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Treatment of lethal cowpox virus respiratory infections in mice with 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine and its orally active diacetate ester prodrug

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Abstract

The acyclic purine nucleoside analog, 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine (S2242) and its orally active diacetate ester prodrug (HOE961) were reported to be potent inhibitors of vaccinia virus replication in cell culture and in infected mice. These compounds were evaluated further, using infections with the related cowpox virus. Against a wild-type (WT) cowpox virus strain in mouse C127I cell culture, 50% effective concentrations (EC_{50}, determined by plaque reduction assays) of S2242 and cidofovir (a positive control) were 3.5 and 1.0 μM, respectively. EC_{50} values obtained against a cidofovir-resistant strain of the virus were 33 and 230 μM, respectively. Compounds were at least ten-fold less potent against WT virus in Vero cells than C127I cells. S2242 and cidofovir were 50% inhibitory to the proliferation of uninfected C127I cells at 340 and 180 μM, respectively, but neither compound inhibited Vero cell growth at 1000 μM. Mice were lethally infected with cowpox virus by intranasal inoculation, followed 24 h later by antiviral treatment for 5 consecutive days. Once or twice daily intraperitoneal (i.p.) treatments with either S2242 or HOE961 at 100 mg/kg per day resulted in ≥ 70 survival compared with no survivors in the placebo group. Lower doses of these compounds (10 and 30 mg/kg per day) were not protective, however. Cidofovir was 100% protective at 30 mg/kg per day. A 10-day course of treatment gave comparable survival results and demonstrated the oral efficacy of HOE961. Treatments with S2242 (100 mg/kg per day) and cidofovir (30 mg/kg per day) each reduced lung and nasal virus titers by approximately ten-fold, whereas, HOE961 (100 mg/kg per day) was less active. Overall, S2242 and HOE961 were found to be effective against cowpox virus infections in mice but were less potent than cidofovir. Since, HOE961 was orally active, it may have advantages over the other parenterally administered compounds for treating orthopoxvirus infections.

Keywords: Antiviral; Cowpox virus; Orthopox virus; Acyclic purine; Cidofovir

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1. Introduction

Antiviral compounds are being sought for the treatment of orthopoxvirus (smallpox, monkeypox) infections that may be acquired as a result of biowarfare/bioterrorist activities (Breman and Henderson, 1998; Hooper, 1998; Orent, 1998), for treating natural infections such as molluscum contagiosum (Meadows et al., 1997; Davies et al., 1999; Ibarra et al., 2000; Toro et al., 2000), or for therapy of vaccinia infections in immunosuppressed individuals (Kesson et al., 1997). Treatments targeted against parapoxviruses would also be desirable (Nettleton et al., 2000). A number of compounds have been identified over the years that are effective against orthopoxviruses (as reviewed by De Clercq, 2001). Of those that have been more extensively studied to date, cidofovir is the most potent and promising in animal models (Neyts and De Clercq, 1993; Bray et al., 2000; Smee et al., 2000b). Ribavirin has been found to be weaker than cidofovir in its activity (Smee et al., 2000a) but potentially useful in certain human infections (Kesson et al., 1997). In a preliminary report, 5-iodo-2′-deoxyuridine was effective in treating vaccinia virus infections in mice (Neyts et al., 2001). The diacetate ester prodrug (compound HOE961) of 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine (compound S2242) was also reported to be active against vaccinia virus infections in mice (Neyts and De Clercq, 2001).

Beside its efficacy against vaccinia virus infections, S2242 (or HOE961) is a potent and selective inhibitor of herpesviruses (human cytomegalovirus, herpes simplex types 1 and 2, and varicella-zoster virus) (Neyts et al., 1994, 1995). Thus, the potential use of either of these compounds for treatment of several different indications makes them desirable candidates for further evaluation. The orally active prodrug, HOE961, provides a more convenient treatment option than compounds such as cidofovir that require intravenous administration (Wachsman et al., 1996).

