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In vitro activity of potential anti-poxvirus agents

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Abstract

The potential use of variola or another orthopoxvirus such as monkeypox as a weapon of bioterrorism has stimulated efforts to develop new drugs for treatment of smallpox or other poxvirus infections. At the present time only cidofovir is approved for use in the emergency treatment of smallpox outbreaks. Although cidofovir is very active against the orthopoxviruses in vitro and in animal model infections, it is not active when given orally and must be administered with precaution so as to avoid renal toxicity. In an attempt to identify alternative treatment modalities for these infections we have determined the anti-poxvirus activity in vitro of most of the approved antiviral agents as well as a number of cidofovir analogs and prodrugs. From these studies, we have identified the nucleotide analog, adefovir dipivoxil, some alkoxyalkyl esters of cidofovir and a number of prodrugs of cidofovir that warrant further investigation as potential therapies for smallpox or other orthopoxvirus infections.

Keywords: Orthopoxvirus; Smallpox; Monkeypox; Cidofovir; Adefovir dipivoxil

1. Introduction

There has been little interest or incentive in developing antiviral therapies or new vaccines for smallpox or other diseases due to poxviruses since variola virus, the causative agent of smallpox was considered to be eradicated about 25 years ago. A low level of effort in identifying new agents with activity against this group of viruses has continued over the years and a few agents such as methisazone, ribavirin, idoxuridine, and related analogs, interferons and interferon inducers, adenine arabinoside, cytosine arabinoside, S2242, cidofovir (CDV) and a number of phosphonate nucleotides have been reported to be active (Woodson and Joklik, 1965; De Clercq and De Somer, 1968; Sidwell et al., 1972; De Clercq et al., 1976, 1987; Naesens et al., 1997; Nettleton et al., 2000; De Clercq, 2001; Neyts and De Clercq, 2001; Kern et al., 2002). In recent years, however, the potential of the use of variola virus or another orthopoxvirus such as monkeypox virus as a bioterrorism weapon has heightened our awareness as to our vulnerability to this disease since vaccination for smallpox was discontinued in 1980 (Breman and Henderson, 1998; Henderson, 1998; Heymann et al., 1998; O'Toole, 1999; Hutin et al., 2001). This potential threat has resulted in a resurgent effect to identify and develop more agents that can be used in an emergency situation to treat these candidate viral diseases. Since there is little incentive for industry to spend hundreds of millions of dollars to develop a drug against a disease that currently does not exist, the emphasis has been on identifying antiviral agents that are already approved for another indication. One such compound, cidofovir (CDV) (De Clercq, 1993; Hitchcock et al., 1996), is approved for intravenous use in the treatment of cytomegalovirus (CMV) retinitis in HIV-infected patients (Safrin et al., 1997). The drug is very active in tissue culture cells against all the orthopoxviruses that have been tested including vaccinia, monkeypox, and variola viruses (De Clercq et al., 1986, 1987; De Clercq, 2001; Huggins et al., 2002; Kern et al., 2002). The activity of CDV against orthopoxviruses is of particular interest since the compound has been shown to be active when given parenterally or by aerosol in animal models infected with vaccinia virus or cowpox virus (De Clercq et al., 1989; Neyts and De Clercq, 1993; Bray et al., 2000, 2002; Smee et al., 2000, 2001, 2002).

Although CDV is a highly effective inhibitor of orthopoxvirus replication in tissue culture cells and highly effective in preventing mortality in vaccinia or cowpox virus infected mice when given parenterally, it is absorbed poorly when administered orally to humans (Cundy et al., 1996a,b). The lack of oral bioavailability is a major limitation to the use of this drug in a large-scale emergency situation such as a smallpox outbreak. While this does not preclude the use of CDV under those conditions, it does present some challenging logistical problems.

