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Antiviral Research 57 (2003) 41–52

Antiviral Research

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# A review of compounds exhibiting anti-orthopoxvirus activity in animal models

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Received 3 June 2002; accepted 6 December 2002

**Abstract**

Several animal models using mice (most frequently), rabbits, or monkeys have been used to identify compounds active against orthopoxvirus infections. The treatment of vaccinia virus infections has been well studied in models involving infection of scarified skin or eyes, or resulting from intravenous, intraperitoneal, intracerebral, or intranasal virus inoculation. Cowpox virus has been used in intranasal or aerosol infection studies to evaluate the treatment of lethal respiratory infections. Rabbitpox, monkeypox, and variola viruses have been employed to a lesser extent than the other viruses in chemotherapy experiments. A review of the literature over the past 50 years has identified a number of compounds effective in treating one or more of these infections, which include thiosemicarbazones, nucleoside and nucleotide analogs, interferon, interferon inducers, and other unrelated compounds. Substances that appear to have the greatest potential as anti-orthopoxvirus agents are the acyclic nucleotides, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (cidofovir, HPMPC) and 1-[((*S*)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl]cytosine (cyclic HPMPC), and the acyclic nucleoside analog, 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine (S2242). Other classes of compounds that have not been sufficiently studied in lethal infection models and deserve further consideration are thiosemicarbazones related to methisazone, and analogs of adenosine-$N^1$-oxide and 1-(benzyloxy)adenosine.


*Keywords:* Smallpox; Bioterrorism; Vaccinia virus; Cowpox virus; Monkeypox virus; Nucleoside; Thiosemicarbazone; Cidofovir

## 1. Introduction

Because of the increasing concern over the use of either variola (smallpox) or monkeypox virus in biowarfare or bioterrorism (Breman and Henderson, 1998; Hooper, 1998; Orent, 1998; Peters, 2002), efforts are being made to identify antiviral agents that will be effective in treating these diseases. This is being done in conjunction with the scale-up of conventional smallpox vaccines using live attenuated vaccinia virus. Smallpox vaccination was discontinued in the early 1980s, thus most individuals have never been vaccinated, and those who were vaccinated over 20 years ago presumably have lost their immunity.

In dealing with the threat of smallpox, there are two major concerns: the prophylaxis and treatment of smallpox itself, and the problem of progressive vaccinia as a complication of vaccination in immunodeficient individuals. These are very different types of illnesses, and it is clear that different animal models may be useful to test potential therapies. A brief discussion will be given of the available (and potentially available) animal models used to evaluate anti-orthopoxvirus agents. The main emphasis of this article will be directed toward lethal infections models that may be applicable to variola and monkeypox virus infections in humans. Lesser attention will be given to models of orthopoxvirus infections in immunodeficient animals that may mimic progressive vaccinia disease in humans.

Over the past 50 years, a number of antiviral substances have been evaluated for efficacy against orthopoxviruses in the various animal models. In this review, compounds that were found to be effective against these infections are presented. This represents a more comprehensive list than what was recently published (De Clercq, 2001). We have excluded reports of other compounds that were tested but found to be inactive.

## 2. Human therapy of smallpox and other orthopoxvirus infections

Smallpox was eradicated at the time when antiviral chemotherapy was in its infancy. Compounds showing

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0166-3542/02/$ – see front matter 
PII: S0166-3542(02)00199-7

efficacy in cell culture and in animal models lacked sufficient potency or safety to be effective in treating humans. The first compound to be extensively studied in humans infected with variola virus was methisazone (Marboran®, 1-methylisatin 3-thiosemicarbazone). This compound was found to have no therapeutic benefit against the infection (Rao et al., 1966a), but initially was considered to have a marked prophylactic effect in treating household members and other contacts of smallpox victims. This was determined in field trials conducted in India (Bauer et al., 1963, 1969; Bauer, 1965; Rao et al., 1966b) and in Brazil (Do Valle et al., 1965) using orally-administered drug. In those studies, the numbers of contacts going on to develop smallpox and the numbers of deaths were compared between treated and untreated groups. The studies took into account vaccination status at the time of enrollment. Later, field trials conducted in Pakistan indicated no statistically significant prophylactic effect of methisazone treatment (Heiner et al., 1971). Heiner et al. pointed out that in all trials there was a reduction in attack rate by methisazone treatment, but also a substantial number of drug failures in each study (individuals who developed smallpox in spite of treatment). In addition, methisazone did not have an impact on the severity of illness in those individuals contracting the disease. A related thiosemicarbazone, 4-bromo-3-methylisothiazole-5-carboxaldehyde thiosemicarbazone (M&B 7714) was found to be essentially inactive in both therapeutic and prophylactic field trials conducted in India (Rao et al., 1965, 1966a,b). Both methisazone and M&B 7714 induced nausea and vomiting in 10–25% of treated patients in the India and Pakistan trials and 66% in the Brazil study.

