Original article

Antiviral activities and phosphorylation of 5-halo-2′-deoxyuridines and *N*-methanocarbathymidine in cells infected with vaccinia virus

Donald F Smee, Daniel E Humphreys, Brett L Hurst and Dale L Barnard*

Institute for Antiviral Research, Department of Animal, Dairy and Veterinary Sciences, Utah State University, Logan, Utah, USA

*Corresponding author: E-mail: dsmee@cc.usu.edu

Background: The antipoxviral activities and phosphorylation of *N*-methanocarbathymidine ([*N*]-MCT) and four 5-halo-2′-deoxyuridines, namely 5-fluoro- (FdU), 5-chloro- (ClduU), 5-bromo- (BrdU), and 5-iodo- (IdU) derivatives, were explored.

Methods: Antiviral activities and nucleoside metabolism were determined in C127I mouse, LLC-MK$_2$ monkey, and A549 human cells infected with thymidine-kinase-containing and -deficient (TK$^+$ and TK$^-$) vaccinia (WR strain) viruses.

Results: The antiviral potencies of ClduU, BrdU and IdU were increased 16–26-fold in LLC-MK$_2$ cells infected with TK$^+$ compared with TK$^-$ virus infections, but enhancement of activity was much less in the other cell lines. (*N*)-MCT was nearly equally active against TK$^+$ and TK$^-$ viruses in the three cell lines. Antiviral activity of FdU was associated with cytotoxicity. Uninfected and infected cells metabolized compounds to mono-, di- and triphosphates. The thymidine, BrdU and IdU triphosphate levels were higher in C127I and LLC-MK$_2$ cells infected with TK$^+$ than with TK$^-$ virus. (*N*)-MCT monophosphate levels were much higher in TK$^+$ virus-infected cells, but without corresponding increases in (*N*)-MCT triphosphate. Furthermore, TK$^+$ virus infections did not appreciably alter (*N*)-MCT triphosphate levels in other mouse (L929), monkey (MA-104 and Vero) and human cell lines (A549). Antiviral potency of the compounds was greater in C127I than in LLC-MK$_2$ cells, yet lower intracellular triphosphate levels were found in C127I cells.

Conclusion: We conclude that viral TK plays an important role in increasing the antiviral potencies of these compounds in some cell lines, but minimally in others. These findings may have implications in treating infected animals with compounds that are dependent upon poxvirus TK for their activation, because viral TK activity may vary greatly due to cell type.

Introduction

It has long been recognized that herpes simplex virus thymidine kinase (TK) is able to phosphorylate certain nucleoside analogues to their monophosphate forms in infected cells, whereas host cells do this poorly [1,2]. This effect has led to the identification of selective antiviral compounds, some of which have been developed as drugs, including idoxuridine, acyclovir, ganciclovir and penciclovir [3]. The herpes simplex virus possesses a type I TK that has a broader substrate specificity than the host cell type II TK and orthopoxvirus type II TK [4,5]. Because of the narrow substrate specificity of poxvirus TK, there have been few discoveries of compounds that are selectively phosphorylated by poxvirus TK. Prichard and colleagues [6,7] presented evidence that the antiviral potencies of 5-bromo-2′-deoxyuridine (BrdU), 5-iodo-2′-deoxyuridine (IdU) and *N*-methanocarbathymidine [(*N*)-MCT] were greater against TK-containing (TK$^+$) cowpox virus than against TK-deficient (TK$^-$) cowpox virus. This suggested that the compounds were activated by cowpox virus TK and gave an indication that the poxvirus TK differs slightly from host cell TK in substrate specificity. This feature of the virus may be exploited to identify selective new poxvirus inhibitors. Indeed, Prichard and colleagues [8] later reported a series of 2′-deoxyuridine derivatives whose antiviral activity was highly dependent upon poxvirus TK.

The antipoxvirus activities of certain compounds may be dependent upon the cell line in which the assay was conducted. For example, we found that BrdU was more potent against TK$^+$ than TK$^-$ vaccinia virus, but this effect was only seen in Vero monkey cells and A549

human cells and not in C127I mouse cells [9]. The antiviral potency of (*N*)-MCT in each of these three cell lines differed markedly, but the minimal inhibitory concentrations against TK^+ and TK^- viruses in a particular cell line were nearly the same [9].

Differences in antiviral potency of nucleoside analogues associated with the cell line used for assay are often due to differences in intracellular phosphorylation of such compounds. For example, ribavirin and cidofovir are more potent against poxviruses in mouse cells than in monkey cells, a result of greater phosphorylation in mouse cells ([10] and unpublished observations). In the present study, we endeavoured to better understand how poxvirus TK contributes to antiviral potency in cell culture, and whether differences in antiviral potency in cell lines were related to different degrees of intracellular phosphorylation. We used commercially available radioactive BrdU and IdU and custom-synthesized (*N*)-MCT as probes of nucleoside metabolism, working on the premise that the nucleoside triphosphate form is primarily the active antiviral moiety of these substances [11–13].

