoffman (1973)
Hypothermia	Arensman et al. (1977)
Lung virus assayed by hemagglutinin	Davies et al. (1964)
Lung virus assayed by virus-induced plaques or cytopathic effect	Finter (1970)
Lung virus assayed by allantois-on-shell assay	Tisdale and Bauer (1976)
Histopathological changes in lung	Arensman et al. (1977)

[a] Initial report of use in antiviral experiment.

The most acceptable antiviral experiment should use multiple parameters to assess the efficacy of test drugs. Usually, such experiments will use two groups of infected mice treated with each dose of test compound or with placebo; one group is monitored the duration of the experiment for daily occurrence of death, weight loss, SaO_2 decline, etc.; mice are killed in the second group at varying times after virus exposure and their lungs removed for assay of consolidation and virus titer and their serum α_1-AG quantified.

An example of an experiment using multiple disease parameters to illustrate the concepts discussed is seen in Table 3. In the experiment, orally administered oseltamivir was evaluated against influenza A/NWS/3 (H1N1) virus infections in mice. The drug was administered in a single dose of 20 mg/kg per day twice daily for 5 days beginning 4-h pre-virus exposure. The animals were challenged with different concentrations of virus, ranging 10-fold from 10^{-2} ($10^{5.75}$ cell culture 50% infectious doses ($CCID_{50}$) per ml). Four mice in each group were killed on day 6 and their lungs removed, assigned a consolidation score ranging from 0 (normal) to 4 (maximal plum coloration), weighed, homogenized, the homogenates centrifuged at $2000 \times g$ for 10 min, and varying 10-fold dilutions of the supernate assayed for virus titer in MDCK cells using CPE produced after a 96-h incubation at 37°C as endpoint. The serum taken from the mice on day 6 was assayed for α_1-AG using single radial immunodiffusion kits. Eight additional mice in each group were observed daily for death for 21 days, and their SaO_2 values determined by pulse oximeter (Sidwell et al., 1992) on days 3, when SaO_2 decline usually begins to occur, through day 11, when the values are seen to decline to the maximum degree or the animals died. Animals dying of obvious influenzal pneumonia were assigned SaO_2 values of 75% in order to compare mean differences late in the infection.

The infection induced by the highest virus challenge dose was very severe, with the animals all dying, the mean day to death (MDD) being 4.9 days. Marked lung consolidation was seen on day 6 (mean weight of 460 mg compared with normal control weight of 150 ± 24 mg), high virus titers

Table 3
Influence of influenza challenge dose of influenza A (H1N1) virus[a] on the efficacy of orally administered[b] oseltamivir[c] in mice

Treatment	Viral challenge dose ($CCID_{50}$/mouse)	Infected, treated mice						
		Surv/Total	MDD[d] ± S.D.	Day 11 SaO_2[e] (% ± S.D.)	Day 6 disease parameter			
					Lung Score ± S.D.	Lung Weight (mg ± S.D.)	Lung Virus titer[f] (log_{10}/g)	Serum α_1-AG[g] (μg/ml ± S.D.)
GS4104	$10^{4.75}$	8/8[h]	>21.0 ± 0.0[h]	88.1 ± 1.8[h]	0.4 ± 0.8[h]	200 ± 22[h]	5.3 ± 0.5[h]	222.5 ± 29[h]
H_2O		0/8	4.9 ± 0.7	75.0 ± 0.0	4.0 ± 0.0	460 ± 0.0	7.3 ± 0.0	450.0 ± 0
GS4104	$10^{3.75}$	8/8[h]	>21.0 ± 0.0[h]	87.1 ± 1.2[h]	0.1 ± 0.2[h]	165 ± 19[h]	5.1 ± 0.5[j]	130.0 ± 63[h]
H_2O		0/8	7.8 ± 3.9	76.0 ± 2.8	3.5 ± 0.6	435 ± 50	6.8 ± 0.5	435.0 ± 19
GS4104	$10^{2.75}$	8/8[i]	>21.0 ± 0.0[h]	87.1 ± 1.5[j]	0.0 ± 0.0[h]	165 ± 24[h]	5.3 ± 0.5[i]	121.3 ± 41[j]
H_2O		4/8	8.0 ± 2.4	80.5 ± 6.0	2.8 ± 0.9	360 ± 58	6.1 ± 0.4	361.3 ± 107
GS4104	$10^{1.75}$	8/8	>21.0 ± 0.0	86.9 ± 1.6	0.0 ± 0.0[j]	152 ± 10[h]	5.1 ± 0.4[j]	110.0 ± 29[h]
H_2O		8/8	>21.0 ± 0.0	86.8 ± 2.0	1.1 ± 0.5	248 ± 17	6.6 ± 0.6	383.8 ± 24