Cytosine arabinoside (ara-C) was originally thought to be effective in the treatment of smallpox (Hossain et al., 1972). However, in a subsequent controlled double-blind study conducted in Ethiopia, the drug did not alter the natural course of the disease when given intravenously for 4 days (Dennis et al., 1974). That study investigated the effect of drug treatment on progression, distribution, and density of rash in uncomplicated smallpox (no deaths occurred). A related nucleoside, adenine arabinoside (Ara-A) was judged to be ineffective against smallpox in a double-blind study conducted in Bangladesh (Koplan et al., 1975). Treatment with ara-A intravenously for 7 days failed to impact mortality, number of febrile days, or the ability to recover virus from lesions relative to placebo. However, there was a statistically significant 3-day shortening in the time to scab formation, suggesting moderate efficacy.

Other compounds developed for clinical use after the above early studies may have greater promise in treating smallpox infections. Of these, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (cidofovir, HPMPC) was found to be effective in the treatment of molluscum contagiosum (Meadows et al., 1997; Davies et al., 1999; Ibarra et al., 2000; Toro et al., 2000), a disease caused by another type of orthopoxvirus. Topical cidofovir was also shown to be active in the treatment of a severe skin lesion caused by orf virus (a parapoxvirus) in an immunodeficient patient (Geerinck et al., 2001). Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) combined with immune globulin arrested the spread of vaccinia virus in an immunocompromised individual (Kesson et al., 1997). Admittedly, these infections are less severe than variola or monkeypox, and predicting efficacy from these studies may not be prudent. Evaluating compounds for the treatment of severe lethal infections in animal models (Neyts and De Clercq, 1993; Bray et al., 2000; Smee et al., 2001a,b) offers some credibility of the usefulness of present day compounds against variola or monkeypox infections.

Since there is no clinically approved treatment for smallpox, monkeypox, or other orthopoxvirus infections, the need exists to identify one or more candidate drugs. Because orthopoxvirus infections in the general population are rare, there is little incentive for pharmaceutical companies to search for new inhibitors that may be effective. Thus, existing compounds, particularly those approved for clinical use in other indications, and related derivatives have provided most of the materials for present chemotherapy studies.

## 3. Orthopoxvirus animal models available for chemotherapy studies

Members of the orthopoxvirus family that have been discovered to date include buffalopox (a vaccinia subspecies), camelpox, cowpox, ectromelia (mousepox), monkeypox, rabbitpox (a vaccinia subspecies), raccoonpox, taterapox (gerbilpox), vaccinia, variola, volepox, and possibly the virus causing Uasin Gishu disease (African horsepox) (Francki et al., 1991). Of these, the viruses that have been used for chemotherapy experiments in mice (Tables 3–6) include cowpox, vaccinia, rabbitpox, and to a lesser extent variola. There are many different types of vaccinia strains that have been used in these animals, some of which cause only mild (non-lethal) infections and two others (IHD and WR strains) that cause lethal infections. Some experiments with vaccinia virus have also been conducted in rabbits and monkeys (Tables 1–2), as have fatal respiratory infections of rabbits with rabbitpox (Table 5). No published information is available for small animal model infections with buffalopox, camelpox, raccoonpox, taterapox, volepox, or African horsepox virus. Ectromelia virus causes severe or lethal infections in particular strains of mice and could be used as an infection model (Fenner and Buller, 1997). Because of the high potential of this virus to spread throughout an animal colony (Fenner, 1982), there has been a reluctance to use this animal model for chemotherapy experiments. The National Institute of Allergy and Infectious Diseases of the National Institutes of Health is presently supporting research in this area, however. Studies with monkeypox virus in monkeys (Table 5) have been restricted to laboratories at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) or at the Centers for Disease Control

Table 1
Compounds active in eye (keratitis) infection animal models of orthopoxvirus infection

| Animal model | Test compound | Effective treatment regimen | Reference |
|---|---|---|---|
| Vaccinia keratitis in rabbits | Poly I:C | Topical treatment with 50 or 200 μg per eye 2 or 8 times a day for 8, 12 or 14 days starting 24 h before or up to 1 h after infection | Chowchuvech et al., 1970; Moschini and Oh, 1972 |
| | Ribavirin | Topical treatment with 5% ointment hourly from 8 a.m. to 7 p.m. daily for 7 days starting 1 day after infection | Sidwell et al., 1973b |
| | Ara-A, ara-HxMP | Topical treatment with 0.02 or 0.2% ointment hourly from 8 a.m. to 7 p.m. or 7 days starting 1 day after infection | Sidwell et al., 1975 |
| | Ara-A, 5-iodo-dUrd | Topical treatment with 5% Ara-A or 0.1% 5-iodo-dUrd every 2 h from 7 a.m. to 11 p.m. starting 36 h after infection | Hyndiuk et al., 1976a |
| | Trifluorothymidine (TFT), 5-iodo-dUrd | Topical treatment with 0.5 or 5% TFT or 0.5% 5-iodo-dUrd every 4 h from 7 a.m. to 11 p.m. starting 36 h after infection | Hyndiuk et al., 1976b |
| Vaccinia keratitis in monkeys | Interferon | Topical treatment with 15–20,000 units per eye once or twice daily for 2–3 days starting 15 h before or simultaneously at the time of infection | Neumann-Haefelin et al., 1975 |

