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ScienceDirect
Antiviral Research 73 (2007) 69–77
Antiviral Research
www.elsevier.com/locate/antiviral

Cell line dependency for antiviral activity and in vivo efficacy of *N*-methanocarbathymidine against orthopoxvirus infections in mice

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Received 23 December 2005; accepted 13 April 2006

Abstract

A novel carbocyclic thymidine analog, *N*-methanocarbathymidine [(*N*)-MCT], was evaluated for inhibition of orthopoxvirus infections. Efficacy in vitro was assessed by plaque reduction assays against wild-type and cidofovir-resistant strains of cowpox and vaccinia viruses in nine different cell lines. Minimal differences were seen in antiviral activity against wild-type and cidofovir-resistant viruses. (*N*)-MCT's efficacy was affected by the cell line used for assay, with 50% poxvirus-inhibitory concentrations in cells as follows: mouse = 0.6–2.2 μM, rabbit = 52–90 μM, monkey = 87 to >1000 μM, and human = 39–220 μM. Limited studies performed with carbocyclic thymidine indicated a similar cell line dependency for antiviral activity. (*N*)-MCT did not inhibit actively dividing uninfected cells at 1000 μM. The potency of (*N*)-MCT against an S-variant thymidine kinase-deficient vaccinia virus was similar to that seen against S-variant and wild-type viruses in mouse, monkey, and human cells, implicating a cellular enzyme in the phosphorylation of the compound. Mice were intranasally infected with cowpox and vaccinia viruses followed 24 h later by intraperitoneal treatment with (*N*)-MCT (twice a day for 7 days) or cidofovir (once a day for 2 days). (*N*)-MCT treatment at 100 and 30 mg/kg/day resulted in 90 and 20% survival from cowpox virus infection, respectively, compared to 0% survival in the placebo group. Statistically significant reductions in lung virus titers on day 5 occurred in 10, 30, and 100 mg/kg/day treated mice. These same doses were also active against a lethal vaccinia virus (WR strain) challenge, and protection was seen down to 10 mg/kg/day against a lethal vaccinia virus (IHD strain) infection. Cidofovir (100 mg/kg/day) protected animals from death in all three infections.

Keywords: Vaccinia virus; Cowpox virus; (*N*)-MCT; Carbocyclic thymidine; Cidofovir; Antiviral; Thymidine kinase

1. Introduction

Due to concerns about using variola (smallpox) or monkeypox viruses as bioterrorism agents, treatment options for diseases caused by these viruses are being sought (Breman and Henderson, 1998, 2002). Such treatments may also have application in treating complications due to smallpox vaccination (live vaccinia virus) (Bray, 2003), molluscum contagiosum (Davies et al., 1999; Silverberg, 2003; Baxter and Highet, 2004), or parapoxvirus virus infections (Nettleton et al., 2000; De Clercq, 2002). A number of compounds have shown efficacy against orthopoxvirus infections (reviewed by Smee and Sidwell, 2003). Cidofovir is the most potent agent tested to date in mouse models of infection with cowpox and vaccinia viruses (Bray et al., 2000; Smee et al., 2001a,b; Quenelle et al., 2003), but suffers from renal toxicity and very low oral bioavailability (Wachsman et al., 1996). Orally active prodrugs of cidofovir exhibiting reduced toxicity are being investigated that hold promise against poxvirus infections (Quenelle et al., 2004; Smee et al., 2005).

Carbocyclic thymidine was reported to exhibit antiviral activity against vaccinia virus in cell culture (Beres et al., 1990). A related compound, *N*-methanocarbathymidine [(*N*)-MCT] (Fig. 1), was synthesized and found to inhibit herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in vitro (Marquez et al., 1996; Zalah et al., 2002; Russ et al., 2003; Huleihel et al., 2005). The activity of the compound was due to phosphorylation by the herpes thymidine kinase (Zalah et al., 2002; Marquez et al., 2004; Schelling et al., 2004) and subsequent inhibition of viral DNA polymerase activity (Marquez et al., 2004).

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doi:10.1016/j.antiviral.2006.04.010

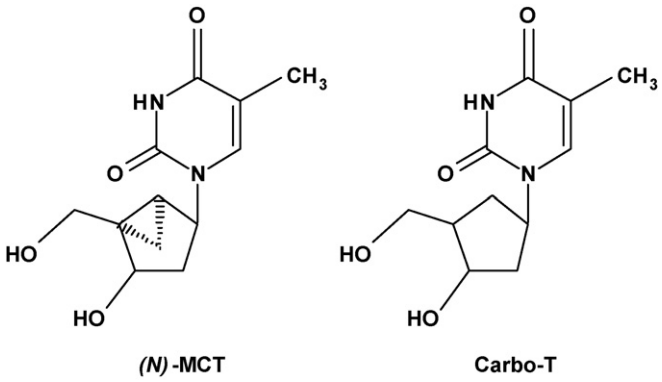

Fig. 1. Structures of *N*-methanocarbathymidine [(*N*)-MCT] and carbocyclic thymidine (Carbo-T).

A recent publication by Prichard et al. (2006a) demonstrated the activity of (*N*)-MCT against the orthopoxviruses vaccinia and cowpox in vitro, as well as its efficacy in mice infected intranasally with these viruses. In comparing the cell culture antiviral activity of (*N*)-MCT against thymidine kinase positive and negative cowpox viruses, the investigators concluded that (*N*)-MCT is phosphorylated by this viral enzyme (Prichard et al., 2006a), which may be the basis for its selectivity since the compound is not toxic to cells well above virus-inhibitory concentrations.