[a] A/NWS/33.
[b] Bid × 5 beginning 4 h pre-virus exposure.
[c] 20 mg/kg per day.
[d] Mean day to death.
[e] Arterial oxygen saturation as determined by pulse oximeter (Sidwell et al., 1992).
[f] Determined by assay of supernates from centrifuged lung homogenates in MDCK cells using CPE after 96-h incubation as endpoint.
[g] α_1-Acid glycoprotein, measured by radial immunodiffusion kit (Saikin Kagaku Inst. Co. Ltd., Sendai, Japan).
[h] $P<0.001$, compared with H_2O controls receiving the same virus challenge.
[i] $P<0.05$.
[j] $P<0.01$.

were recovered from the lungs, and the α_1-AG value at this time was 450 μg/ml compared with a mean normal control value of 100 ± 18 μg/ml. The lowest viral challenge dose was not lethal to the mice, but still induced a 65% increase in lung weight, high lung virus titers were seen, and α_1-AG levels were 284% higher than normal. SaO_2 levels declined in a virus dose–responsive manner; the day 11 SaO_2 values, when the decline was greatest, are also shown in Table 3.

Treatment with oseltamivir was highly effective using every disease evaluation parameter, which was rather surprising since the in vivo efficacy of antiviral compounds can sometimes be overwhelmed by excessively high viral challenge (Sidwell, 1985). This compound has been well-tolerated in mice at dosages of at least 1000 mg/kg per day, and the minimum effective dose is approximately 10 mg/kg per day (Sidwell et al., 1998), so the 20 mg/kg per day dose used in this study was relatively low, yet was still highly effective. It was interesting that the influenza A/NWS/33 virus was quite insensitive to treatment by GS4071, when assayed in vitro (Table 1), yet in this animal experiment, using the identical virus pool, oseltamivir was highly inhibitory to the infection. The reason for the difference between in vitro and in vivo sensitivity is currently being investigated.

Once efficacy is seen against an influenza virus infection in mice, it is important to use then a variety of other strains of the virus in follow-up evaluations to ascertain if the antiviral effect seen can be applicable to hopefully all viral strains encountered in the clinic. Oseltamivir has been evaluated by us against infections in mice induced by a spectrum of two influenza A (H1N1) viruses, two influenza A (H3N2) viruses, and two influenza B viruses (Table 4). All were inhibited significantly by oral treatment with this drug, although the efficacy appeared to vary with the virus, the A/Victoria/3/79 (H3N2) virus infection appearing least sensitive.

Table 4
Spectrum of influenza viruses used in in vivo[a] antiviral evaluations with oseltamivir[b]

Virus	Dose (mg/kg per day)	Survivor increase (%)	Day 10 mean SaO_2 increase (%)[c]	Maximum mean lung weight decrease (mg)	Maximum mean lung virus titer reduction[c] (log_{10})
A/NWS/33 (H1N1)	10	88[d]	8.6[d]	124[d]	4.6[d]
	1	75[d]	8.5[d]	124[d]	1.9[d]
	0.1	13	5.2[e]	134[d]	nd
A/PR/8/34 (H1N1)	100	45[d]	5.2[d]	50[e]	1.5[d]
	10	45[d]	5.5[d]	30	1.2[d]
	1	5	2.8	10	1.0
A/Shangdong/09/93 (H3N2)	100	100[d]	8.8[d]	56[e]	1.0[e]
	10	90[d]	7.8[d]	20	0.2
	1	30[e]	7.0[d]	22	0.7
A/Victoria/3/79 (H3N2)	10	69[d]	5.6[d]	22	0.3
	1	32	2.5	0	0.0
	0.1	8	0.0	0	0.7
B/Hong Kong/5/72	10	78[d]	8.5[d]	42[d]	1.3[d]
	3.2	79[d]	6.9[d]	nd	nd
	1	0	2.5	nd	nd
B/Lee/40	10	26	4.0[e]	40[e]	1.8[d]

[a] All studies run in BALB/c mice.
[b] Treatment were p.o. bid × 5 beginning 4 h pre-virus exposure.
[c] Compared with saline-treated controls.
[d] $P < 0.01$.
[e] $P < 0.05$.