Table 2
Compounds active in skin lesion animal models of orthopoxvirus infection

| Animal model | Test compound | Effective treatment regimen[a] | Reference |
|---|---|---|---|
| Vaccinia skin lesions in rabbits | Polyacrylic acid | Single i.p. injections of 25 mg/kg 1 or 8 days before virus infection | De Clercq and De Somer, 1973 |
| | Ara-A | Daily i.p. injections of 150, 300 or 600 mg/kg/day for 5 days starting on the day of infection, or 300 or 600 mg/kg/day starting 1 day after infection | Klein et al., 1974 |
| | Ara-A | Topical treatment with 5 or 20% ointment twice daily for 15 days starting 4 h after infection. | Sloan, 1975 |
| | Phosphonoacetic acid | Topical treatment with 2% ointment applied twice daily for 4 days starting 1 day after infection | Friedman-Kien et al., 1976 |
| | Poly (ICLC) | Topical treatment with 0.17% ointment applied once daily for 5 days starting 8 h before or 3 days after infection | Levy and Lvovsky, 1978 |
| Vaccinia skin lesions in monkeys | Interferon | Daily i.m. or i.v. injections of 500,000 units/kg from days −1 to +7 of the infection | Weimar et al., 1980 |

[a] i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous.

and Prevention (CDC) due to biosecurity reasons. Such experiments may also be carried out in some specialized laboratories in other countries, such as at Vector in Novosibirsk, Russia. Besides being lethal to certain primate species, monkeypox virus will also cause disease and death in cotton rats under certain conditions (Shelukhina and Shenkman, 1998). In recent studies at the CDC, variola virus was found to infect and cause a fatal disease in monkeys when administered by intravenous (i.v.) injection (Anonymous, 2001). This new model in under intense investigation.

## 4. Activities of compounds in non-lethal orthopoxvirus infections of the eyes

Several antiviral substances have been tested in a vaccinia keratitis model in rabbits (Table 1). In this model, the cornea is scarified with a needle and virus is applied to the eye. Lesions spread slowly and are limited to the vicinity of the inoculated area (Darrell and Vrabec, 1971). Compounds found to be effective by topical application in this model include polyinosinic-polycytidylic acid (poly I:C, an interferon inducer), ribavirin, ara-A, 9-β-D-arabinofuranosylhypoxanthine 5′-monophosphate (ara-HxMP), trifluorothymidine (TFT), and 5-iodo-2′-deoxyuridine (5-iodo-dUrd). Poly I:C had no therapeutic benefit against the infection, but was effective if given before (or 1 h after) virus exposure. Compounds showing marked inhibition of keratitis when treatment was started after infection included ribavirin, ara-A, and TFT, each used at a 5% concentration. 5-Iodo-dUrd was less effective than the other nucleosides, but was only used at a maximum concentration of 0.5% (as the commercially available product). In a single report interferon was used to treat vaccinia keratitis in monkeys (Table 1). Treatment starting 15 h before virus challenge was effective against the infection.

## 5. Activities of compounds in non-lethal orthopoxvirus infections of the skin

Vaccinia virus skin lesions can be induced in rabbits (Klein et al., 1974) and monkeys (Weimar et al., 1980) similar to human vaccinations (Table 2). Ara-A was effective in