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Table 1
Properties of an ideal anti-orthopoxvirus drug

Active against vaccinia, monkeypox and variola virus
Should be active orally for ease of administration
Long intracellular half-life is desirable so dosing can be infrequent
Must be stable for long periods under adverse storage conditions
Needs to be inexpensive, so large amounts can be stockpiled
Must have a tolerable safety profile also for children and immunocompromised individuals

There are a number of issues that should be considered in the development of a drug for use in a situation such as smallpox when the target population includes all members of society including children and immunocompromised patients. The desirable characteristics for a drug to be used in this situation are listed in Table 1. In addition to being active orally, the drug should have a long intracellular half-life so administration can be infrequent and of course have a toxicity profile that is acceptable for all individuals. Additionally, since the drug will need to be stockpiled for a future potential need, it needs to be inexpensive, and highly stable under a variety of storage conditions.

2. Activity of licensed antiviral drugs against orthopoxvirus replication

In our attempts to identify additional drugs that might be used for a smallpox outbreak we first tested many of the licensed antiviral drugs that are approved for other indications for their ability to inhibit vaccinia virus (VV) or cowpox virus (CV) replication in cell culture assays. These materials were first evaluated using a cytopathic effect (CPE) inhibition assay using human foreskin fibroblast (HFF) cells. Active compounds in this assay system were confirmed using a classical plaque reduction assay in HFF cells (Kern et al., 2002). Selected compounds were also tested in Vero 76 cells. These include drugs approved primarily for herpesvirus or human immunodeficiency virus (HIV) infections. For the HIV inhibitors we tested representative compounds for the nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). In addition, one compound approved for cancer chemotherapy (gemcitabine) was included. The compounds that exhibited activity against the two poxviruses were CDV, gemcitabine, idoxuridine, trifluridine, and vidarabine (Table 2). As mentioned previously, CDV is the most promising candidate on this list. Although long-term administration results in significant nephrotoxicity (Safrin et al., 1997), its use for a smallpox outbreak would be infrequent and short-term and, in these conditions, its toxicity may not be a big issue (De Clercq, 2002), but its need for parenteral administration remains a logistical limitation. The compound gemcitabine was extremely active against both vaccinia virus and cowpox virus in vitro, but also very toxic. It did, however, have a selectivity index of >200, suggesting that additional preclinical studies are warranted to determine the potential of this drug for treatment of poxvirus infections. Idoxuridine and trifluridine, which are both approved for topical treatment of herpes simplex virus (HSV) keratitis probably do not have a sufficient toxicology database to support parenteral use. Vidarabine, the first parenteral therapy approved for serious herpesvirus infections, is not active orally and was not active in murine models for vaccinia or cowpox virus infections (data not presented).

Table 2
Activity of licensed antiviral drugs against orthopoxvirus replication

Drug	Indication	Sponsor	EC_{50} (μM)	
			VV	CV
Acyclovir	HSV	Glaxo SmithKline	>300	>300
Brivudin	Herpes	Berlin-Chemie	>100	66
Cidofovir	CMV	Gilead Sciences	33	43
Famciclovir	HSV	Novartis	>300	>300
Ganciclovir	CMV	Roche	>300	>300
Gemcitabine	Cancer	Eli Lilly	0.013	0.017
Foscarnet	HSV/HIV	Astra-Zeneca	>300	>300
Idoxuridine	HSV	Glaxo SmithKline	6.0	2.0
Lamivudine	HIV/HBV	Glaxo SmithKline	>400	>400
NRTI[a]	HIV	Various	>300	>300
NNRTI[b]	HIV	Various	>300	>300
Protease inhibitors[c]	HIV	Various	>300	>300
Penciclovir	HSV	Novartis	>300	>300
Ribavirin	RSV	ICN Pharmaceuticals	280	>400
Trifluridine	HSV	Glaxo SmithKline	1.7	1.5
Vidarabine	HSV	Parke Davis	12	45
Viread	HIV	Gilead Sciences	>300	>300

[a] Abacavir, didanosine, stavudine, zalcitibine, zidovudine.
[b] Efavirenez, nevaripine.
[c] Indinavir, nelfinavir, ritonavir, saquinavir.