HOE961 was previously tested in a non-lethal tail pox model in normal mice and in a lethal infection in severe combined immunodeficient mice (Neyts and De Clercq, 2001). In the latter model the time to death could be extended by treatment, but the animals eventually died from the infection. This result was to be expected, since, SCID mice do not possess an immune system necessary for eradication of the virus. S2242 was not evaluated in those studies. We were interested in using the lethal cowpox virus respiratory infection model in normal mice to determine how effective S2242 and HOE961 would be in relation to cidofovir. This model may be more applicable to human infections acquired through biowarfare or bioterrorism than those reported by Neyts and De Clercq (2001). The results presented here indicate that all three compounds were effective, but that cidofovir was more potent in its action.

2. Materials and methods

2.1. Antiviral compounds

S2242 and HOE961 were kindly provided by Juergen Puenter of Aventis Pharma (Frankfurt, Germany). Cidofovir, a positive control for the studies, was from Norbert Bischofberger, Gilead Sciences (Foster City, CA). The compounds were dissolved in cell culture medium for in vitro studies, or in saline or water for intraperitoneal (i.p.) or oral treatments of mice.

2.2. Virus and cells

Cowpox virus (Brighton strain), both wild-type (WT) and cidofovir-resistant (R) forms, were provided by John Huggins at the US Army Medical Research Institute of Infectious Diseases (Ft. Detrick, Frederick, MD). Cowpox-R, a twice plaque purified syncytium forming virus, was originally prepared in cell culture by multiple replicative cycles in the presence of increasingly higher concentrations of cidofovir. The cowpox-WT virus was propagated in African green monkey kidney (MA-104) cells for infections in mice. Murine C127I and African green monkey kidney (Vero) cells were used for cell culture antiviral assays. Titrations of virus

from tissue samples were performed in Vero cells. The MA-104 cells were obtained from BioWhittaker (Walkersville, MD), whereas, the other two cell lines were from the American Type Culture Collection (Manassas, VA). C127I and MA-104 cells were grown in Eagle's medium containing 5% fetal bovine serum, whereas, the Vero cells were cultured in Medium 199 containing 5% serum. Eagle's medium containing 2% serum and gentamicin (50 μg/ml) was used for antiviral assays and virus titrations.

2.3. Cell culture antiviral and toxicity studies

The antiviral activities of S2242 and cidofovir were determined by plaque reduction assays in 12-well plates of C127I and Vero cells. Briefly, microwells were infected with 50–75 plaque forming units (PFU) of virus followed 1 h later by addition of S2242 or cidofovir at varying half-$\log_{10}$ concentrations. After 4 days the plates were fixed and stained with 0.1% crystal violet in 1% buffered formalin. Plaques were counted with the aid of a light box. Fifty percent effective concentrations (EC_{50} values) of compounds were determined by plotting concentration versus percent of untreated control on semi-$\log_{10}$ graph paper. Means of three independent assays ± standard deviations (S.D.) are reported.

Cytotoxicity studies were conducted using actively dividing uninfected cells seeded at about 1×10^4 cells per well in 24-well microplates. After an overnight incubation, the cells were exposed to the test compounds (0.5 ml per well) in growth medium (Eagle's medium containing 5% serum and 50 μg gentamicin per ml) at varying half-$\log_{10}$ concentrations for 3 days. Then 0.34% neutral red in phosphorous buffer saline (PBS) was added (0.2 ml per well) for an additional 2 h incubation at 37 °C. Cells were rinsed twice with PBS, the wells were aspirated dry, and 0.2 ml per well of 50% Sörensen's citrate buffer/50% ethanol was added to solubilize the neutral red. After 30 min, 100 μl from each well was transferred to a 96-well plate in order to be read by an ELISA plate reader at 540 and 405 dual wavelengths. Fifty percent inhibitory concentrations (IC_{50} values) were determined similar to the above EC_{50} calculation method, and means from three independent assays are reported.