Table 3
Compounds active in tail lesion animal models of orthopoxvirus infection

| Animal model | Test compound | Effective treatment regimen[a] | Reference |
|---|---|---|---|
| Vaccinia tail lesions in mice induced by pricking the tail with an infected needle | Isothiazole thiosemicarbazone | Repeated s.c. doses of 100 mg/kg/day for 4 days starting 1 h after infection | Rao et al., 1965 |
| Vaccinia tail lesions in mice following i.v. injection of vaccinia virus | Thiosemicarbazones, amantadine, 5-Iodo-dUrd, cephalosporin C, caprochlorone | Repeated s.c. doses for 7 days, methisazone active at 6.25–400 mg/kg/day; others active at 50–100 mg/kg/day. Treatment started 24 h after infection | Boyle et al., 1967 |
| | Interferon | Five repeated i.p. doses of 10,000 or 100,000 units per mouse within 24 h before infection | De Clercq and De Somer, 1968; Tignor et al., 1992 |
| | Polyacrylic acid | Single i.p. doses of 33 mg/kg from 4 weeks to 1 day before infection | De Clercq and De Somer, 1968 |
| | 2′- and 3′-C-methyladenosine, 3′-C-methylcytidine, methisazone | Two i.p. doses (50–200 mg/kg) 3 h before and 18 h after infection | Walton et al., 1969 |
| | Rifampicin | Single p.o. dose of 250 mg/kg 24 or 6 h before infection, or repeated 250 mg/kg/day doses for 5 days starting 1 day before infection | Mückter et al., 1971; Tignor et al., 1992 |
| | Ara-C, ribavirin, 5-iodo-dUrd, 5-ethyl-dUrd, 5-thiocyano-dUrd | Repeated i.p. doses of 4, 20, or 100 mg/kg/day for 4 days starting immediately after infection | De Clercq et al., 1976 |
| | Thiosemicarbazones | Repeated s.c. doses of 10, 20, or 40 mg/kg/day for 6 days starting 3 h after infection | Walter et al., 1981 |
| | Carbocyclic 3-deazadenosine | Repeated i.p. doses of 250 mg/kg/day for 4 days starting immediately after infection | De Clercq et al., 1984 |
| | Ofloxacin | Single p.o. treatments of 0.1, 1, or 10 mg/kg at the time of infection, or repeated p.o. treatments with the same doses on days 0–4 of the infection | Ikeda et al., 1987 |
| | HPMPA | Repeated i.p. or s.c. doses of 5, 10, 20, 50, or 100 mg/kg per day for 5 days starting immediately after infection | De Clercq et al., 1989 |
| | 3-Deazaneplanocin A ($c^3$-NpA), ara-A | Repeated s.c. doses of 4 or 8 mg/kg/day ($c^3$-NpA) or 300 mg/kg/day (Ara-A) daily for 7 days starting 1 day before infection | Tseng et al., 1989 |
| | 3′-Fluoro-3′-deoxyadenosine | Repeated i.p. dose of 100 mg/kg/day twice-daily for 5 days starting 1 h before virus infection | Van Aerschot et al., 1989 |
| | EICAR, ribavirin | Repeated i.p. doses of 10 or 25 mg/kg given daily for 6 days starting immediately after infection | De Clercq et al., 1991 |
| | Adenosine-$N^1$-oxide, Ara-A, ribavirin, and 5′-deoxyadenosine dialdehyde | Repeated i.p. doses given daily for 6 days starting 1 day before infection. Doses (mg/kg/day) were not reported | Tignor et al., 1992 |
| | Quinolinamine | Repeated i.p. doses of 10 or 300 mg/kg/day given daily for 6 days starting 1 day before infection | Tignor et al., 1992 |
| | Ampligen and poly ICLC | Repeated i.p. doses of 25–100 μg (ampligen) or 20 μg (poly ICLC) given daily for 6 days starting 1 day before infection | Tignor et al., 1992 |
| | Analogs of adenosine-$N^1$-oxide and 1-(benzyloxy)adenosine | Repeated i.p. doses given daily for 7 days starting 1 day before infection. Doses (mg/kg/day) were not reported | Kwong et al., 1998 |
| | Interleukin-18 | Three i.p. doses (1 or 5 μg/injection) given on days 0, 2 and 4 after infection | Tanaka-Kataoka et al., 1999 |
| | H961 (or HOE961, the diacetate ester prodrug of S2242) | Repeated p.o. or s.c. doses of 100 mg/kg/day for 10 days, starting immediately after infection | Neyts and De Clercq, 2001 |
| Vaccinia tail lesions in immunocompromised (SCID) mice following i.v. injection of vaccinia virus (mice later die from the infection) | Cidofovir | Single s.c. dose of 100 mg/kg given 7 days or 1 day before infection, or 20 mg/kg given 1 day before infection | Neyts and De Clercq, 1993 |

[a] i.p., intraperitoneal; i.v., intravenous; p.o., oral; s.c., subcutaneous.

Table 4
Compounds active in lethal encephalitis animal models of orthopoxvirus infection