(*N*)-MCT was evaluated in our laboratory for antiviral activity against two orthopoxviruses, cowpox and vaccinia, in cell culture and in mice. Comparisons were also made in vitro using vaccinia virus thymidine kinase positive and negative strains. The conclusions we draw regarding the animal studies are similar to those of Prichard et al. (2006a), but differ in terms of the role that poxvirus thymidine kinase may play relative to phosphorylation of the compound. In addition, we present the efficacy of (*N*)-MCT in a variety of cell lines and point out concerns due to the low efficacy of the compound in cells of higher animal species.

2. Materials and methods

2.1. Antiviral compounds

N-methanocarbathymidine or (north)-methanocarbathymidine (Marquez et al., 1996; Zalah et al., 2002), also referred to as *N*-methanocarbathymine or (north)-methanocarbathymine (Russ et al., 2003), was provided through the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). The synthesis of the compound has been published (Marquez et al., 1996). Carbocyclic thymidine was synthesized by the published method (Ötvos et al., 1987). 5-Bromo-2′-deoxyuridine was purchased from Sigma (St. Louis, MO). Mick Hitchcock of Gilead Sciences (Foster City, CA) kindly provided cidofovir. The compounds were dissolved in cell culture medium or in saline for injection into mice. Saline served as the placebo control for animal studies.

2.2. Viruses and cells

Cowpox virus (Brighton strain) was obtained from John Huggins at the US Army Medical Research Institute of Infectious Diseases (USAMRIID, Ft. Detrick, Frederick, MD). Joseph Esposito of the Centers for Disease Control and Prevention (Atlanta, GA) originally provided this virus to USAMRIID. A syncytium-forming variant of the virus was isolated by plaque isolation as previously described (Smee et al., 2002b). Vaccinia virus (WR and IHD strains) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Vaccinia virus (WR S-variant strain) and vaccinia virus (WR S-variant vTK-79 strain, a thymidine kinase-deficient form of the WR S-variant strain) were also obtained from ATCC. High titer virus pools were prepared in African green monkey kidney (MA-104) cells purchased from BioWhittaker (Walkersville, MD). Cidofovir-resistant viruses were originally prepared by cell culture passage in the presence of increasingly higher concentrations of the drug in Vero 76 cells (for syncytium-forming cowpox virus) or Vero cells (for vaccinia virus), as described previously (Smee et al., 2002b, 2005). The African green monkey kidney (Vero) cells used in the present studies were obtained from ATCC. Other cell lines used for the research obtained from ATCC were mouse mammary tumor (C172I) cells, mouse connective tissue (NCTC clone 929 or L929) cells, rabbit kidney (RK-13) cells, African green monkey kidney (CV-1) cells, rhesus monkey kidney (LLC-MK_2), human lung carcinoma (A549) cells, and human embryonic lung (MRC-5) cells. Cell culture medium for propagating C127I, L929, RK-13, CV-1, LLC-MK_2, and MA-104 cells was MEM with 10% fetal bovine serum (FBS) and 0.18% sodium bicarbonate. MEM with 5% FBS and 0.18% bicarbonate was used to grow Vero cells. Ham's F12K medium containing 10% FBS, 2 mM glutamine, and 0.15% bicarbonate was used for A549 cells. Basal media eagle (BME) containing 10% FBS and 0.18% bicarbonate was the growth medium for MRC-5 cells. For antiviral assays the appropriate medium for each type of cell culture contained 50 μg/ml of gentamicin, and the FBS concentration was reduced to 2%.

2.3. Cell culture antiviral and cytotoxicity studies

Sensitivities of the poxviruses to (*N*)-MCT in the nine cell lines were determined by plaque reduction assays in 12-well microplates. Cells were infected with about 100 plaque-forming units (PFU) of virus per well; the virus in 0.2 ml volume was adsorbed for 0.5–1 h then removed, and two-fold dilutions of compound were applied to wells. Incubation times were generally 2 days for A549, CV-1, MA-104, and MRC-5 cells, and 3 days for C127I, L929, RK-13, LLC-MK_2, and Vero cells to obtain comparably sized plaques. Cells were fixed and stained in 10% buffered formalin/0.2% crystal violet for 10 min. Plaques were counted using a Plaque Viewer (Bellco, Vineland, NJ). The concentration of compound causing a 50% reduction in virus plaque numbers (EC_{50}) was determined by plotting concentration versus percent of control on semilog graph paper.

Cytotoxicity determinations were made using uninfected cells treated with compound in growth medium appropriate for each cell line (5% FBS for Vero cells, 10% FBS for the other cells). The actively dividing cell assay consisted of seeding 24-well microplates with approximately 1–2 × 10^4 cells/well and letting them attach overnight. Then compounds were added at varying half-$\log_{10}$ dilutions. After 3 days of incubation the cells were subjected to 0.011% neutral red staining for 2 h as previously described (Smee et al., 2001c). The eluted dye from each well was transferred to a 96-well plate in order to be read optically for determination of 50% inhibitory concentration (IC_{50}) values. This assay is much more sensitive to detect toxicity than the stationary cell monolayer toxicity assay frequently used by researchers.

Data for the above in vitro studies represent mean values ± standard deviations for three independent assays, except for experiments with carbocyclic thymidine performed in Vero and A549 cells. The results from these evaluations are from two independent assays due to limited amounts of compound, also since concentrations up to 1000 μM were tested.