Methods

Compounds

Thymidine, 5-fluoro-2′-deoxuridine (FdU), 5-chloro-2′-deoxyuridine (CldU), BrdU and IdU were purchased from Sigma (St. Louis, MO, USA). (*N*)-MCT or (north)-methanocarbathymidine [13,14], also referred to as *N*-methanocarbathymine or (north)-methanocarbathymine [15], was provided through the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD, USA). The synthesis of this compound has been published elsewhere [16]. [Methyl-^{3}H]thymidine (24.6 Ci/mmol), [6-^{3}H] BrdU (24.5 Ci/mmol), [6-^{3}H]IdU (16.2 Ci/mmol) and [methyl-^{3}H](*N*)-MCT (4.7 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA, USA). Non-radioactive compounds were combined with their respective radioactive forms to obtain 5 and 100 μM concentrations used for phosphorylation experiments.

Viruses and cells

Three forms of vaccinia virus (WR strain) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were wild-type (WT), S-variant (S-Var) and S-Var vTK-79 strains. The vTK-79 strain is TK^-, as indicated in ATCC literature, that was derived from the S-Var virus by passage in the presence of 5-bromo-2′-deoxyuridine, whereas the other two strains are TK^+. The WT virus produces large plaques and the two forms of the S-Var virus produce small plaques in cell culture. High titre virus pools were prepared in African green monkey kidney (MA-104) cells (BioWhittaker, Walkersville, MD, USA). Other cell lines used for the research included mouse mammary tumour (C172I), mouse connective tissue (NCTC clone 929 or L929), African green monkey kidney (Vero), rhesus monkey kidney (LLC-MK_2) and human lung carcinoma (A549) cells, all obtained from ATCC. Cell culture medium for propagating C127I, L929, 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. 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%. For antiviral and phosphorylation assays, the appropriate medium for each type of cell culture contained 2% FBS and 50 μg/ml of gentamicin. Cell proliferation assays used cell culture medium containing full-strength FBS (as indicated above) and gentamicin.

Plaque reduction, cytotoxicity and virus yield reduction assays

Determinations of the antiviral activities of the four 5-halo-2′-deoxyuridine compounds were made in 12-well microplates of confluent C127I, LLC-MK_2 and A549 cells by plaque reduction assay, as previously described, using multiple half-$\log_{10}$ dilutions of test compound [9]. Cytotoxicity assays were performed using semi-confluent cell monolayers in 24-well microplates. Approximately 2×10^4 cells were seeded and allowed to attach overnight followed by treatment with compounds for 3 days. Determinations of percent inhibition of cell replication were made by neutral red staining [9]. (*N*)-MCT has already been shown to inhibit vaccinia virus strains by plaque reduction assay and to be non-cytotoxic at 1,000 μM to replicating cells [9]. To confirm the observations of antiviral activity of (*N*)-MCT, virus yield reduction assays were performed in C127I, LLC-MK_2 and A549 cells. Approximately 400 plaque-forming units (PFU) of virus were used to infect each well of 12-well microplates. After 1 h of virus adsorption, (*N*)-MCT was added at varying half-$\log_{10}$ dilutions. Virus titration plates were run in parallel to get an accurate count of input virus. At 3 days, the virus-containing plates were frozen at -80°C. The frozen plates were later thawed, the cells and supernate were sonicated for 45 s each and the entire yield of virus (intracellular and extracellular) from each well was titrated in 12-well plates of Vero cells, followed 3 days later by fixing, staining (0.2% crystal violet in 10% buffered formalin for 15 min) and counting of the plaques. Final results were converted to $\log_{10}$ PFU/ml of virus that were normalized to 400 PFU of input virus per cell culture.

Because the C127I cells required about 1,000-fold more S-Var and TK- viruses to infect with the same relative PFU as the other cell lines, we were concerned that this input virus might persist and be detected later during virus titrations. Subsequently, we discovered by titrating virus from cells after only a 6 h incubation period that we could recover this virus by Vero cell titration, whereas WT virus could not be detected (that is, it had uncoated). In virus yield reduction assays, the results showed about 10^3 PFU of S-Var and TK- viruses recovered from C127I cells treated with all concentrations from 10 to 1,000 μM. We believe this to be the input virus that could not initially infect the cells rather than progeny virus. Thus, the graphs represent plots where 10^3 PFU was subtracted from the titres to eliminate this background virus.

Preparation of cell extracts for nucleotide analysis

Cells were infected with virus at a multiplicity of infection (MOI) of approximately 5 PFU per cell. After 1 h of virus adsorption, infected and uninfected cells in T-25 flasks were exposed to ^{3}H-(*N*)-MCT (10 μCi/ml), ^{3}H-BrdU (5 μCi/ml), ^{3}H-IdU (5 μCi/ml) or ^{3}H-thymidine (5 μCi/ml) for 12 h. Because of the high MOI used, degradation of the cells and decreases in radioactivity were evident at later time points. After incubation, the medium was removed and the cells in the flasks were treated with 0.5 ml of 3.5% perchloric acid. After 5 min at 4°C, the acid extracts were neutralized with 0.25 ml of 1 N KOH/1 M imidazole. This resulted in the formation of a precipitate that was later pelleted by centrifugation. The resulting supernates were frozen at -80°C until analysed. For each experiment, untreated flasks of cells were trypsinized prior to the start of each infection and the cells were counted using a haemacytometer in order to calculate viral MOI, which was necessary for determining picomoles of nucleoside mono-, di- and triphosphates per 10^6 cells.