| Animal model | Test compound | Effective treatment regimen[a] | Reference |
|---|---|---|---|
| Intracerebral (i.c.) inoculation of mice with rabbitpox virus | Isatin β-thiosemicarbazone | Repeated s.c. doses of 2 mg/mouse twice daily for 5 days starting 4 h after infection | Bauer and Sheffield, 1959 |
| | Thiosemicarbazones | Repeated i.p. doses of 31-250 mg/kg/day either once or twice daily for 9 days starting 1 day before infection | Sidwell et al., 1968 |
| Intracerebral inoculation of mice with vaccinia virus | Benzaldehyde thiosemicarbazones | Repeated p.o. treatments of 0.01–0.1% of the diet starting 2 days before infection. Treatment duration not indicated | Thompson et al., 1951a |
| | Dichlorophenoxythiouracil | Repeated p.o. treatments of 0.75% of the diet for 10 days starting on the day of infection | Thompson et al., 1951b |
| | Thiosemicarbazones | Repeated p.o. treatments of 0.04–0.12% of the diet for 10 days starting 1 day before infection; or daily i.p. injections of 2.5, 5, or 10 mg/mouse for 1–3 days starting on day 0 or day 1 of the infection | Minton et al., 1953 |
| | Netropsin | Repeated i.p. treatments of 70 mg/kg/day twice daily for 5 days starting 30–60 min after infection | Schabel et al., 1953 |
| | Thiosemicarbazones | Repeated p.o. treatments of 0.015–0.2% of the diet. Starting day and treatment duration not indicated | Thompson et al., 1953a |
| | Thiosemicarbazones | Repeated p.o. treatments of 0.04–0.16% of the diet for 9–13 days starting 2 days before infection | Thompson et al., 1953b |
| | Isatin β-thiosemicarbazone | Repeated s.c. injections of 1.25 or 2.5 mg/mouse twice daily for 5 days starting 2 h after infection | Bauer, 1955 |
| | 4-Bromo-3-methylisothiazole-5-carboxaldehyde thiosemicarbazone | Repeated p.o. doses of 120, 250, or 500 mg/kg/day once daily for 4 days starting on the day of infection; or 1000 mg/kg/day starting 1–2 days after infection | Slack et al., 1964 |
| | Isothiazole thiosemicarbazone | Repeated s.c. injections of 125, 250, or 500 mg/kg/day once daily for 4 days starting 1 h after infection, or up to 3 days after infection | Rao et al., 1965 |
| | *N*-Ethylisatin β-thiosemicarbazone | Single s.c. injection of 1, 2, or 4 mg/mouse given 2 h after infection | Lieberman et al., 1966, 1967 |
| | Methisazone | Repeated s.c. doses of 6.25–400 mg/kg/day once daily for 7 days starting 24 h after infection | Boyle et al., 1967 |
| | Ara-A, thiosemicarbazones | Repeated i.p. doses of 31–250 mg/kg/day either once or twice daily for 9 days starting 1 day before infection | Sidwell et al., 1968 |
| | Ara-A, thiosemicarbazone | Repeated i.p. doses of 62–1000 (Ara-A) or 31–125 (thiosemicarbazone) mg/kg/day, or p.o. doses of Ara-A at 500–1000 mg/kg/day, once daily for 7 or 9 days starting 1 day after infection | Dixon et al., 1969 |
| | Ara-AMP, cyclic ara-AMP | Single i.c. treatment with 2.5 or 40 mg/kg at 6 h after infection | Sidwell et al., 1973a |
| | Ara-HxMP | Single i.c. treatment with 40 mg/kg at 6 h after infection | Allen et al., 1975 |
| | Thiosemicarbazones | Repeated s.c. doses of 5, 10, or 20 mg/kg/day twice daily for 5.5 days starting 3 h after infection | Zgórniak-Nowosielska et al., 1976 |
| | Thiosemicarbazones | Repeated s.c. doses of 1 mmole/kg once daily for 5 days starting 3 h after infection | Veckenstedt and Zgórniak-Nowosielska, 1979 |
| | Hetarylhydrazone | Repeated s.c. doses of 1 mmole/kg once daily for 5 days starting 3 h after infection | Veckenstedt et al., 1979 |
| Intracerebral inoculation of 2–6-day-old mice with variola virus | *N*-Ethylisatin β-thiosemicarbazone | Repeated s.c. doses of 1.25–25 mg/kg/day once daily for 5 days starting at the time of infection or 6 h after infection | Bauer and Sadler, 1960 |
| | Isothiazole thiosemicarbazone | Repeated p.o. doses of 0.125 or 1 mg/mouse once daily for 10 days starting 1 h after infection | Rao et al., 1965 |

[a] i.c., intracerebral; i.p., intraperitoneal; p.o., oral; s.c., subcutaneous.

preventing lesion development in rabbits either by topical or systemic route of administration. Phosphonoacetic acid was also active in the same model by topical treatment. The immunostimulating agents (either through interferon induction or other mode of action) polyacrylic acid and poly (ICLC) and were active against vaccinia skin lesions in rabbits by intraperitoneal and topical routes, respectively. Human interferon was effective in preventing lesion development in monkeys when treatments were started prior to virus infection (Table 2).

## 6. Activities of compounds in non-lethal orthopoxvirus infections of the tail

Dermal lesions have been induced in mice by applying vaccinia virus to the tail then pricking the virus-exposed part of the tail several times with a needle (Salaman and Tomlinson, 1957). This results in a lesion developing at and spreading from the wound site. In a single-reported study using this model, an isothiazole thiosemicarbazone completely prevented lesion development (Table 3). A more commonly used dermal tail lesion model in mice is the i.v. injection of vaccinia virus in the tail vein (Boyle et al., 1967), which causes dermal lesions to develop up and down the tail. Ampligen, poly ICLC, murine interferon, interleukin-18, polyacrylic acid, quinolinamine, and rifampicin were effective if treatments started before or very early in the infection (Table 3). Several nucleoside analogs and thiosemicarbazones were active when given parentally over several days starting a day before or within a day after infection. Methisazone was active in suppressing tail lesion formation over a wide range of doses, as was (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA). H961 or HOE961, a prodrug of 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl] purine [S2242] was found to be orally active in the model. Other substances (amantadine, caprochlorone, cephalosporin C, and ofloxacin) were only moderately effective. Vaccinia tail lesions have also been induced in severe combined immunodeficient (SCID) mice (Neyts and De Clercq, 1993) (Table 3). Cidofovir was very effective in preventing tail lesion development. All mice died, however, from generalized viral infections due to their immunocompromised status. This is because drug therapy alone cannot completely clear virus from SCID mice.

## 7. Activities of compounds in lethal orthopoxvirus encephalitis infections

The early chemotherapy studies involving lethal infections of mice with rabbitpox or vaccinia virus used encephalitis models resulting from i.c. virus inoculation (Thompson et al., 1951a,b; Bauer and Sheffield, 1959) (Table 4). Certain thiosemicarbazones showed efficacy against rabbitpox in this model. Thiosemicarbazones, ara-A and derivatives, netropsin, dichlorophenoxythiouracil, and a hetarylhydrazone compound were reported active in the neurovaccinia virus model under a variety of treatment conditions. Methisazone was effective over a broad range of doses as low as 6.25 mg/kg/day, whereas ara-A required 10-fold and higher doses for efficacy. Baby mice less than 7 days old can be lethally infected i.c. with variola minor (alastrim) virus (Bauer and Sadler, 1960). Two reports indicated that thiosemicarbazones were active in preventing death in this model (Table 4).