2.4. Animal experiments

Female 13–15 g specific pathogen-free BALB/c mice were obtained from B&K Universal, Fremont, CA. They were quarantined 48 h prior to use and maintained on Wayne Lab Blox and tap water in the AAALAC-accredited Laboratory Animal Research Center at Utah State University.

For intranasal infections the mice were anesthetized with Ketamine (100 mg/kg) by i.p. injection. They were infected intranasally with approximately 5×10^5 PFU of cowpox virus per mouse in a 50 μl inoculum volume. One day later the animals were treated with compound or placebo. Treatments were given i.p. or by oral gavage once or twice daily for 5 or 10 days starting 24 h after virus exposure. Mice were weighed every 2–3 days and mean weights recorded.

Five additional mice in each treatment group in the 10-day treatment study were sacrificed on day 6 of the infection. Lungs and nasal (nose/sinus) tissue were removed, weighed and frozen for subsequent virus titer determinations. Homogenization of lungs and nasal tissue was done in 1 ml medium using sterilized mortars and pestles. Samples were serially diluted and plaque titrated in Vero cells. Plaques were counted with the aid of a light box at 3 days. Plaque numbers were converted to PFU/g of tissue. The homogenization and titration procedures used were previously published (Smee et al., 2000a).

2.5. Statistical evaluations for animal studies

Survivor number increases were evaluated by the two-tailed Fisher exact test. The two-tailed Mann–Whitney *U*-test was used to analyze differences in the mean day of death, reductions in tissue virus titers, and area under the curve differences in animal weights (for days 8 through 20 of the infection). All comparisons were made between drug-treated and placebo groups, except where indicated.

3. Results

3.1. In vitro antiviral and cytotoxic activities

S2242 and cidofovir were evaluated for antiviral activity against cowpox virus in C127I and Vero cell culture (Table 1). Both compounds were highly active against the WT virus in C127I cells, with cidofovir being about three-fold more potent than S2242. The potencies of both compounds were much less in Vero cells, with S2242 being slightly more potent than cidofovir. Against the cidofovir-resistant virus, both compounds were much less active than they were versus the WT virus in C127I cells. Interestingly the resistant virus was inhibited by S2242 in Vero cells at a concentration less than two-fold higher than the concentration inhibiting WT virus, whereas, cidofovir was ineffective against the resistant virus.

In uninfected C127I cells, cidofovir and S2242 inhibited cell proliferation, with IC_{50} values of 180 ± 70 and 340 ± 110 μM, respectively. The compounds did not inhibit the proliferation of Vero cells at ≤ 1000 μM, however. Thus, these compounds were not toxic to cells at concentrations inhibiting WT virus replication, as was reported previously in herpesvirus infections (Snoeck et al., 1988; Neyts et al., 1994).

3.2. Efficacies of compounds in infected mice using 5-day treatment regimens

Mouse studies were first conducted using once or twice daily i.p. treatments of infected mice for 5 consecutive days (Table 2). For animals treated once a day, S2242 and HOE961 were both effective at the 100 mg/kg per day dose in preventing mortality in 70 and 80% of the animals, respectively. Lower doses were not protective, however, except that the 30 mg/kg per day dose of either compound delayed the mean day of death significantly. Cidofovir was 100% protective at a dose of 30 mg/kg per day. Treatments twice a day with S2242 and HOE961 were 100 and 80% protective at 100 mg/kg per day, respectively, but the 30 mg/kg per day dose of either compound was still largely ineffective in preventing mortality. Cidofovir was 100% protective at 30 mg/kg per day when given twice a day. Animal weights during the infection are shown in Fig. 1 for groups treated with the highest dose of each inhibitor. Areas under each curve were statistically analyzed, showing that the mean weight of the cidofovir-treated group during days 8–20 of the infection were significantly higher than those of the S2242 and HOE961 groups ($P < 0.001$). Some improvement in body weight was seen in the S2242 group treated twice a day (Fig. 1A), compared with the group treated once a day (Fig. 1B). The results could not be compared statistically with assurance of accuracy since, they represented data from two separate experiments.