5. The chicken model

The chicken has been used in a number of elegant antiviral studies, early reports oriented primarily towards the study of amantadine and rimantadine resistance (Bean et al., 1985; Webster et al., 1985; Bean et al., 1989). In later work, zanamivir, administered by intratracheal drops, had only a weak inhibitory effect on an infection in chickens induced by a highly pathogenic strain of avian influenza virus, and failed to inhibit the infection induced by two other avian strains of the virus (McCauley et al., 1995). It was suspected by the authors of this latter study that the virus had spread from the respiratory tract to other locations in the body not reached by the drug. Such viral replication in areas other than the respiratory tract makes the chicken model less acceptable as a model for predicting efficacy against human influenza.

This animal model is valuable particularly when an avian influenza virus is to be studied and in vivo virulence is to be ascertained. It also offers an interesting model for study of effects of therapy on transmission of the virus to other birds via contact.

6. Utilization of the mouse model for study of drug-resistant viruses

The potential for influenza viruses to develop resistance to antiviral drugs has been well established in clinical studies with amantadine and rimantadine (Hayden et al., 1989; Hayden and Couch, 1992; Monto and Arden, 1992; Englund et al., 1998). Such resistance development has also been demonstrated in mice. Oxford et al. (1970) reported achieving this in vivo resistance using the A/Singapore/1/57 (H2N2) influenza virus in amantadine-treated mice by serial passage of the virus through the mice. Resistance was seen after a single passage in mice and increased with subsequent passages through the treated animals. We have seen similar rapid emergence of the influenza A/Port Chalmers/1/73 (H3N2) virus by single passage of the virus through mice receiving either amantadine (0.5, 1, 2, or 10 mg/ml) or rimantadine (0.125, 0.25 mg/ml) in the drinking water. Virus recovered from lungs of the continuously treated mice 5 days after virus exposure was assayed for in vitro sensitivity to the respective drugs, comparing the sensitivity to virus passaged in parallel in placebo-treated mice. As seen in Table 5, marked decrease in sensitivity to the respective drugs was seen, the EC_{50} values of virus recovered from the treated mice being 100- to 1000-fold higher than seen using the control (wild-type) virus. It is noted that the viruses were completely cross-resistant to both drugs.

A similar study was run to determine if passage of influenza A/Shangdong/09/93 (H3N2) virus through mice treated with oseltamivir would also develop resistance, in this case, to the parent compound GS4071. Mice were infected with a lethal dose of the virus and treated p.o. with 1, 10,

Table 5
Development of resistance to amantadine or rimantadine by influenza A/Port Chalmers/1/73 (H3N2) virus after single passage through treated mice[a]

Compound (mg/ml)	EC_{50}[b] (μg/ml) against virus recovered[c] from mice treated with				
	Rimantadine at the rate doses of		Amantadine at the rate doses of		
	0.25	0.125	2	0.5	H_2O
Amantadine	5.4	15	>32	>32	<0.032
Rimantadine	3.3	4.3	>32	>32	<0.032

[a] Treatment with each drug in the drinking water at the concentration shown beginning 2 h (amantadine) or 24 h (rimantadine) prior to virus exposure.

[b] 50% Effective (virus-inhibitory) concentration; data obtained from visually determined CPE inhibition..

[c] Lungs taken 5 days after virus exposure.

