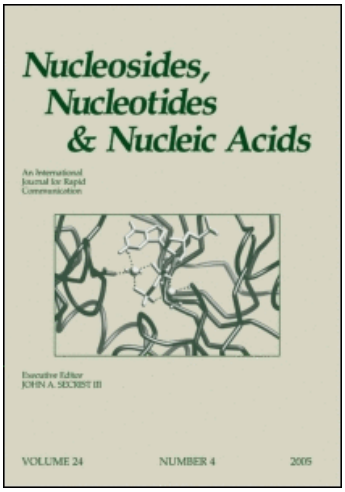

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Anti-cowpox Virus Activities of Certain Adenosine Analogs, Arabinofuranosyl Nucleosides, and 2'-Fluoro-arabinofuranosyl Nucleosides

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Anti-cowpox Virus Activities of Certain Adenosine Analogs, Arabinofuranosyl Nucleosides, and 2′-Fluoro-arabinofuranosyl Nucleosides[†]

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ABSTRACT

Nucleoside analogs were investigated for their potential to inhibit cowpox virus (a surrogate for variola and monkeypox viruses) in cell culture and in lethal respiratory infections in mice. Cell culture antiviral activity was determined by plaque reduction assays, with cytotoxicity determined by cell proliferation assays. Selectivity indices (SI's, 50% cytotoxic concentration divided by 50% virus-inhibitory concentration) were determined for 15 compounds. Three arabinofuranosyl (Ara) nucleosides showed activity in mouse mammary tumor (C127I) cells: guanine (Ara-G), thymine (Ara-T), and adenine (Ara-A) with SI's of 113, 61, and 95, respectively. The 2′-fluoro-Ara nucleosides of 5-F-cytosine (FIAC), 5-methyluracil (FMAU), and 5-iodouracil (FIAU) exhibited SI's of 148, 77, and 29, respectively. Other potent compounds included cidofovir (a positive control) and 3′-*O*-methyladenosine, with SI values of 164 and 56, respectively. In general, assays performed in African green monkey kidney (Vero) cells produced lower SI's than in C127I cells, except for 5-iodo-2′-deoxyuridine (IDU) which had an SI of > 71 in Vero cells and 3.1 in C127I cells. Intranasal infection of mice with cowpox virus was followed a day later by twice daily intraperitoneal treatment with compounds for 5 days. Ara-A was active at 300 mg/kg/day (40% survival), FMAU at 100 mg/kg/day (70% survival), and cidofovir (given for 1 day

[†]In honor and celebration of the 70th birthday of Professor Leroy B. Townsend.

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only) at 100 mg/kg (80–100% survival). None of the other compounds, including IDU, prevented death nor delayed the time to death. Cidofovir had the best potential for treating orthopoxvirus infections of those tested.

Key Words: Antiviral; Nucleosides; Cidofovir; Idoxuridine; Fialuridine; Vidarabine; Cowpox virus; Bioterrorism.

INTRODUCTION

Antiviral substances are being sought for the treatment of orthopoxvirus infections, including variola (smallpox) and monkeypox that may be used as bioterrorism agents.[1,2] Treatments are also sought for complications due to administration of the smallpox vaccine (which is live vaccinia virus) to immunosuppressed individuals,[3] and for other pox virus infections such molluscum contagiosum[4,5] or those caused by orf virus.[6] Over the years a number of compounds have been evaluated in various animal models of orthopoxvirus infection. Those compounds found active in these models were recently reviewed.[7] Some of the animal models (such as tail, skin, or eye infections) that were used previously were not lethal. Recently more severe respiratory infection models have been developed using cowpox[8] or vaccinia[9,10] (WR strain) viruses in mice.