Groups of treated uninfected mice were evaluated for signs of toxicity during treatment (Table 2). None of the animals died over 21 days. Mice treated with S2242 (100 mg/kg per day) or cidofovir (30 mg/kg per day) did not gain as much weight as placebo controls between the onset and end of treatment. Otherwise the treatments were well tolerated.

Table 1
Antiviral activities of S2242 and cidofovir against wild-type (WT) and cidofovir-resistant (R) cowpox virus infections in cell culture

	50% Effective concentration (μM)			
	S2242		Cidofovir	
Virus	C127I cells[a]	Vero cells	C127I cells	Vero cells
Cowpox-WT	3.5 ± 0.5[b]	35 ± 11	1.0 ± 0.5	53 ± 15
Cowpox-R	33 ± 16	54 ± 3	230 ± 90	>1000

[a] Cell line used to determine antiviral activity.
[b] Mean values ± S.D. were determined from at least three independent plaque reduction assays.

Table 2
Effects of once or twice daily treatments for 5 consecutive days with S2242, HOE961, or cidofovir on a cowpox virus respiratory infection in mice

Treatment (mg/kg per day)[a]	Uninfected toxicological controls		Infected, treated			
	Once-daily treatments		Once daily treatments		Twice daily treatments	
	Survivors/total	Host weight change (g)[b]	Survivors/total	Mean day of death[c]	Survivors/total	Mean day of death[b]
S2242 (100)	3/3	+1.0	7/10***	17.0 ± 3.6**	10/10***	–
S2242 (30)	3/3	+2.2	1/10	15.0 ± 3.9***	2/10	17.8 ± 2.4**
S2242 (10)	–[d]	–	0/10	10.4 ± 1.5**	0/10	9.8 ± 1.6
HOE961 (100)	3/3	+1.9	8/10***	17.5 ± 2.1**	8/10***	12.5 ± 3.5
HOE961 (30)	3/3	+1.7	0/10	10.3 ± 1.1**	0/10	10.8 ± 2.0
HOE961 (10)	–	–	0/10	9.8 ± 0.9	0/10	8.9 ± 0.6
Cidofovir (30)	3/3	+1.0	10/10***	–	10/10***	–
Placebo	3/3	+2.5	0/10	8.8 ± 0.9	0/10	10.1 ± 2.1

[a] Intraperitoneal treatments started 24 h after virus exposure.
[b] Difference between initial weight (day 0) and weight on day 6.
[c] Of mice that died prior to day 21.
[d] Not determined.
**, $P<0.01$; ***, $P<0.001$.

3.3. Efficacies of compounds in infected mice using 10-day treatment regimens

One encouraging finding from the 30 mg/kg per day S2242 group (Table 2) was the very long time to death in animals that died. These results suggested that a longer treatment course might be beneficial in promoting survival and overall well-being during the infection. It also provided an opportunity to evaluate HOE961 orally. Consequently, an experiment was conducted to determine the effects of once daily treatment for 10 days on the infection (Table 3). Lung and nasal virus titers were quantified, in addition to looking for indicators of survival. The 100 mg/kg per day i.p. dose of S2242 protected 100% of mice from death, as did the 30 mg/kg per day dose of cidofovir. HOE961 was 80% protective when given by oral gavage. Both S2242 and cidofovir reduced lung and nasal virus titers to the same extent. HOE961 was weakly inhibitory to lung virus titers compared with the placebo control, but was more suppressive of nasal virus titers. Mean animal weights during the infection are shown in Fig. 2. Mice treated with cidofovir had significantly higher body weights on days 8–20 than the other two treated groups ($P<0.001$). S2242 was more protective than HOE961 in preventing weight loss ($P<0.001$). Since, HOE961 was most likely not completely orally absorbed, it is understandable that its internal effective dose following metabolism to S2242 was less than that of S2242, resulting in a weaker antiviral effect.