Table 6
Comparison of the virulence of two GS4071-resistant influenza A (H3N2) viruses[a] in mice[b]

Virus designation	Maximum virus dilution to induce day 3 infection seen by			Maximum virus dilution to induce day 7 infection seen by		
	Lung score[c]	Increased lung weight[d]	Lung virus titer[e]	Lung score	Increased lung weight	Lung virus titer
Wt	<1:4	1:4000	1:40 000	1:400 000	1:400 000	1:40 000
12-S3 (BS3)	<1:4	<1:4	<1:4	1:40	1:40	<1:4
12-B1 (BB1)	1:4	<1:4	1:40 000	1:400 000	1:400 000	1:400 000

[a] A/Victoria/3/75; Wt, Wild-type (parental) virus; 12-S3 (BS_3), passaged 12 times in vitro in presence of GS4071, contains two mutations (A28T in HAI and R124M in HA2) in HA gene, one mutation (R292K) in neuraminidase gene; 12-B1 (BB_1), passaged eight times in vitro in presence of GS4071, contains two mutations (A28T in HA1 and R124M in HA2) in tis HA gene, but has wild-type neuraminidase.

[b] Female, 8–10-g *BALB/c* mice.

[c] Consolidation score of 1.0 or greater.

[d] Statistically significant ($P<0.05$) lung weight increase compared with normal controls. Normal control mean weights on day 3, 97 ± 11 mg; on day 7, 133 ± 24 mg.

[e] Mean lung virus titer of 1.0 $\log_{10}$ or greater.

or 100 mg/kg per day of oseltamivir twice daily for 5 days beginning 4-h pre-virus exposure. On day 6, 48 h after final treatment, virus recovered from the lungs of the drug- or placebo-treated mice was tested for in vitro sensitivity to GS4071 using the same method as used for the amantadine/rimantadine study described above. The sensitivity to GS4071 of the viruses recovered from the oseltamivir-treated animals did not significantly ($P > 0.05$) differ from that of the virus harvested from placebo-treated animals (Mendel and Sidwell, 1998). A second passage of the virus through mice treated with oseltamivir yielded virus which again did not differ in its sensitivity to GS4071 (unpublished data). These data indicate virus resistance to GS4071 does not readily develop in vivo after exposure to oseltamivir.

The mouse model has been used widely to study the relative virulence of drug-resistant viruses. Zanamivir- and oseltamivir-resistant influenza viruses have exhibited generally a significant reduction in infectivity to mice compared with the concomitant wild-type (parental) viruses (review, Gubareva et al., 2000). This lessening of virulence can be seen in mice using several approaches. Primarily, mice are infected in parallel with the parental and the mutant viruses using equal concentrations of each as determined by assay in vitro. One assay of virulence is to remove the lungs early in the infection (e.g. day 3, Gubareva et al., 1997) and the virus titer in each determined with the titers required of the challenge viruses to induce a 50% infection ascertained. Another method is to quantify the amount of virus produced in the lungs by equivalent concentrations of the viruses (Tai et al., 1998).

If the parental virus is animal-adapted, the titration in mice can then also utilize disease parameters to demonstrate the relative virulence of the mutant virus. In our work with the amantadine- and rimantadine-resistant viruses, the mutant virus was found to maintain its virulence as determined both by titer of virus induced in the lungs, which was comparable to that induced by the parental virus, and by production of lung consolidation as measured by increased lung weight and decreased SaO_2%, which was similar to that induced by the wild-type parental virus (Sidwell et al., 1995).

A similar assay for virulence was run using variants of influenza A/Victoria/3/75 (H3N2) virus with reduced sensitivity to the NA inhibitor GS4071 through selection in vitro after eight and 12 passages in the presence of the compound in work done by Gilead researchers (Tai et al., 1998). The eighth passage variant, designated 12-

B1 (BB_1), contained two amino acid substitutions in the HA (A28T in HA1 and R124M in HA2) but no changes in NA. The twelfth passage variant, designated 12-S3 (BS_3) contained the same HA mutations and a Lys substitution for the conserved Arg292 of the NA. We assayed each virus, as well as the wild-type parental virus for virulence in mice by inoculating 8–10 g female BALB/c mice with 90 μl of 10-fold serial dilutions of each virus using six animals per dilution. Three and 7 days later, the lungs of three animals were weighed, assigned a consolidation score ranging from 0 (normal) to 4 (maximal coloration) and assayed for virus titer. As seen in Table 6, the infectivity of the wild-type and the 12-B1 variant were relatively similar in their ability to induce consolidation and produce infectious virus in the lungs; the 12-S3 variant, in contrast, was very weakly virulent to the mice. The results indicated the HA mutations did not influence virulence of the virus, but the R292K NA mutation, despite the presence of the HA mutations, markedly lessened the virulence.