From studies in the more severe infection models, only two compounds have been reported that have sufficient potency to prevent death in animals. These are 1-[(S)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine (cidofovir)[8–11] and 2-amino-7-[1,3-dihydroxy-2-propoxy)methyl]purine (compound S2242 and its orally active diacetate ester prodrug HOE961).[12,13] Other compounds that may have potential but have not been thoroughly investigated in the more severe orthopoxvirus infection models include certain adenosine analogs, arabinofuranosyl nucleosides, and 2′-fluoro-arabinofuranosyl nucleosides. 8-Methyladenosine (8-Me-Ado) and 3′-*O*-methyladenosine (3′-*O*-Me-Ado) were both reported to be very active against poxvirus infections in cell culture.[14,15] 9-β-D-Arabinofuranosyladenine (Ara-A, vidarabine) and 1-β-D-arabinofuranosylcytosine (Ara-C) have shown some positive effects in animal infections with vaccinia virus,[16,17] as has 5-iodo-2′-deoxyuridine (IDU).[17,18] Other arabinofuranosyl compounds are commercially available but have not been tested. 2′-Fluoro-arabinofuranosyl nucleosides are reported to inhibit herpes viruses in animals,[19] but have not been evaluated against orthopoxviruses. Thus, the purpose of the present studies was to evaluate a number of these compounds both in cell culture and in mice to determine their potential against cowpox virus, a member of the orthopoxvirus family that is closely related to vaccinia, variola, and monkeypox viruses. Such studies might lead to identifying compounds besides cidofovir and S2242 that could have potential to treat serious orthopoxvirus infections in humans.

EXPERIMENTAL

Antiviral compounds. The following compounds were purchased from Sigma Chemical Company (St. Louis, MO): 8-bromoadenosine (8-Br-Ado), Ara-C,

1-β-D-arabinofuranosyl-5-chlorocytosine (5-Cl-Ara-C), 1-β-D-arabinofuranosyl-5-fluorocytosine (5-F-Ara-C), and IDU. Bristol Myers Squibb provided 2′-fluoro-2′-deoxy-1-β-D-arabinofuranosyl-5-iodocytosine (FIAC), 2′-fluoro-2′-deoxy-1-β-D-arabinofuranosyl-5-iodouracil (FIAU, fialuridine), and 2′-fluoro-2′-deoxy-1-β-D-arabinofuranosyl-5-methyluracil (FMAU). Cidofovir was obtained from Mick Hitchcock of Gilead Sciences (Foster City, CA). 1-β-D-arabinofuranosylthymine (Ara-T), and Ara-A were purchased from ICN Pharmaceuticals (Costa Mesa, CA). RI Chemical (Orange, CA) provided 8-Me-Ado and 9-β-D-arabinofuranosylguanine (Ara-G). 3′-*O*-Me-Ado was obtained from ChemGenes Corp. (Ashland, MA). 8-Chloroadenosine (8-Cl-Ado) was synthesized and kindly provided by Benjamin Philmus and Bradley Davidson from the Department of Chemistry, Utah State University (Logan, UT).

The compounds were dissolved in cell culture medium for in vitro experiments or in physiological saline solution (PSS) for treatment of mice. Compounds with low solubility were suspended in PSS and sonicated for injection of animals.

Virus and cells. Cowpox virus (Brighton strain) was obtained from John Huggins, U.S. Army Medical Research Institute of Infectious Diseases (Ft. Detrick, Frederick, MD) as a twice plaque-purified isolate. The virus originated from the Centers for Disease Control and Prevention, (Atlanta, GA). The virus was propagated in African green monkey kidney (Vero) cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA). Mouse mammary tumor (C127I) cells, used in antiviral studies, were also obtained from ATCC. Vero cells were cultured in Medium 199 containing 5% fetal bovine serum (FBS) and 0.1% sodium bicarbonate. C127I cells were grown in Eagle's medium containing 5% FBS and 0.1% sodium bicarbonate. Antiviral studies were done in maintenance medium (Eagle's medium with 2% FBS, 0.18% sodium bicarbonate, and 50 μg gentamicin/ml).

Cell culture antiviral studies. Twelve-well microplates containing confluent monolayers of Vero or C127I cells were infected with cowpox virus at 50–100 plaque forming units (PFU) per well. The plates were rocked every 5–10 min for about 45 min. Then medium containing compound in half-$\log_{10}$ dilution increments was applied (two microwells per concentration) following removal of the infectious inoculum. At three days the cells were fixed and stained with 10% buffered formalin containing 0.2% crystal violet. After 5–10 min the stain was removed and the plates were rinsed with water. Air-dried plates were then counted for plaque numbers in this standard plaque reduction assay.[12] Concentrations of compound reducing plaque numbers by 50% (EC_{50} values) were determined by dose-response plots graphed on semi-$\log_{10}$ paper. Each value in Table 1 represents a mean ± standard deviation for at least three independent assays.