## 8. Activities of compounds in lethal orthopoxvirus respiratory infections

Mice can be lethally infected with cowpox virus when infected by the i.n. route (Bray et al., 2000; Martinez et al., 2000) (Table 5). Cidofovir was highly protective in this model with just a single parenteral treatment administered as early as 16 days before infection or up to 4 days after infection. Treatment with ribavirin was effective in moderately severe infections to prevent mortality, and was effective in severe infections when followed by single injections of cidofovir. S2242 and its prodrug (HOE961) were both active against respiratory cowpox virus infections, but were not as potent as cidofovir and required daily administration.

Cowpox virus is also lethal to mice by aerosol infection route (Bray et al., 2000, 2002) (Table 5). Aerosolized or subcutaneous (s.c.) injections with either cidofovir or its cyclic prodrug, 1-[((*S*)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl]cytosine (cyclic HPMPC), were both protective against the infection. Cidofovir was more active on a mg/kg basis than cyclic HPMPC, however.

Ectromelia virus is lethal to susceptible strains of mice by multiple injection routes, including i.n. instillation (Fenner and Buller, 1997). Chemotherapy studies with this virus are in progress (M.L. Buller, unpublished).

Monkeys infected with aerosolized monkeypox virus at a sufficiently high dose die from infection (Zaucha et al., 2001). This model has been used to demonstrate the efficacy of i.v. cidofovir in preventing mortality and decreasing lung infection parameters (Table 5).

Rabbitpox virus causes a generalized infection in rabbits when inoculated by the i.n. route (Westwood et al., 1966). Rao et al. (1965) reported that an isothiazole thiosemicarbazone protected rabbits from this otherwise fatal infection (Table 5).

The WR and IHD strains of vaccinia virus can cause lethal infections in mice when given i.n. (Nelson, 1938; Turner, 1967), whereas other strains are attenuated. Curiously, the mice are not prone to die following i.p. injection with the WR and IHD strains, except at high virus challenge doses (Ramirez et al., 2002). Chemotherapy studies using the intranasal vaccinia model were done in the early 1950s (Table 5). More detailed antiviral studies were published

Table 5
Compounds active in lethal orthopoxvirus respiratory infection animal models

| Animal model | Test compound | Effective treatment regimen[a] | Reference |
|---|---|---|---|
| Intranasal (i.n.) or aerosolized cowpox virus infection in mice | Cidofovir | Single s.c. dose of 100 mg/kg up to 16 days before or as late as 4 days after infection | Bray et al., 2000; Smee et al., 2002b |
| | Cidofovir | Single i.n. dose of 2.5–40 mg/kg at 24 h after infection | Smee et al., 2000b |
| | Ribavirin followed by cidofovir | Repeated s.c. injections of ribavirin (100 mg/kg/day) on days 1–5 after infection, followed by single s.c. injection of cidofovir (75 mg/kg) on day 6 or 7 after infection | Smee et al., 2000a |
| | Cidofovir, cyclic HPMPC | Single aerosol treatment of 1–5 mg/kg, or single s.c. treatments with 10–100 mg/kg cidofovir or 75–100 mg/kg cyclic HPMPC at 1 day before infection or 2 h after infection | Bray et al., 2002 |
| | S2242 and HOE961 (or H961, the diacetate ester prodrug of S2242) | Repeated i.p. treatments once or twice daily with 100 mg/kg/day for 5 days, or oral treatment with HOE961 (100 mg/kg/day) once daily for 10 days, starting 1 day after infection | Smee et al., 2002a |
| Aerosolized monkeypox virus infection in monkeys | Cidofovir | Single i.v. treatment of 5 mg/kg on the day of infection | Huggins et al., 1998 |
| Intranasal rabbitpox virus infection in rabbits | Isothiazole thiosemicarbazone | Repeated p.o. treatments of 100 or 200 mg/kg/day for 4 days starting 1 h after infection | Rao et al., 1965 |
| Intranasal vaccinia virus infection in mice | Thiosemicarbazones | Repeated p.o. (in the diet) treatments of 5–45 (unsubstituted derivative) or 700–1450 ($N^4$-isobutyl derivative) mg/kg/day for 13 days starting 2 days before infection | Hamre et al., 1951 |
| | Dichlorophenoxythiouracil | Repeated p.o. treatments of 0.75% of the diet for 15 days starting 2 days before infection | Thompson et al., 1951b |

[a] i.n., intranasal; i.p., intraperitoneal; i.v., intravenous; p.o., oral; s.c., subcutaneous.

50 years later using the WR strain (Smee et al., 2001a,b). Single injections with cidofovir given a day after infection were highly protective in preventing mortality and in reducing virus titers in many tissues in this model.