Recently, an extensive review of influenza virus resistance to NA inhibitors was published (McKimm-Breschkin, 2000). Infectivity of many HA mutants was compromised in mice; some of these viruses demonstrated reduced sensitivity to inhibition by zanamivir. Viruses containing both HA and NA mutations are extremely compromised in their ability to replicate in mice.

7. Utilization of the influenza mouse model to evaluate potential immunomodulatory effects of test compounds

The immune system of the mouse has been well studied in the laboratory; a major advantage to the use of these animals is the wide assortment of commercial kits available for measurement of immune factors. This has great value when immunomodulating agents are to be considered as potential drugs for the treatment of influenza infections.

It has been established that $CD8^+$ T cells play a major role in the clearance of influenza virus during primary infection (Lightman et al., 1987; Eichelberger et al., 1991); $CD4^+$ T-cells apparently also play a role in the immune response to primary influenza virus infections (Lightman et al., 1987; Eichelberger et al., 1991; Bender et al., 1992). IgA antibodies also act as primary mediators of resistance to influenza virus infections, acting both to inhibit replication of the virus intracellularly and to assist in the clearance of virus from epithelial cells (Renegar and Small, 1991; Mazanec et al., 1992). Interferon, particularly interferon-α, may also play a significant role in the primary immune response (Green et al., 1982); both interferon and interferon inducers provide resistance to influenza infection in mice (Takano et al., 1963; Came et al., 1969; Gerone et al., 1971), but have not been found useful in the treatment of humans infected with the virus (Hill et al., 1972; Merigan et al., 1973; Treanor et al., 1987). The role of other cytokines in the primary response to influenza virus infection is unclear, although Hennet et al. (1992) showed interleukin (IL)-6, IL-1, tumor necrosis factor-α, colony-stimulating factor, and leukotriene B4 to be stimulated relatively early (1–3 days) after influenza virus infection in mice.

Thus immune modulators, which would stimulate these primary host defense factors may have potential in the treatment of influenzal disease. Numerous studies have been reported on the use of such immune modifying agents in treating experimental influenza virus infections in mice; with the exception of the interferon inducers referred to above, none have demonstrated sufficient activity to warrant clinical study.

8. Evaluation of antiviral efficacy in immunocompromised hosts

Influenza virus infections can be especially challenging in individuals whose immune system is compromised (Rocha et al., 1991; Whimbey and Bodey, 1992; Ljungman et al., 1993; Klimov et al., 1995; Sable and Hayden, 1995). It would seem appropriate, therefore, to have an influenza model, in which the animal's immune system is compromised, for the study of potential therapeutic agents. Aged mice, having reduced cytotoxic

lymphocyte activity, have been shown to have an increased sensitivity to influenza virus infection (Bender and Small, 1993; Dong et al., 2000), and cyclophosphamide treatment has been reported also to significantly compromise the immune response in mice, making them more susceptible to influenza virus infection (Hurd and Heath, 1975; Mastino et al., 1991).

Using influenza A (H1N1) virus-infected mice whose immune system was compromised by intraperitoneal cyclophosphamide injections (100 mg/kg) administered every 4 days for ten treatments, we have been able to demonstrate that the NA inhibitor RWJ-270201 was able to protect the mice from dying of the infection (Sidwell et al., 2000). Treatment using 100, 10, and 1 mg/kg per day of the compound was begun 8 days after virus exposure and continued twice daily for 5 days.

9. Summary

Many approaches have been utilized for the in vitro and in vivo study of potential influenza virus inhibitors. No single in vitro or in vivo evaluation system has gained universal approval. In considering the use of any animal model, species differences in drug pharmacology and metabolism must be taken into account when evaluating the differences seen in the antiviral activity demonstrated. This review has attempted to describe the systems, which have been used most frequently by antiviral investigators and particularly in our own laboratory, illustrating certain concepts by examples of experiments run with compounds, which have been found efficacious in clinical trials. The selection of the evaluation model for study of potential new anti-influenza virus agents and for follow-up considerations of resistance development or immunological effects will depend upon background and preference of the investigator.

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