Cytotoxicity assays were conducted using rapidly proliferating uninfected Vero or C127I cells in 24-well microplates. Approximately 1×10^4 cells were seeded into each well in growth medium and allowed to attach overnight. Then compounds at varying dilutions were applied for 3 days. After that, 0.1% (final concentration) neutral red was added for 2 h. The plates were rinsed twice with phosphate buffered saline, then the remaining neutral red in the cells was solubilized in 0.2 ml 50% Sörensen's citrate buffer (pH 4.0)/50% ethanol for 30 min. A 100 μl volume from each well

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was transferred to a 96-well microplate and quantified with an ELISA plate reader as described.[12] The extent of neutral red dye uptake is proportional to cell number. Fifty percent cell growth-inhibitory concentrations (IC_{50} values) were determined in a manner similar to that described above for EC_{50} determinations. At least three independent cytotoxicity assays were run with each compound. Selectivity indices (SI values) were determined by dividing IC_{50} by EC_{50} for each set of data. We consider selectivity indices of 10 or greater as indicative of compounds meriting further investigation.

Antiviral experiments in animals. Female BALB/c mice weighing about 15 grams each were obtained from Simonsen Labs (Gilroy, CA). The animals were anesthetized by intraperitoneal (i.p.) injection of ketamine (100 mg/kg) followed by intranasal administration of 5×10^5 plaque forming units (PFU) per animal of cowpox virus in a 50 µl volume. The standard treatment regimen was i.p. administration of compound or placebo (saline) in a 100 µl volume twice a day (in half-daily doses) for 5 days starting 24 h after virus challenge. The positive control compound used in all studies was cidofovir given as a single 100 mg/kg/day i.p. injection at 24 h post-infection. There were 10 treated animals per dosage group and 10 placebo controls in each study. Uninfected toxicity control mice (5/group) were treated with compounds or placebo using the same doses and regimen.

All of the compounds were not tested in the same experiment, but cidofovir was evaluated each time as the positive control. The adenosine analogs were evaluated as a group, as were the arabinofuranosyl compounds, and the 2′-flouro-arabinofuranosyl compounds. IDU was tested with FMAU in a special follow-up study with the latter compound. In each experiment the placebo group had no survivors.

Statistical evaluations for animal experiments. Statistical interpretations of increases in numbers of survivors were determined by the Fisher exact test. The Mann-Whitney U-test statistically analyzed increases in mean day of death. All statistical evaluations were two-tailed except as indicated in Table 2, and compared compound-treated groups to the placebo control group.

RESULTS AND DISCUSSION

Antiviral Activity in Cell Culture

Compounds were evaluated for antiviral activity in plaque reduction assays performed in mouse (C127I) and monkey (Vero) cells (Table 1). Of the 8-substituted adenosine analogs, the compounds showed antiviral activity but also exhibited cytotoxicity. Both antiviral activity and cytotoxicity were more pronounced in C127I cells than in Vero cells. 8-Br-Ado was the only 8-substituted adenosine analog with moderate selectivity in C127I cells (SI of 9). 8-Me-Ado was previously reported as having potency against vaccinia virus with minimal toxicity resulting in a high selectivity index.[14] This may be true in stationary cell monolayers cultures that were used previously for assessing toxicity, but the compound was definitely cell growth-inhibitory at low concentrations in actively dividing cells. Many compounds appear to

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Table 1. Antiviral activities of nucleoside analogs against cowpox virus in cell culture.