## 9. Activities of compounds in lethal systemic orthopoxvirus infections

Systemic infections can be induced in mice with ectromelia virus (by footpad or i.p. injection route), and with i.p. cowpox virus or vaccinia (WR strain) virus (at high virus challenge doses). Ectromelia virus replication occurs at high levels in the liver and spleen (Fenner and Buller, 1997). Chemothapy studies with this virus have not been published, except for a report of the lack of efficacy of a thiosemicarbazone (Rao et al., 1965). Cowpox virus is as virulent by i.p. route as it is by i.n. instillation (D.F. Smee, unpublished), resulting in less virus replication in the lungs than in the liver and spleen compared to the i.n. infection. No chemotherapy studies have been published in this model. In a single-reported study, 10 μg of a monoclonal antibody directed against the intracellular mature form of vaccinia virus was administered i.p. 1 day before virus exposure. This treatment completely prevented death of the mice, whereas the same antibody given a day after infection was not protective (Ramirez et al., 2002).

## 10. Activities of compounds in lethal models of orthopoxvirus infection in immunosuppressed mice

Immune-deficient mice are subject to lethal infections with cowpox (Bray et al., 2000) or vaccinia virus (Worthington and Conliffe, 1977; Neyts and De Clercq, 1993) (Table 6). Mice immunosuppressed by anti-thymocyte serum and infected with vaccinia virus were effectively treated with high doses of ara-A. The treated animals survived the infection, probably because the mice eventually recovered their immunity. Severe combined immunodeficient (SCID) mice infected with either cowpox or vaccinia viruses had a prolongation of life due to antiviral treatment with cidofovir, H961 (S2242 prodrug) or 5-iodo-dUrd, but eventually died from the infection. Cessation of antiviral treatment rapidly leads to virus rebound in SCID mice.

## 11. Relevance of the animal models to predicting efficacy against variola or monkeypox infection in humans

Because of the severe nature of variola and monkeypox virus infections in humans, animal models should be equally severe in order to test the treatment potential of new agents. For this reason, the non-lethal animal infection models (Tables 1–3) probably are not relevant in predicting

Table 6
Compounds active in lethal orthopoxvirus infection models in immunocompromised mice

| Animal model | Test compound | Effective treatment regimen[a] | Reference |
|---|---|---|---|
| Intravenous (i.v.) infection of immunosuppressed (by anti-thymocyte treatment) mice with vaccinia virus | Ara-A | Repeated i.p. doses of 250, 500, or 1000 mg/kg/day once daily for 5 days starting 1, 3, or 5 days after infection | Worthington and Conliffe, 1977 |
| Intranasal (i.n.) infection of immunocompromised (SCID) mice with cowpox virus | Cidofovir (HPMPC) | Repeated s.c. doses of 100 mg/kg once daily every 3 or 6 days starting immediately after infection | Bray et al., 2000; Smee et al., 2002b |
| Infection of immunocompromised (SCID) mice with vaccinia virus by i.n., i.p., or i.v. routes | Cidofovir | Single s.c. dose of 100 mg/kg 7 days or 1 day before infection, or repeated s.c. doses of 1, 5, or 20 mg/kg/day, once daily for 5 days starting 2 h after infection | Neyts and De Clercq, 1993 |
| | H961 (or HOE961, the diacetate ester prodrug of S2242) | Repeated p.o. or s.c. doses of 100 mg/kg/day for 10 days, starting immediately after infection | Neyts and De Clercq, 2001 |
| | 5-Iodo-dUrd | Repeated s.c. dose of 150 mg/kg/day for 5 days with 2 days off, then 75 mg/kg/day for 5 days, starting 2 h after infection | Neyts and De Clercq, 2001 |

[a] i.n., intranasal; i.p., intraperitoneal; i.v., intravenous; p.o., oral; s.c., subcutaneous.

efficacies of compounds against variola and monkeypox virus infections in humans. This premise is supported by the reported failure of some drugs such as ara-C (found active in non-lethal animal infection models) against smallpox infections in man, as reviewed earlier.

Some of the lethal infection models may be more relevant than others for predicting anti-variola or anti-monkeypox virus activity in humans. Injection of rabbitpox, vaccinia, or variola virus by i.c. route was conducted for many years as a means of inducing lethal infections. This was done even though reports at the time indicated that the less traumatic i.n. vaccinia infection route would also induce fatal disease and could be used for antiviral testing (Hamre et al., 1951; Thompson et al., 1951b). The last reported use of the i.c. infection model was in 1979 (Veckenstedt et al., 1979). Systemic models using ectromelia, cowpox, or vaccinia (WR strain) viruses may be useful for antiviral evaluations. However, the i.n. models of orthopoxvirus infection are more relevant for studying the treatment of lethal orthopoxvirus infections in mice than the systemic or i.c. infection models. This is because the virus inoculation route and nature of the infection more closely approximate human smallpox disease acquired in a biowarfare/bioterrorism scenario. Intranasal administration of rabbitpox to rabbits produces fatal infections, which could also be utilized for efficacy testing of compounds. Unfortunately, the rabbit model requires greater quantities of compounds for evaluation, more space for housing, more costs for animal purchases, and more feed than mouse models. Rabbit-to-rabbit transmission of the infection throughout an animal facility is also a potential problem.