4. Discussion

S2242 and HOE961 were found to be active against cowpox virus infections, both in cell culture and in mice. Potencies in cell culture were cell line dependent, with greater antiviral effects seen in mouse (C127I) cells than in monkey (Vero) cells. The potency of S2242 was three-fold less than that of cidofovir in mouse cell culture against WT virus, but was superior to cidofovir in monkey cells. In addition, the cidofovir-resistant form of cowpox virus was not as cross-resistant to S2242 in Vero cells than it was in C127I cells. The cytotoxicities of the two compounds were greater in C127I cells than in Vero cells. These results

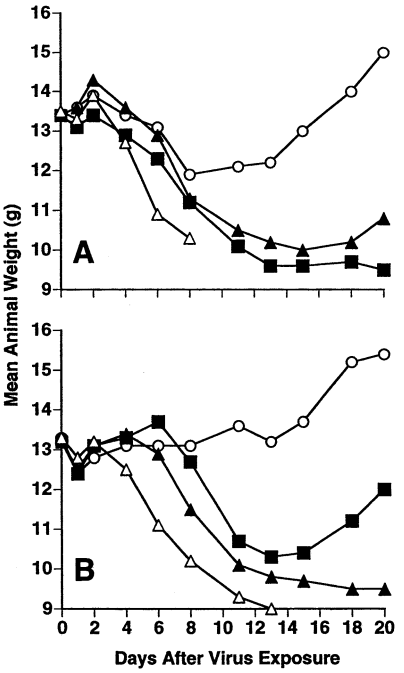

Fig. 1. Mean animal weights following i.p. treatment (A, once daily for 5 days; B, twice daily for 5 days) of a cowpox virus respiratory infection in mice with S2242, HOE961 or cidofovir. Data accompany the results in Table 2. Symbols, ○, cidofovir (30 mg/kg per day); ■, S2242 (100 mg/kg per day); ▲, HOE961 (100 mg/kg per day); △, placebo.

probably reflect a differential degree of metabolism of either compound to the active antiviral triphosphate form in these cell lines.

In the animal experiments we demonstrated

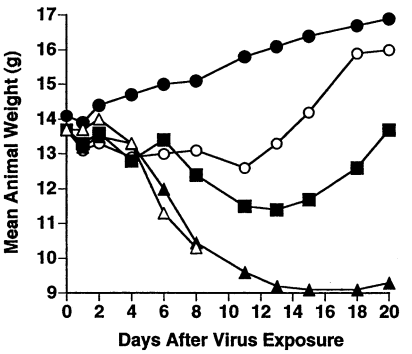

Fig. 2. Mean animal weights following treatment (once daily for 10 days) of a cowpox virus respiratory infection in mice with S2242, HOE961 or cidofovir. S2242 and cidofovir were given i.p., whereas HOE961 and placebo were administered orally. Data accompany the results in Table 3. Symbols, ●, uninfected; ○, cidofovir (30 mg/kg per day); ■, S2242 (100 mg/kg per day); ▲, HOE961 (100 mg/kg per day); △, placebo.

that S2242 and HOE961 were effective in preventing mortality in mice infected intranasally with a lethal cowpox virus challenge, but only at the 100 mg/kg per day dose. S2242 was active by i.p. inoculation, whereas, HOE961 was effective by both i.p. and oral routes, as expected. The compounds were not as potent, nor as effective as cidofovir, which was used at 30 mg/kg per day in these experiments. The lesser potency of S2242 compared with cidofovir in mice correlated with potency differences seen in mouse cell culture assays.