In special studies performed in C127I and LLC-MK_2 cells, thymidine or 2′-deoxycytidine (both obtained from Sigma) at varying half-$\log_{10}$ concentrations were added to the culture medium of infected cells treated with 3.2 μM (C127I cells) or 1000 μM (LLC-MK_2 cells) of (*N*)-MCT. Then standard plaque reduction assays were performed. The concentrations of (*N*)-MCT that were chosen were previously shown to be 80–100% inhibitory to virus plaque formation. These assays were done to determine whether these natural nucleosides could reverse the antiviral activity of (*N*)-MCT.

2.4. Mouse infection studies

BALB/c mice (13–15 g) were purchased from Charles River Labs (Wilmington, MA). They were quarantined 48 h before use. Experiments with the mice were done using an infectious virus challenge of 5 × 10^5 PFU of the normal form of cowpox virus (producing rounded-cell cytopathology as opposed to syncytia) (Smee et al., 2002b) or 1 × 10^5 PFU/mouse of vaccinia virus (WR and IHD strains) (Smee et al., 2001a,b, 2004), administered intranasally in a 50 μl volume following anesthesia with ketamine (100 mg/kg given by intraperitoneal (i.p.) injection). Infection under anesthesia (as opposed to application to unanesthetized animals) allowed the infectious fluid to penetrate deeper into the lungs, and less virus was required for achieving a 50% lethal dose. Treatments with compounds were given starting 24 h after virus exposure. (*N*)-MCT was administered i.p. twice a day for 7 days and cidofovir was given once a day i.p. for 2 days. Short-term treatments with cidofovir are sufficient to provide protection from these infections (Smee et al., 2001a,b, 2004). There were 10 drug-treated mice per group held for death and 5 mice per group for virus titer determinations. The placebo groups had 10–20 mice held for death. On day 5 of the infection, lungs from sacrificed mice were collected, weighed, and frozen at −80 °C for later virus titrations. Virus titers from these samples were determined by plaque assay in Vero cells, as described previously (Smee et al., 2001a).

2.5. Statistical methods

Statistical comparisons were made of the compound-treated groups to the placebo control by two-tailed analyses. The Fisher exact test was used to interpret differences in numbers of survivors. Mean day of death and lung virus titer differences were statistically analyzed by the Mann–Whitney *U*-test. Calculations were made using the InStat computer program (GraphPad Software, San Diego, CA).

3. Results

3.1. Antiviral activity and cytotoxicity of (N)-MCT

(*N*)-MCT was evaluated for antiviral activity in nine cell lines of mouse, rabbit, monkey, and human origin (Table 1). The compound was highly active in two mouse cell lines, with EC_{50} values of 0.6–2.2 μM against wild-type and cidofovir-resistant cowpox virus and vaccinia viruses. (*N*)-MCT was much less effective in rabbit and human cells, with EC_{50} values against the four viruses varying from 52 to 90 μM in rabbit cells, and 44 to 220 μM in human cells. The least antiviral potency was seen in monkey cells, with no inhibition of virus seen in MA-104 cells and minimal activity seen in Vero cells. EC_{50}'s in rhesus monkey kidney (LLC-MK_2) cells (87–183 μM) were lower than in the three African green monkey kidney (CV-1, MA-104, and Vero) cell lines (250 to >1000 μM). The degree of difference in antiviral activity of (*N*)-MCT in mouse cells compared to monkey cells (particularly MA-104 and Vero) was remarkable. Overall, vaccinia virus was inhibited to a greater degree than cowpox virus, except in mouse cells, where nearly the same inhibition values were measured and in MA-104 and Vero cells where little activity was seen. For each cell line, minimal differences were observed in inhibition of wild-type versus cidofovir-resistant viruses. The greatest difference was seen with cowpox virus in L929 cells where wild-type virus was inhibited at 0.6 μM and the resistant virus was inhibited at 1.7 μM. Because many of the other cell lines showed minimal differences in inhibition of the two viruses, it is concluded that the cidofovir-resistant viruses are not cross-resistant to (*N*)-MCT.

In cytotoxicity assays, (*N*)-MCT was not inhibitory to the growth of actively dividing cells at 1000 μM (Table 1). Stationary cell monolayers were also unaffected by compound treatment as judged microscopically. Because of the low cytotoxicity of (*N*)-MCT, selectivity index (SI) values were high against these poxviruses in mouse cells, but considerably lower in the other cell lines (Table 1).

3.2. Activities of compounds against a TK-deficient vaccinia virus

(*N*)-MCT was reported to be phosphorylated by HSV-1 thymidine kinase (TK) (Zalah et al., 2002; Marquez et al., 2004; Schelling et al., 2004). It was also shown to be more potently inhibitory to wild-type than to thymidine kinase-deficient cowpox virus (Prichard et al., 2006a), suggesting a role of the orthopoxvirus enzyme in (*N*)-MCT phosphorylation. To further

Table 1
Antiviral activity and cytotoxicity of (*N*)-MCT in various cell lines and against wild-type (WT) and cidofovir-resistant (R) strains of cowpox and vaccinia viruses