Aerosol exposure of mice to cowpox virus is another severe infection model, whereas vaccinia virus has not yet been reported to be aerosolized into mice. Aerosolization is a less efficient infection route than i.n. delivery, requiring a larger amount of virus to infect and special methods for biocontainment of the aerosol. Thus, it is used only by specialized facilities such as USAMRIID. The monkeypox virus infection model in monkeys is available to a limited number of investigators (at USAMRIID, CDC, and Vector). This is perhaps the most definitive but most expensive test system for evaluating compounds with potential efficacy against lethal variola or monkeypox virus infections in humans. The monkey model of variola virus infection will require further study to validate its utility and relevance to human smallpox.

Because variola or monkeypox virus may most likely be disseminated via an aerosol by a bioterrorist, animal models using the same or an i.n. infection route may be preferred over other routes of infection. The exception is variola in monkeys, which requires i.v. inoculation to cause symptoms and death similar to smallpox (Anonymous, 2001). Infection of monkeys with variola virus by aerosol route leads to non-lethal infections (LeDuc and Jahrling, 2001).

Studies of the treatment of orthopoxvirus infections in immunosuppressed mice should be useful in determining efficacies of compounds targeted toward progressive vaccinia in immunodeficient humans as a complication of vaccination. However, there are limitations in using these models to extrapolate the information to the treatment of lethal infections in normal animals. The measured parameter of efficacy in the SCID mouse infection model is delay in the mean day of death, rather than survival from the infection. One concern with this model is that drug effects on the immune system cannot be assessed as they can in normal mice. Compounds may be antiviral, yet exert an immunosuppressive effect that will not allow normal mice to clear the virus infection (thus, they will ultimately die from the

infection). Such compounds may show a beneficial effect in delaying the time to death in SCID mice. For example, we have tested 5-iodo-dUrd in the cowpox virus respiratory infection model in normal mice and found it to be inactive (D.F. Smee and R.W. Sidwell, unpublished), even though the compound prolonged the lives of SCID mice infected with vaccinia virus (Neyts et al., 2002). In another example, treatment with ribavirin (known to be immunosuppressive; Jolley and Hinshaw, 1980) at 100 mg/kg/day delayed the time to death by 5 days in mice infected with a relatively high cowpox virus challenge dose, yet the animals died anyway from the infection (Smee et al., 2000a).

## 12. Future directions for studying antiviral agents in animal models

Many of the compounds that appear in Tables 1–6 have already been tested in lethal respiratory infection models in mice using either cowpox or vaccinia virus. Data regarding the less active and inactive compounds have yet to be published, however. Certain classes of compounds have not been studied to date due to limited amount of compound available for testing. They include thiosemicarbazones and analogs of adenosine-$N^1$-oxide and 1-(benzyloxy)adenosine. Methisazone showed relatively good potency in the i.c. infection model (Boyle et al., 1967) yet failed in some of the human trials of smallpox infection (Rao et al., 1966a; Heiner et al., 1971). Because of these disappointing results, methisazone lost favor as an anti-smallpox drug. In order to correlate the utility of the current lethal infection models in mice with results from the former smallpox trials, it will be important to know how compounds such as methisazone and related thiosemicarbazones perform in these models. It may be possible to better optimize effective treatment regimens with these and other antiviral agents using the lethal respiratory infection models, or at least understand the limitations of the compounds.

Ribavirin showed limited efficacy in the lethal cowpox virus infection model (Smee et al., 2000a) and was active in combination with vaccinia immune globulin in treating a progressive vaccinia virus infection in an immunocompromised individual (Kesson et al., 1997). Its activity in other severe infection models are under investigation, including effects against vaccinia virus (IHD strain) in mice (D.F. Smee, unpublished) and against monkeypox virus in monkeys (J.W. Huggins, USAMRIID, unpublished).

Cidofovir is clearly the most potent and potentially useful anti-orthopoxvirus agent discovered to date (De Clercq, 2002). It is an attractive drug candidate because it is already approved for human use in treating cytomegalovirus retinitis. Because cidofovir is only effective by i.v. infusion (Wachsman et al., 1996), orally active prodrugs of cidofovir should be developed and tested for efficacy in orthopoxvirus animal models. Recently, novel alkoxyalkyl ester prodrugs of cidofovir have been reported which exhibit enhanced antiviral potency in cell culture (Kern et al., 2002) and do have oral bioavailability (Huggins et al., 2002; Winegarden et al., 2002). These types of compounds will undoubtedly be evaluated in greater detail for efficacy against orthopoxviruses.

S2242 is perhaps the next most potent agent after cidofovir that has been evaluated in lethal poxvirus infections, both in normal and in immunodeficient mice. More studies with S2242 (and prodrug HOE961) could be done to better understand the potential utility of this compound.

Limited drug combination studies have been conducted to date in lethal mouse infection models (Smee et al., 2000a); more of these additional experiments using two or more active agents could be considered. These and investigations involving single agents in mice should lead to future efficacy studies of the most potent compounds in the monkeypox (or variola) model in monkeys, prior to being considered for clinical development.

## Acknowledgements

This work was supported by Contract N01-AI-15435 from the Virology Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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