Table 3
Antiviral activity of compounds against orthopoxvirus replication with phase II/phase III history

Drug	Indication	Sponsor	EC_{50} (μM)	
			VV	CV
Adefovir	HIV/HBV	Gilead Sciences	>300	>300
Adefovir dipivoxil	HIV/HBV	Gilead Sciences	5.1	13
Amdoxovir	HIV	Triangle	>300	>300
Clevudine	HBV	Triangle	>300	>300
Coactinon	HIV	Triangle	>300	>300
Coviracil	HBV/HIV	Triangle	>81	>81
Emivirine	HIV	Triangle	>300	>300
Entecavir	HBV	Bristol-myers squibb	53	85
Fialuridine	HBV-CMV	Eli Lilly	1.5	0.24
Lobucavir	HIV/HSV	Bristol-myers squibb	27	66
Mozenavir	HIV	Triangle	>100	>100
Sorivudine	HSV/VZV	Bristol-myers squibb	>100	>100

3. Anti-pox virus activity of compounds with phase II/phase III history

In addition to numerous compounds that are approved for use in humans, we also evaluated a number of compounds that have undergone phase II/phase III clinical trials and therefore have a reasonably complete toxicology profile. These results, summarized in Table 3 indicated that adefovir dipivoxil and fialuridine were the most active candidates tested in this group. Although fialuridine is very active in tissue culture, its previous toxicity history in treatment of hepatitis in humans probably precludes it as a serious candidate. Adefovir dipivoxil, on the other hand, does appear to be a serious candidate in that it is very active against vaccinia and cowpox virus replication in tissue culture and has good oral bioavailability (Barditch-Crovo et al., 1997). It needs to be evaluated against monkeypox virus and variola virus before its potential is known. Additionally, animal model studies using vaccinia and cowpox need to be performed. Lobucavir had moderate activity in cell culture which might justify evaluation in animal model infections.

4. Activity of cidofovir, cyclic cidofovir and their alkoxyalkyl analogs against vaccinia, cowpox, monkeypox, and variola viruses

As pointed out above, a major limitation to the use of CDV in the management of a poxvirus outbreak is its lack of activity when given orally. Cyclic CDV (Bischofberger et al., 1994) has activity against vaccinia and cowpox virus that is similar to that seen with CDV (Kern et al., 2002) but also is not sufficiently orally bioavailable. An additional approach to the development of new but closely related entities for use in poxvirus infections has been to synthesize prodrugs of CDV that would increase their oral bioavailability. It has been reported previously that alkylglycerol phosphate or alkoxypropyl phosphate esters of acyclovir and ganciclovir had greater oral bioavailability in rodents and were active orally in animal models of herpes simplex virus, murine cytomegalovirus and woodchuck hepatitis virus infections (Hostetler et al., 1997, 2000, 2001). To obtain better oral activity with CDV, similar methodology was utilized to produce 3-hexadecyloxy-l-propanol CDV or cyclic CDV (HDP-CDV, HDP-cCDV) (Kern et al., 2002). A comparison of the activity against vaccinia, cowpox, monkeypox, and variola viruses between the parent nucleotides, CDV, cCDV, and the alkoxyalkyl ester analogs is summarized in Table 4. In general, it took 25–50 uM of CDV or cCDV to inhibit the replication of the four orthopoxviruses tested, whereas the HDP-CDV and HDP-cCDV analogs were active at levels of 50- to 200-fold less than the parent molecules. Although the cytotoxicity of the analogs was also increased, the selectivity index of HDP-CDV or HDP-cCDV was increased considerably over that of the parent compounds (Kern et al., 2002). The HDP-CDV is very active orally in mice resulting in plasma levels that are well below those required to inhibit orthopoxvirus replication in vitro (Winegarden et al., 2002). The HDP-CDV has been evaluated in animal models using vaccinia virus and cowpox virus infection of mice and was active orally (Kern, unpublished results) and in cowpox virus-infected mice when given orally or by aerosol (Huggins et al., 2002).