	Activity in C127I cells			Activity in Vero cells		
Compound	EC_{50}[a]	IC_{50}[b]	SI[c]	EC_{50}	IC_{50}	SI
Adenosine analogs						
8-Cl-Ado	32 ± 3.0	45 ± 25	1.4	>100	18 ± 11	<1
8-Br-Ado	7.6 ± 3.9	68 ± 25	9	650 ± 130	415 ± 35	<1
8-Me-Ado	12.7 ± 4.5	5.2 ± 1.6	<1	>1000	515 ± 150	<1
3′-*O*-Me-Ado	4.9 ± 3.4	275 ± 100	56	24 ± 3	400 ± 125	17
Arabinofuranosyl nucleosides						
Ara-A	0.9 ± 0.2	55 ± 25	61	6.1 ± 2.2	300 ± 170	49
Ara-C	0.16 ± 0.03	0.4 ± 0.3	2.5	0.3 ± 0.08	0.8 ± 0.6	2.7
5-Cl-Ara-C	4.9 ± 1.0	65 ± 30	13	100 ± 44	>1000	>10
5-F-Ara-C	0.11 ± 0.01	0.3 ± 0.2	2.7	0.22 ± 0.03	0.6 ± 0.3	2.7
Ara-G	1.2 ± 0.3	135 ± 60	113	197 ± 45	>1000	>5
Ara-T	1.0 ± 0.3	95 ± 30	95	123 ± 6	>1000	>8
2′-Fluoro-arabinofuranosyl nucleosides						
FIAC	0.8 ± 0.14	118 ± 40	148	39 ± 3	>1000	>26
FIAU	0.16 ± 0.01	4.7 ± 3.0	29	27 ± 10	400 ± 130	15
FMAU	0.22 ± 0.08	17 ± 10	77	19 ± 5	240 ± 175	13
Control compounds						
Cidofovir	1.1 ± 0.6	180 ± 70	164	53 ± 15	>1000	>19
IDU	0.8 ± 0.3	2.5 ± 1.7	3.1	14 ± 3	>1000	>71

[a]Fifty percent effective concentration (μM, plaque reduction assay).
[b]Fifty percent cell-inhibitory concentration (μM, cell proliferation assay).
[c]Selectivity index (IC_{50} divided by EC_{50}).

be well tolerated in stationary monolayers where cells are in a non-replicative state but exhibit much greater cytotoxic or cytostatic effects in proliferating cell cutures.[20] Because of this, we feel that cytotoxicity determinations in rapidly dividing cells are a better predictor of toxicity that might be encountered in animals. This proved to be the case for 8-Me-Ado. 3′-*O*-Me-Ado showed the greatest degree of potency and selectivity of the adenosine analogs in both cell lines.

The arabinofuranosyl nucleosides and 2′-fluoro-arabinofuranosyl nucleosides showed varying degrees of potency and selectivity in cell culture (Table 1). Activities were greater in C127I cells than in Vero cells. Compounds with the greatest selectivity in C127I cells and in order of potency were FIAC > Ara-G > Ara-T > Ara-A > FMAU > FIAU. Cidofovir was the most selective compound in C127I cells but was much less so in Vero cells. Interestingly, IDU exhibited greater selectivity in Vero cells than it did in C127I cells. This was attributed to its higher degree of toxicity in the latter cell line. IDU was the most selective of the compounds tested in Vero cells, followed by Ara-A, FIAC, and cidofovir.

We have previously compared the activities of selected antiviral agents against orthopoxvirus infections in Vero and mouse cells.[13,21] In each case the degree of antiviral potency exhibited in mouse cells was greater than that seen in Vero cells. For two compounds studied, ribavirin and cidofovir, phosphorylation to the active antiviral state was greater in mouse cells.[21] This largely explained the differences in antiviral

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effects of the two compounds observed in the two cell lines. Other compounds, such as those reported in Table 1, presumably are also poorly phosphorylated in Vero cells compared to mouse cells.

Antiviral Activity in Cowpox Virus-Infected Mice

Animals were infected intranasally with cowpox virus, then were treated i.p. twice a day for 5 days with compounds (Table 2). 8-Cl-Ado was not evaluated in the assays because it was judged to have insufficient activity against the virus in cell culture (Table 1) to warrant animal testing. 8-Me-Ado was also ineffective in cell culture, but was tested in mice because of the prior report claiming remarkable selectivity in vitro.[14] The activities of compounds are reported in Table 2 at doses that did not induce excessive weight loss in the mice. Higher doses may have been tested in some cases, but are not reported on the tables because they caused toxicity (the mice did not survive the

Table 2. Antiviral activities of nucleoside analogs against cowpox virus infections in mice.