Cell line (origin)	Virus	EC_{50}[a] (μM)	IC_{50}[b] (μM)	SI[c]
C127I (mouse)	Cowpox-WT[d]	1.0 ± 0.3	>1000	>1000
	Cowpox-R[d]	2.2 ± 0.9		>455
	Vaccinia-WT	0.9 ± 0.3		>1110
	Vaccinia-R	1.7 ± 0.4		>590
L929 (mouse)	Cowpox-WT	0.6 ± 0.1	>1000	>1670
	Cowpox-R	1.7 ± 0.5		>590
	Vaccinia-WT	0.8 ± 0.2		>1250
	Vaccinia-R	0.8 ± 0.2		>1250
RK-13 (rabbit)	Cowpox-WT	90 ± 44	>1000	>11
	Cowpox-R	85 ± 26		>12
	Vaccinia-WT	63 ± 16		>16
	Vaccinia-R	52 ± 10		>19
CV-1 (monkey)	Cowpox-WT	447 ± 42	>1000	>2.2
	Cowpox-R	472 ± 30		>2.1
	Vaccinia-WT	250 ± 114		>4
	Vaccinia-R	263 ± 78		>3.8
LLC-MK_2 (monkey)	Cowpox-WT	167 ± 42	>1000	>6
	Cowpox-R	183 ± 50		>5.5
	Vaccinia-WT	105 ± 23		>9.5
	Vaccinia-R	87 ± 31		>11
MA-104 (monkey)	Cowpox-WT	>1000	>1000	0
	Cowpox-R	>1000		0
	Vaccinia-R	>1000		0
	Vaccinia-WT	>1000		0
Vero (monkey)	Cowpox-WT	>1000	>1000	0
	Cowpox-R	>1000		0
	Vaccinia-WT	620 ± 60		>1.6
	Vaccinia-R	670 ± 95		>1.5
A549 (human)	Cowpox-WT	130 ± 58	>1000	>6
	Cowpox-R	220 ± 80		>4.5
	Vaccinia-WT	207 ± 21		>4.8
	Vaccinia-R	216 ± 32		>4.6
MRC-5 (human)	Cowpox-WT	67 ± 17	>1000	>15
	Cowpox-R	72 ± 25		>14
	Vaccinia-WT	44 ± 5		>23
	Vaccinia-R	39 ± 10		>26

[a] Fifty percent effective (virus-inhibitory) concentration ± S.D. determined by plaque assay.
[b] Fifty percent cell-inhibitory concentration ± S.D. of actively dividing uninfected cells.
[c] Selectivity index (IC_{50} divided by EC_{50}).
[d] Syncytium-forming cowpox virus as described in Section 2.

test this hypothesis, (*N*)-MCT was evaluated for activity in three cell lines infected with an S-variant TK-deficient vaccinia virus (vTK-79 strain) and its activity compared to efficacy against wild-type WR strain virus and the S-variant virus from which the TK-deficient virus was derived. 5-Bromo-2′-deoxyuridine, reported to be phosphorylated by cowpox virus TK by virtue of being much less inhibitory to a TK-deficient strain of the virus than wild-type virus (Prichard et al., 2006b), was also tested in parallel as a control to validate the TK-deficient nature of the vTK-79 strain of vaccinia virus. Cidofovir was also included in the experiments, since it is not dependent upon monophosphorylation for activity (the drug already contains a phosphonate substituent as part of its structure). The activity of (*N*)-MCT was similar against vaccinia wild-type, S-variant, and TK-deficient viruses when tested in the three different cell lines (Table 2). These results implicate a cellular enzyme in the phosphorylation of (*N*)-MCT and not viral TK. 5-Bromo-2′deoxyuridine was less potent against TK-deficient virus than against wild-type and S-variant viruses when assayed in monkey and human cells. However, 5-bromo-2′-deoxyuridine showed similar inhibitory activity against these three virus types when assayed in mouse cells, suggesting that phosphorylation by cellular enzymes was sufficient for antiviral activity in murine cells. Cidofovir was similarly active against the three viruses, although the potency in mouse cells was greater than in other cells, as was expected based upon the published literature (Smee et al., 2001c, 2002a, 2004).

3.3. Effects of thymidine and 2′-deoxycytidine on the antiviral activity of (N)-MCT

An experiment was conducted to determine whether thymidine or 2′-deoxycytidine could reverse the antiviral activity of (*N*)-MCT in murine cell cultures. Such a study would determine whether (*N*)-MCT was acting as a thymidine analog, a 2′-deoxycytidine analog, or both, with regard to being phosphorylated or acting as an enzyme inhibitor. Fig. 2A depicts the effects of thymidine present in the culture medium of C127I mouse cells on (*N*)-MCT activity. In the absence of thymidine, 3.2 μM (*N*)-MCT completely inhibited virus plaque formation. As the thymidine concentration increased, the ability of (*N*)-MCT to inhibit viral plaques decreased. A similar study was conducted with 2′-deoxycytidine combined with 3.2 μM (*N*)-MCT. In this experiment there was no reversal of viral plaques (i.e., (*N*)-MCT was 100% inhibitory to virus plaque formation), even when (*N*)-MCT-treated cells were simultaneously exposed to 100 μM 2′-deoxycytidine (data not shown).

A similar experiment was performed using monkey LLC-MK_2 cells, requiring a high (*N*)-MCT concentration of 1000 μM to reduce plaque numbers adequately (Fig. 2B). However, in this experiment low doses of thymidine caused reversal of (*N*)-MCT activity. In contrast, up to 100 μM of 2′-deoxycytidine did not reverse the antiviral activity of the compound (data not shown). The results in both mouse C127I and monkey LLC-MK_2 cells suggest that (*N*)-MCT behaves as a thymidine analog.