Table 4
Activity of cidofovir, cyclic cidofovir and their alkoxyalkyl derivatives against vaccinia, cowpox, monkeypox and variola viruses

Compound	EC_{50} (μM)			
	Vaccinia[a]	Cowpox[a]	Monkeypox[b]	Variola[b]
CDV	46.2 ± 11.9	44.7 ± 6.3	4.6	27.3
HDP-CDV	0.8 ± 0.4	0.6 ± 0.3	0.07	0.1
CCDV	50.6 ± 13.1	48.3 ± 8.0	63.6	45.8
HDP-cCDV	3.8 ± 1.5	2.1 ± 1.9	1.8	0.9

[a] Kern et al. (2002).
[b] Huggins et al. (2002).

Table 5
Efficacy and cytotoxicity of acyclic nucleoside phosphonates against vaccinia virus and cowpox virus in HFF cells

Compound	GS number[a]	Vaccinia virus EC_{50} (μM)	Cowpox virus EC_{50} (μM)	Cytotoxicity CC_{50} (μM)
HPMPC (CDV)	GS 0504	33 ± 9.1	43 ± 2.5	278 ± 9.2
Cyclic HPMPC (cHPMPC)	GS 0930	38 ± 11	48 ± 8.0	>302 ± 0
Butyl salicyl cyclic HPMPC	GS 3857	32 ± 13	34 ± 4.2	>213 ± 22
Phenethyl alaninyl cyclic HPMPC—mixture	GS 7356	7.1 ± 0.3	6.8 ± 1.8	207 ± 18
Ethyl alaninyl cyclic HPMPC—isomer I	GS 7357	15 ± 13	27 ± 6.5	>276 ± 23
Ethyl alaninyl cyclic HPMPC—isomer II	GS 7358	15 ± 11	26 ± 3.5	>278 ± 0
HPMPA	GS 0577	3.5 ± 2.8	5.0 ± 4.7	269 ± 21
PMEA	GS 0393	>366 ± 0	>366 ± 0	>366 ± 0
Bis(pivaloyloxymethyl)PMEA	GS 0840	5.1 ± 0.7	13 ± 8.8	117 ± 27
Bis(butylalaninyl)PMEA	GS 8357	4.4 ± 0.2	10 ± 8.2	100 ± 27
PMEDAP	GS 0573	204 ± 15	>347 ± 0	>339 ± 12
PME(N^6-cyclopropyl)DAP	GS 8369	23 ± 6.9	28 ± 13	>263 ± 59
Bis(butylalaninyl) PME(N^6-cyclopropyl)DAP	GS 8361	0.08 ± 0.01	0.26 ± 0.2	49 ± 33
PMPA	GS 1278	>300	>300	>300
Bis(isopropoxycarbonyloxymethyl)PMPA	GS 4331	>157	>157	>157
Isopropyl alaninylphenyl PMPA	GS 7340	23.5	98.9	>143
PMEG	GS 8358	4.0 ± 0.7	11.4 ± 1.3	88

[a] Gilead sciences.