Compound	Maximum dose tested (mg/kg/day)[a]	Percent survival[b]	Days increase in MDD[c]
Adenosine analogs			
8-Cl-Ado	nd[d]	nd	nd
8-Br-Ado	100	0	–
8-Me-Ado	30[e]	0	–
3′-O-Me-Ado	100	0	–
Arabinofuranosyl nucleosides			
Ara-A	300	40*	4.6***
Ara-C	30[e]	0	–
5-Cl-Ara-C	100	0	–
5-F-Ara-C	30[e]	0	–
Ara-G	100	0	–
Ara-T	100	0	–
2′-Fluoro-arabinofuranosyl nucleosides			
FIAC	30[f]	0	–
FIAU	30[f]	0	–
FMAU	100	70**	3.2***
Positive control compounds			
Cidofovir[g]	100	80–100***	3.9***
IDU	150	0	–

*P < 0.05 (one-tailed analysis), **P < 0.01, ***P < 0.001, compared to placebo group.
[a]Intraperitoneal treatments were given twice a day for 5 days starting 24 hours after virus exposure. Cidofovir was administered one time only at 24 hours.
[b]Compounds were tested in separate experiments by category along with cidofovir. In each study the placebo group had no survivors.
[c]Mean day of death of mice that died prior to day 21.
[d]Not determined.
[e]A higher dose (100 mg/kg/day) induced excessive weight loss.
[f]Insufficient compound available for evaluating a higher (100 mg/kg/day) dose.
[g]Average results from 4 separate experiments.

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infections anyway at higher doses). Quantities of FIAC and FIAU were limited, thus precluding the testing of higher doses. For all of these compounds tested in the model, only Ara-A and FMAU prevented death of animals, as did the positive control cidofovir. It is interesting that Ara-A yielded a slightly greater increase in the time to death compared to cidofovir and FMAU, suggesting that further treatment may lead to greater survival. We have done such studies, treating out to 10 days with no enhancement of survival (unpublished). The inactive compounds did not even cause an increase in the time to death. Although IDU has shown protection of mice in certain vaccinia virus infection models,[17,18] it was not protective to mice infected with cowpox virus. Many doses of IDU were tested, ranging from 25 to 150 mg/kg/day, but none showed activity in terms of increasing the number of survivors or delaying the time to death.

Various treatment regimens with Ara-A other than the one reported here (e.g., starting treatment 1 h prior to infection and/or using lower doses) have failed to provide 100% protection to cowpox virus-infected animals (unpublished data). Previously, Ara-A was found to be protective in an intracerebral vaccinia virus infection model in mice using a micronized preparation of the compound to facilitate solubilization.[22] In those studies the drug was administered for 9 days. Our preparation was not micronized, and only 5 days of treatment were given. Both factors may have contributed to a moderate antiviral effect in our model. FMAU showed better efficacy than Ara-A against cowpox virus in mice. It is probable that FMAU exhibits mitochondrial toxicity similar to FIAU, which caused the death of humans treated for hepatitis B infection.[23] Thus, the enthusiasm for pursuing FMAU as an anti-orthopoxvirus agent is minimal.

To date, the compounds showing the greatest potential for treating orthopoxvirus infections include cidofovir,[8–11] S2242,[12,13] and their orally-active prodrugs.[12,13,24] Many of the other nucleoside analogs reported here have historically been identified as inhibitors of orthopoxviruses, but showed little promise in cowpox virus-infected mice. In these animal studies, cidofovir was clearly superior to the other compounds tested.

CONCLUSIONS

Fifteen compounds representing adenosine analogs, arabinofuranosyl nucleosides, 2′-fluoro-arabinofuranosyl nucleosides, and control compounds (cidofovir and IDU) were evaluated for antiviral activity in cell culture, with 14 of them further evaluated in a mouse model for antiviral activity against cowpox virus. Compounds exhibiting the greatest selectivity in mouse C127I cells and their order of potency were: cidofovir > FIAC > Ara-G > Ara-T > FMAU > Ara-A > 3′-*O*-Me-Ado > FMAU. Selectivity indices for these compounds were lower in Vero cells than in C127I cells. IDU was the most selective compound in Vero cells, followed by Ara-A, FIAC, and cidofovir. However, in animal studies only cidofovir, FMAU, and Ara-A treatments gave any protection from death. Of these, cidofovir clearly emerged as the most viable candidate for further investigation.

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