3.4. Antiviral activity and cytotoxicity of carbocyclic thymidine

Since (*N*)-MCT is an analog of carbocyclic thymidine and this compound was reported to possess anti-vaccinia virus activity (Beres et al., 1990), we determined whether the latter compound also had the highly unusual cell line-dependent antiviral profile exhibited by (*N*)-MCT. One cell line each of mouse, monkey, and human origin was used to assess the antiviral activity of carbocyclic thymidine (Table 3). A marked antiviral activity in mouse cells was observed in these studies, with activity seen at <0.1 μM. Carbocyclic thymidine was much more toxic to

Table 2
Comparative antiviral activities of (*N*)-MCT, 5-bromo-2′-deoxyuridine (5-Brd-Urd), and cidofovir against wild-type (WT), S-variant, and thymidine kinase-deficient (S-variant TK⁻) strains of vaccinia (WR strain) virus

Compound	Cell line (origin)	EC_{50}[a] (μM) against			Activity ratio[b]
		WT virus	S-variant virus	S-variant TK⁻ virus	
(*N*)-MCT	C127I (mouse)	0.9 ± 0.3	0.5 ± 0.1	0.6 ± 0.1	1.2
	Vero (monkey)	620 ± 60	480 ± 70	310 ± 170	0.6
	A549 (human)	207 ± 21	156 ± 26	147 ± 29	0.9
5-Br-dUrd	C127I	0.9 ± 0.5	0.7 ± 0.4	0.5 ± 0.2	0.7
	Vero	5.8 ± 1.4	10.8 ± 1.3	48 ± 22	4.4
	A549	4.8 ± 1.8	5.0 ± 0.6	30 ± 8	6.0
Cidofovir	C127I	4.9 ± 1.7	3.7 ± 2.5	1.7 ± 0.2	0.5
	Vero	79 ± 9	46 ± 5.3	61 ± 8	1.3
	A549	65 ± 9	35 ± 9	45 ± 8	1.3

[a] Fifty percent effective (virus-inhibitory) concentration ± S.D. determined by plaque assay.
[b] EC_{50} of S-variant TK⁻ virus divided by EC_{50} of S-variant virus.

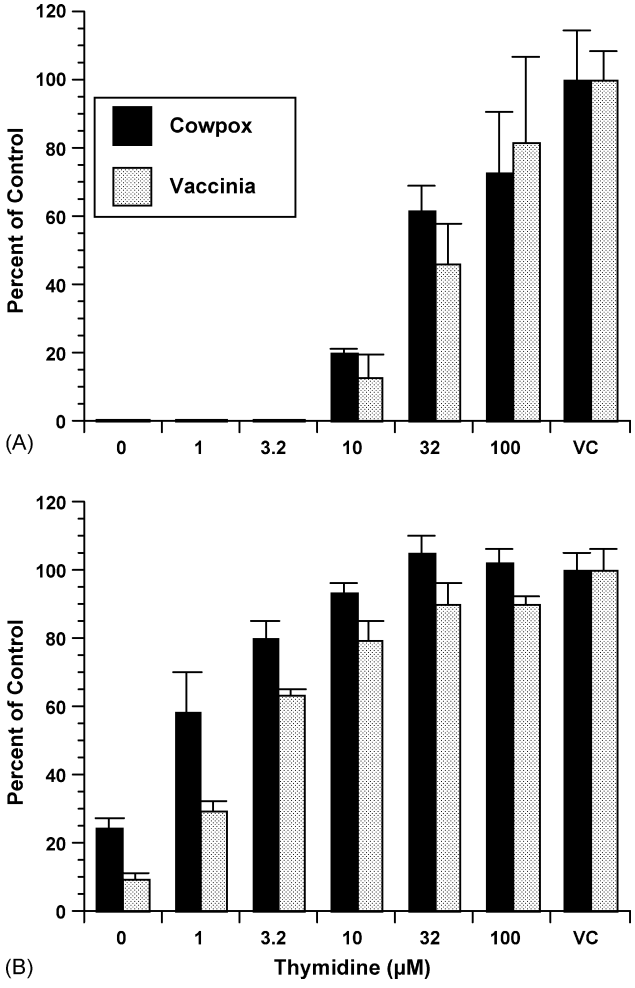

Fig. 2. Reversal of the antiviral activity of (*N*)-MCT by thymidine in C127I (A) and LLC-MK_2 (B) cells. (*N*)-MCT was present in the culture medium at 3.2 μM in C127I cells and at 1000 μM in LLC-MK_2 cells, with varying concentrations of thymidine. VC = virus control (no nucleosides present in medium). Results are from separate experiments, with mean values ± S.D. calculated from duplicate wells.

mouse cells than (*N*)-MCT, but still exhibited a selectivity index of 82–174 in those cells because of its high degree of antiviral potency. Viral inhibition in monkey and human cells was considerably less than in mouse cells (Table 3). Toxicity to actively dividing cells was evident at or below the antivirally active doses. These results demonstrate that carbocyclic thymidine and (*N*)-MCT share the common property of being highly active in mouse cells but not in the primate cells studied.

3.5. Treatment of poxvirus-infected mice with (N)-MCT

The efficacy of (*N*)-MCT was evaluated in a cowpox virus respiratory infection in mice (Table 3). Treatment with compound for 7 days at 100 mg/kg/day resulted in a survival rate of 90%, compared to no survival in the placebo group. Lower doses of 30 and 10 mg/kg/day did not significantly protect mice from the lethal infection, although increases in the mean day of death were evident. (*N*)-MCT treatments at the three doses significantly reduced lung virus titers. Cidofovir at 100 mg/kg/day given for 2 days protected all mice from death and significantly reduced lung virus titers. Treatment with (*N*)-MCT was not overtly toxic to uninfected mice (Table 4), although the animals treated

Table 3
Antiviral activity and cytotoxicity of carbocyclic thymidine in three cell lines