5. Evaluation of additional phosphonate nucleotides for activity against vaccinia and cowpox virus in vitro

Since CDV is one of the few well characterized compounds with good activity against the orthopoxviruses, we have also evaluated a large number of other phosphonate nucleotides for their activity against vaccinia and cowpox virus replication in vitro, and the results are presented in Table 5. A number of these compounds have been described previously by De Clercq and his colleagues and are reviewed by De Clercq (2001). In the CDV {HPMPC [(*S*)-1(3-hydroxy-2-phosphonylmethoxypropyl) cytosine]} series, there are a number of prodrugs that have equal or greater activity against vaccinia and cowpox than CDV, however, little is known about their toxicity or pharmacokinetics at the present time. The adenine analog HPMPA [(*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine] was about 10-fold more active than CDV, an observation that confirms earlier data of De Clercq et al. (1987). In the PMEA [9-(2-phosphonylmethoxyethyl)adenine] (adefovir) series, PMEA itself was not active but an orally active prodrug [bis(pivaloyloxymethyl)PMEA] (adefovir dipivoxil) was very active as was bis(butylalaninyl)PMEA. Of special interest is the fact that both HPMPA and the bis(pivaloyloxymethyl)PMEA were recently reported to have activity against monkeypox and variola viruses at concentrations similar to those reported here for vaccinia and cowpox virus (Baker et al., 2002). Similarly, with the 2-phosphonylmethoxyethyl diaminopurine analog of PMEA (PMEDAP), the parent compound was inactive, whereas a prodrug bis(butylalaninyl) PME(N^6-cyclopropyl)DAP was the most active phosphonate we have tested. In the (*R*)-9-(2-phosphonylmethoxypropyl)adenine (PMPA) (tenofovir) series, the parent compound as well as the bis(isopropoxycarbonyloxymethyl)PMPA (tenofovir disoproxil fumarate), which was recently approved for the treatment of HIV infection in humans, were inactive against vaccinia and cowpox viruses. In contrast, the 2-phosphonylmethoxyethyl guanine (PMEG) analog was very active. In this latter group of compounds, it is interesting that compounds such as tenofovir (PMPA) and adefovir (PMEA) that have activity against HIV or HBV are not active against vaccinia or cowpox viruses. However, they may become active if certain modifications are made that either increase oral activity or absorption, as seen with adefovir dipivoxil in the PMEA series but not with the PMPA prodrugs. From our findings it appears that the compounds that merit additional studies include HPMPA, adefovir dipivoxil, some of the prodrugs of PMEA, particularly bis(butylalaninyl) PME(N^6-cyclopropyl)DAP, as well as PMEG.

The major drawback to the development of one of these compounds is that little is known about their potential toxicology or their absorption, distribution, metabolism, or excretion in animals or humans and would require all the same steps necessary for development of a new drug.

6. Summary and comments

We have evaluated over 500 compounds for activity against vaccinia and cowpox viruses, two members of the orthopoxvirus group most often used as surrogate viruses for variola virus. Our attempts to identify antiviral compounds that have potential for use in the event of a smallpox outbreak due to an act of a bioterrorism or perhaps against an outbreak of monkeypox either naturally occurring or spread purposefully, have identified a few candidate compounds.

The most likely candidate is clearly CDV; it has good activity in tissue culture assays and in animal model infections with vaccinia and cowpox virus infections. Importantly, it is

already approved for use in humans for cytomegalovirus infection and would be available for use under an IND in the case of emergency need. Its major limitation is its lack of oral activity, providing some logistical problems in its delivery. The drug has also been shown to be effective when delivered by aerosol in mice infected with cowpox, and this may be one way to improve its delivery in an emergency situation. The toxicity noted in the treatment of cytomegalovirus infections may not be a major issue since the drug would be given only for a few times over a short time period.

A second compound that may warrant development is the acyclic nucleoside phosphonate prodrug adefovir dipivoxil. There is a lot of clinical toxicity data available (Barditch-Crovo et al., 1997; Benhamou et al., 2001) and the drug has been recently approved for HBV. This compound has greater activity in cell culture than CDV, but needs to be evaluated in murine models and in non-human primates.

Of the compounds tested that have not been in clinical trials, it appears that HDP-CDV may be an ideal candidate for development, since it is a prodrug of CDV and has excellent oral bioavailability and activity, and the collection of toxicological information available for CDV would be very useful in the development of HDP-CDV.

Lastly, the PMEDAP prodrug, bis(butylalaninyl)PME-N^6-(cyclopropyl)DAP has very potent activity in cell culture. However, animal studies need to be carried out before the potential of this compound can be realized.

It should be somewhat comforting to know that there is currently a compound, CDV, that could be used immediately in an emergency outbreak of smallpox and that there are additional compounds that may be superior to CDV particularly if given orally, and that can be developed in a fairly rapid time frame.

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