Table 3
Effects of once daily treatments for 10 consecutive days with S2242, HOE961, or cidofovir on a cowpox virus respiratory infection in mice

Treatment (mg/kg per day)	Treatment route[a]	Survivors/total	Mean day of death[b]	Day 6, lung virus titer[c]	Day 6, nasal virus titer[c]
S2242 (100)	i.p.	10/10***	–	6.9 ± 0.3**	6.6 ± 0.5*
Cidofovir (30)	i.p.	10/10***	–	6.9 ± 0.3**	6.5 ± 0.6*
Placebo	i.p.	1/10	10.2 ± 1.9	8.1 ± 0.1	7.4 ± 0.3
HOE961 (100)	Oral	8/10***	16.0 ± 0.0***	7.8 ± 0.2*	6.7 ± 0.3*
Placebo	Oral	0/10	8.9 ± 0.7	8.1 ± 0.02	7.8 ± 0.5

[a] Intraperitoneal or oral gavage treatments started 24 h after virus exposure.
[b] Of mice that died prior to day 21.
[c] $\log_{10}$ PFU/g.
*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

S2242 was able to suppress virus replication in the lungs to the same extent as cidofovir on the day of assay, but the latter compound consistently prevented weight loss during the infection better than S2242. In comparing S2242 (and its prodrug form, HOE961) with other compounds we have evaluated to date, it appears intermediate in activity between cidofovir and ribavirin (Smee et al., 2000a,b). Other compounds, including arabinofuranosyl nucleosides, mycophenolic acid, and other metabolic inhibitors, have been inferior to ribavirin in their efficacy against lethal cowpox infections (unpublished observations). Thus, S2242 (or HOE961) is the second most potent anti-orthopoxvirus agent in animals to be discovered.

Multiple days of treatment with S2242 or HOE961 were required for antiviral activity in mice. We have also evaluated the efficacies of single i.p. and intranasal treatments (100 and 40 mg/kg, respectively) with these compounds, and found them to be inactive (unpublished data). These results suggest that the antiviral form of the compound has a short intracellular half life. Indeed, Neyts et al. (1998) have confirmed this. In contrast, cidofovir is effective with just a single treatment (Bray et al., 2000; Smee et al., 2000b, 2001). Metabolites of cidofovir were shown to have extremely long intracellular half-lives (Ho et al., 1992).

Toxic effects of S2242 and HOE961 were not observed in the animal studies, except for moderate suppression in weight gain by S2242 at 100 mg/kg per day, which was equivalent to that caused by cidofovir (30 mg/kg per day). Neyts and De Clercq (2001) reported that S2242 induced testicular atrophy in mice, similar to the effect of ganciclovir in rodents. They reported that the compound induced no other abnormalities indicative of toxicity.

Neyts et al. (1998) have studied the phosphorylation of S2242 to the triphosphate in cell culture. The enzyme responsible for the initial step of phosphorylation (to S2242 monophosphate) is deoxycytidine kinase. The particular enzyme is able to phosphorylate deoxycytidine, deoxyadenosine and their analogs (Krenitsky et al., 1976). Kinetic studies of S2242 triphosphate interaction with the orthopoxviral DNA polymerase would need to be performed in order to determine whether S2242 is acting as a deoxyadenosine or a deoxycytidine analog.

A murine cytomegalovirus resistant to cidofovir has been shown to be cross-resistant to the closely related adenosine compound HPMPA (Smee et al., 1995). We reported here that a cowpox virus resistant to cidofovir was also resistant to S2242, but more so in mouse cells than in Vero cells. Viruses affecting humans (monkeypox and smallpox) that potentially may be found resistant to cidofovir might be inhibited by S2242, because of the cell lines these types of viruses grow in. Whether orthopox viral resistance to cidofovir or other agents will become a future problem remains to be determined.

Although, S2242 and HOE961 are less potent than cidofovir in mouse models, the one advantage of using HOE961 as a treatment for orthopoxvirus infections is that it is orally active. Cidofovir must be given intravenously for efficacy in human patients (Wachsman et al., 1996), although, a single treatment with the drug should be adequate for a significant protective benefit. Since, HOE961 holds promise for the oral treatment of poxvirus infections, it deserves further study.

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