Cell line (origin)	Virus	EC_{50}[a] (μM)	IC_{50}[b] (μM)	SI[c]
C127I (mouse)	Cowpox-WT[d]	0.098 ± 0.045	8.0 ± 2.0	>82
	Vaccinia-WT	0.046 ± 0.004		>174
Vero (monkey)	Cowpox-WT	550 ± 15	158 ± 88	<1
	Vaccinia-WT	550 ± 15		<1
A549 (human)	Cowpox-WT	305 ± 148	440 ± 14	1.4
	Vaccinia-WT	245 ± 219		1.8

[a] Fifty percent effective (virus-inhibitory) concentration ± S.D. determined by plaque assay. Values are from two (Vero and A549 cells) or three (C127I cells) independent assays.
[b] Fifty percent cell-inhibitory concentration ± S.D. of actively dividing uninfected cells. Values are from two (Vero and A549 cells) or three (C127I cells) independent assays.
[c] Selectivity index (IC_{50} divided by EC_{50}).
[d] Syncytium-forming cowpox virus as described in Section 2.

Table 4
Effects of intraperitoneal treatment with (*N*)-MCT and cidofovir on a cowpox virus respiratory infection in mice

Compound[a] (mg/kg/day)	Treatment days	Uninfected toxicity controls		Infected, treated		
		Survivors/total	Mean host wt. change[b] (g)	Survivors/total	MDD[c]	Mean lung virus titer[d]
(*N*)-MCT (100)	1–7	5/5	+0.2	9/10***	11.0	7.0 ± 0.3**
(*N*)-MCT (30)	1–7	4/4	+0.9	2/10	11.0 ± 1.8**	7.1 ± 0.2**
(*N*)-MCT (10)	1–7	5/5	+1.0	1/10	10.4 ± 1.0**	7.0 ± 0.3**
Cidofovir (100)	1–2	5/5	+1.1	10/10***	>21***	6.7 ± 0.2**
Placebo	1–7	5/5	+1.7	0/10	8.5 ± 0.5	7.9 ± 0.2

[a] (*N*)-MCT was administered twice daily (in half-daily doses given at 8:30 a.m. and 4:30 p.m.) and cidofovir was given once a day, each starting 24 h after virus exposure.
[b] Weight gain between days 0 and 8 of the infection.
[c] Mean day of death ± S.D. of mice that died prior to day 21.
[d] Log_{10} PFU/gram ± S.D., determined on day 5 of the infection.
** $P < 0.01$.
*** $P < 0.001$.

Table 5
Effects of intraperitoneal treatment with (*N*)-MCT and cidofovir on vaccinia virus (WR and IHD strains) respiratory infections in mice

Compound[a] (mg/kg/day)	Treatment days	Survivors/total	MDD[b]	Mean lung virus titer[c]
Vaccinia (WR strain) infection				
(*N*)-MCT (100)	1–7	7/10***	8.3 ± 0.6	7.9 ± 0.2**
(*N*)-MCT (30)	1–7	4/10*	11.2 ± 4.7***	8.2 ± 0.1**
(*N*)-MCT (10)	1–7	1/10	9.6 ± 2.6***	8.3 ± 0.2**
Cidofovir (100)	1–2	9/10***	8.0	7.0 ± 0.2**
Placebo	1–7	1/20	7.9 ± 1.8	8.7 ± 0.1
Vaccinia (IHD strain) infection				
N-MCT (100)	1–7	9/9***	>21***	7.1 ± 0.3**
N-MCT (30)	1–7	10/10***	>21***	8.1 ± 0.1**
N-MCT (10)	1–7	10/10***	>21***	8.3 ± 0.1**
Cidofovir (100)	1–2	10/10***	>21***	4.7 ± 0.6**
Placebo	1–7	4/20	8.2 ± 0.5	8.6 ± 0.2

[a] (*N*)-MCT was administered twice daily (in half-daily doses given 12 h apart) and cidofovir was given once a day, each starting 24 h after virus exposure.
[b] Mean day of death ± S.D. of mice that died prior to day 21.
[c] Log_{10} PFU/g ± S.D., determined on day 5 of the infection.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

with the 100 mg/kg/day dose gained less weight than other mice.

In a second experiment, mice were infected intranasally with vaccinia virus (WR strain) and treated with (*N*)-MCT and cidofovir (Table 5). (*N*)-MCT at 100 and 30 mg/kg/day prevented death with survivor numbers similar to those of the cowpox virus infection. Increases in the mean day of death were seen in the 30 and 10 mg/kg/day groups. Cidofovir treatment kept 90% of animals alive. All three doses of (*N*)-MCT significantly reduced lung virus titers significantly, but not to the same extent as cidofovir. Also reported in Table 5 is a third experiment where compounds were administered to combat a vaccinia (IHD strain) virus infection. The IHD strain infection has previously been shown to be more readily treatable than the WR strain infection (Smee et al., 2004), as was seen here. All three doses of (*N*)-MCT protected mice from death, as did cidofovir at the dosage used. Twenty percent of placebo-treated mice survived, indicating the infection was not as severe as the WR strain infection. As was seen in the WR strain infection, all three doses of (*N*)-MCT significantly reduced lung virus titers significantly, but not to the same extent as cidofovir.

4. Discussion

(*N*)-MCT is an antiviral agent that is active not only against herpes simplex type 1 virus in vitro (Marquez et al., 1996; Zalah et al., 2002; Russ et al., 2003) but also against orthopoxviruses in cell culture and in mice (Prichard et al., 2006a; and this report). The activity of (*N*)-MCT in vitro against cowpox and vaccinia viruses was found to be highly cell culture dependent, with the greatest potency observed in mouse cell lines. The efficacy of (*N*)-MCT was markedly reduced in cells of higher species (rabbit, monkey, and human). Cidofovir also possesses greater potency in mouse cell lines than in monkey cells, but the degree of difference seen was much less dramatic (Table 2; Smee et al., 2001c, 2002a; Smee and Sidwell, 2004) than that observed for (*N*)-MCT. For example, cidofovir was effective against cowpox virus at 3.2 μM in mouse (3T3) cells and at 92 μM in mon-

key (Vero 76) cells (Smee et al., 2001c). The drug was active against vaccinia virus at 3.9 μM in 3T3 cells and at 37 μM in Vero 76 cells. In contrast, (*N*)-MCT exhibited minimal to no activity in Vero cells but was active at 1–2 μM in mouse cells. Because of this unusual observation, we tested three other monkey cell lines and found (*N*)-MCT to exhibit low potency. The antiviral activity of certain compounds was reported to be greater in MA-104 cells than in Vero cells due to higher levels of phosphorylation in MA-104 cells (Smee et al., 2001c), but in this case the activity of (*N*)-MCT was just as poor or worse in MA-104 cells. The unusual cell line dependency of (*N*)-MCT led us to speculate that this pattern of antiviral activity would apply to the related compound, carbocyclic thymidine. Carbocyclic thymidine was reported to exhibit anti-vaccinia virus activity (Beres et al., 1990) in primary rabbit kidney cell culture, but different cell lines were not investigated in that study. We found that carbocyclic thymidine was even more potent than (*N*)-MCT in mouse cells (although with greater toxicity than (*N*)-MCT) but was largely ineffective in monkey and human cells. Thus, the two compounds share a common cell line dependency.

The greater activity of antiviral compounds in mouse cells relative to other cell lines applies to many other antiviral nucleosides and nucleotides (Smee et al., 2001c, 2002a; Smee and Sidwell, 2004). Thus, the antiviral potency of (*N*)-MCT in mouse cells was not surprising. What was surprising, as emphasized above, was the degree of potency difference seen in mouse cells compared to other cell lines of higher animals, which may possibly translate into low efficacy of the compound in the treatment of higher animals infected with poxviruses. Prichard et al. (2006a) reported the activity of (*N*)-MCT to be 2–6 μM against vaccinia and cowpox virus infections in human foreskin fibroblasts, which is lower than what we report for human lung (MRC-5) cells. What we do not know is whether the antiviral potency of (*N*)-MCT in these neonatal cells compares to activity in cells of older human beings. Cell line dependence on potency occurs with other antiviral compounds against other viruses, an example being ribavirin (Huffman et al., 1973; Smee et al., 2001c). Ribavirin is more potent in mouse cells compared to other cells and has good in vivo efficacy, particularly against influenza virus infections in mice (Sidwell et al., 2005). Yet, the compound was not effective enough to be approved for the treatment of human influenza. Thus, lack of activity in cells of higher species might translate into low efficacy in humans.

(*N*)-MCT proved to be similarly effective against both cidofovir-sensitive and cidofovir-resistant viruses. Cidofovir's difference in potency between wild-type and cidofovir-resistant cowpox and vaccinia viruses was reported to be 13- to 22-fold (Smee et al., 2002b, 2005). The resistant viruses have altered DNA polymerases conferring drug resistance. Specific mutations in the DNA polymerase of cowpox virus have not yet been identified by gene sequencing, although the viral polymerase showed less sensitivity to cidofovir diphosphate than wild-type virus (Smee et al., 2002b). The vaccinia virus resistant to cidofovir has been sequenced, but the specific mutations in the viral DNA polymerase have not yet been published. The cell culture data suggest that (*N*)-MCT would most likely be effective in treating infections caused by cidofovir-resistant strains of these orthopoxviruses in mice, or possibly could be used in combination with cidofovir to suppress the emergence of drug resistant viruses in vivo.

The mode of action of (*N*)-MCT against orthopoxviruses is currently under investigation. The reversal studies reported here suggest that (*N*)-MCT is behaving as a thymidine analog, with little apparent association with 2′-deoxycytidine. We acknowledge the possibility that thymidine and (*N*)-MCT may compete extracellularly for the same receptor for cell uptake, and this could account for reduction in antiviral activity of (*N*)-MCT. In monkey LLC-MK_2 cells the amount of thymidine required to reverse the activity of (*N*)-MCT was quite low (Fig. 2B), giving support for our premise that (*N*)-MCT acts as a thymidine analog. The definitive studies to prove this will require evaluation of (*N*)-MCT as a substrate for thymidine kinase and as an inhibitor (as the triphosphate form) of the poxvirus DNA polymerase in competition with thymidine triphosphate.

The reversal studies suggest that cellular deoxycytidine kinase may not be involved in phosphorylation of (*N*)-MCT. Our results comparing the activity of the compound against wild-type and TK-deficient vaccinia viruses indicate that viral TK is not important for antiviral activity, thus implicating a cellular enzyme (presumably cellular TK) in its phosphorylation. In contrast to the results with (*N*)-MCT, 5-bromo-2′-deoxyuridine showed greater activity against the wild-type virus than TK-deficient virus in monkey and human cells, suggesting its dependence for phosphorylation by the viral TK for antiviral potency. Prichard et al. (2006b) reported greater potency of 5-bromo-2′-deoxyuridine against wild-type cowpox virus than against a TK-deficient viral strain in human foreskin fibroblasts. No dependence upon viral TK was seen with 5-bromo-2′-deoxyuridine against the vaccinia viruses we studied in mouse (C127I) cells, suggesting that mouse TK differs from monkey and human TK's in its ability to phosphorylate the nucleoside. We hypothesize that because mouse TK appears to differ from human and monkey TK's in substrate recognition (as evidenced from the data obtained here with 5-bromo-2′-deoxyuridine comparing replication of wild-type and TK-deficient viruses), this difference in cellular enzyme activity accounts for greater phosphorylation of (*N*)-MCT in mouse cells compared to monkey and human cells. This would explain the antiviral potency differences of the compound observed when different cell lines were used for assay. Further work will need to be performed to confirm this hypothesis.

There is the possibility that the S-variant TK-deficient vTK-79 strain of vaccinia virus (Panicali and Paoletti, 1982) that we used may contain one or more mutations in the viral DNA polymerase gene because it was derived by cell culture passage in the presence of 5-bromo-2′-deoxyuridine (Dennis Panicali, Therion Biologics, Cambridge, MA, personal communication). We would argue that if there are such mutations they are insignificant, and do not lead to decreased antiviral potency of 5-bromo-2′-deoxyuridine, as determined in mouse C127I cells. The fact that there are no appreciable differences seen in the activities of (*N*)-MCT and cidofovir against the wild-type, S-variant and S-variant TK-deficient vaccinia (WR strain) viruses also suggest

inconsequential mutations (if any) in the vaccinia virus DNA polymerase.

Regarding the necessity of poxvirus thymidine kinase to phosphorylate (*N*)-MCT, there are conflicting results between our data and those of Prichard et al. (2006a), whose data suggest that cowpox virus TK phosphorylates the compound, whereas our data do not suggest this for vaccinia virus TK. It is possible that cowpox and vaccinia virus TK's differ in their ability to phosphorylate (*N*)-MCT. Their cowpox virus wild-type and TK-deficient strains contain a green fluorescent protein lacZ insert and were assayed colorimetrically (Prichard et al., 2006a). The assay may not be equivalent in sensitivity to the plaque reduction assay that we used, and the insertion of the foreign gene may have altered the properties of these viruses. Thus, further experimentation will be required to sort out the role of poxviral TK in the phosphorylation of (*N*)-MCT.

Phosphorylation of (*N*)-MCT to the monophosphate form is considered a necessary step in metabolism to its antiviral state in infected cells. However, it is not yet known whether phosphorylation to the triphosphate occurs in poxvirus-infected cells. It has been reported that HSV-1 thymidine kinase phosphorylates (*N*)-MCT not only to the monophosphate but also to the diphosphate (Marquez et al., 2004), where cellular enzymes presumably convert the diphosphate to the triphosphate. Then the triphosphate inhibits the HSV-1 DNA polymerase (Marquez et al., 2004). In the case of poxviruses, cellular enzymes apparently would be required to convert (*N*)-MCT to di-, and triphosphate forms. Poxvirus replication not only can be inhibited at the DNA polymerase level by nucleoside triphosphate, but also indirectly via inhibition of cellular thymidylate synthetase by nucleoside monophosphate (De Clercq, 2001). The latter mode of action would also lead to cellular cytotoxicity. Because (*N*)-MCT exhibited a high degree of selectivity by virtue of very low cytotoxicity in our assays, these results tend to argue against inhibition of cellular thymidylate synthetase as a mode of antiviral action of the compound.

When mice were infected intranasally with cowpox and vaccinia viruses, they were effectively treated with (*N*)-MCT. Treatment of the vaccinia (IHD strain) virus infection with (*N*)-MCT was more protective than treatment of the vaccinia (WR strain) infection, as has been documented elsewhere using other test compounds (Smee et al., 2004). Prichard et al. (2006a) recently reported similar results of survival data using (*N*)-MCT in these infection models, although their publication did not include lung virus titer results. Our lung virus titer data indicated antiviral activity of (*N*)-MCT, but not to the same degree as cidofovir. What may be a more important matter to consider is whether (*N*)-MCT has potential for the treatment of human infections, and how this might be investigated based upon the low potency of the compound in human and monkey cells. Currently monkey models of monkeypox and variola (smallpox) virus infections are used to predict potential efficacy of compounds that may be beneficial for humans. Compounds inhibiting cowpox and vaccinia viruses in cell culture generally are similarly inhibitory to these other viruses (Smee et al., 2001c, 2002b; Baker et al., 2003), suggesting that (*N*)-MCT will be poorly active against monkeypox and variola viruses if assayed in monkey and human cells. These studies will undoubtedly be conducted by investigators with approved laboratories for working with these viruses. Their results will impact on whether primate studies with (*N*)-MCT should even be considered. Alternative testing may have to be considered, such as measuring anti-poxvirus activity in primary human cells, testing in poxvirus-infected explanted tissues such as lymph nodes, or testing the drug in SCID mice engrafted with human tissues. In addition, if topically administered (*N*)-MCT were found to be effective in suppressing vaccinia skin lesions in mice, it might also be tested for its ability to do the same in monkeys and compared to topical cidofovir. (*N*)-MCT must be found effective in monkey cells regardless of the activity seen in human cells in order to be pursued for evaluation by systemic administration in non-human primates. The results of the present studies dictate that a variety of human and monkey cell lines or culture systems need to be considered for adequate evaluation of the compound against these viruses.

Acknowledgements

This work was supported in part by Contract NO1-AI-15435 from the Virology Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The contents of this article do not necessarily reflect the position or policy of the government and no official endorsement should be inferred. The investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (National Institutes of Health Publication No. 86-23, revised 1985), and used facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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