Nucleotide analysis

Separation of ^{3}H-nucleosides from their phosphorylated metabolites was done by a modification of a high pressure liquid chromatography (HPLC) method previously described [17]. Briefly, 400 μl samples were analysed using an HPLC system (Varian Chromatography Systems, Woburn, MA, USA) fitted with a Whatman (Clifton, NJ, USA) 10 mm × 25 cm SAX column with a linear gradient of 10 mM (pH 7.0)–1 M ammonium phosphate buffer (pH 3.5) and a 1 ml/min flow rate. The 10 mM buffer was run for the first 5 min, followed by the gradient from 5 to 25 min. Then, the 1 M buffer was run for an additional 15 min prior to re-equilibration at the low salt concentration in preparation for the next analysis. Using the 10 mM buffer at pH 7.0 and allowing it to flow for 5 min prior to starting the gradient were necessary for adequately separating the monophosphate from the parent compound (whose large peak of radioactivity eluted from the column with a long tail). The gradient conditions were worked out using non-radioactive thymidine nucleotides (purchased from Sigma). Determinations of the amount of radioactive metabolites of compounds were made by collecting 1 ml fractions in vials, followed by liquid scintillation spectroscopy. Retention times of the parent compound and the mono-, di- and triphosphates of (*N*)-MCT, BrdU and IdU were approximately those of thymidine metabolites.

Because of the very low level of phosphorylation of (*N*)-MCT in cells, an adjustment had to be made for background counts resulting from the nucleoside itself eluting throughout the entire gradient. In order to quantify the background, so that it could be subtracted out, an injection of approximately the same amount of radioactivity of the nucleoside as was detected in the cell culture-derived samples was made, and vials were collected and counted for radioactivity. Small amounts of radioactivity were found in every fraction, although the vast majority of counts eluted between fractions 4 and 12. A ratio of radioactivity in the sample peak for fractions 4–12 to the corresponding background peak was made. Then the total background counts found in fractions corresponding to where the mono-, di- and triphosphate peaks were found for the test samples were multiplied by this ratio and the resulting value was subtracted from the total counts in the peaks. By this method, a more accurate estimate of phosphate forms of (*N*)-MCT in the cells could be made. Values were reported as picomoles of radioactivity per 10^6 cells.

Results

Antiviral activities and cytotoxicities of nucleosides in cell culture

Plaque reduction assays were performed to determine the activities of compounds in cells infected with TK+ and TK- vaccinia viruses (Table 1). FdU was highly potent against the three viruses in C127I and LLC-MK_2 cells, with inhibition seen at <0.1 μM. Accompanying the antiviral activity of FdU was cytotoxic, resulting in low selectivity index values of 2.2–6.7. The activity ratio of TK- to TK+ virus indicated minimal potency differences or a lack of viral TK involvement in the antiviral effect of the compound in those cells. FdU did not exhibit antiviral activity in A549 cells and was not toxic to those cells.

CldU, BrdU and IdU were similarly potent against the vaccinia virus infections (Table 1). Antiviral potencies of the three compounds in C127I cells ranged from 0.5 to 1.6 μM, and were not appreciably

affected by the TK status of the virus. However, there were large differences (16–26-fold) in antiviral potency of the compounds in LLC-MK_2 cells infected with TK^+ viruses compared with TK^- virus infection. In A549 cells, there was a moderate enhancement of activity in the TK^+ cells ranging from 3.1- to 4.6-fold. In general, these three compounds were most potent against TK^+ virus infections in C127I cells, followed by LLC-MK_2 and A549 cells.

(*N*)-MCT was much more potent in C127I cells (50% effective concentrations of 0.5–0.9 μM) than in LLC-MK_2 (183–273 μM) or A549 (17–29 μM) cells (Table 1). The TK- virus infection was inhibited by approximately the same concentrations as those

Table 1. Antiviral activities of 5-halogenated 2-deoxyuridines and (*N*)-MCT in various cell lines against vaccinia (WR strain) TK^+ and TK^- viruses

Compound	Cell line	Virus	EC_{50}*, μM	IC_{50}†, μM	SI‡	Activity ratio (TK^-/S-Var)
FdU	C127I§	WT	0.009 ±0.004	0.020 ±0.019	2.2	
		S-Var	0.003 ±0.002	0.020 ±0.019	6.7	1.3
		TK^-	0.004 ±0.002	0.020 ±0.019	5.0	
	LLC-MK_2¶	WT	0.034 ±0.014	0.083 ±0.021	2.4	
		S-Var	0.015 ±0.005	0.083 ±0.021	5.5	2.3
		TK^-	0.034 ±0.006	0.083 ±0.021	2.4	
	A549#	WT	>1,000	>1,000	–	
		S-Var	>1,000	>1,000	–	–
		TK^-	>1,000	>1,000	–	
CldU	C127I§	WT	1.6 ±0.3	>1,000	>625	
		S-Var	0.5 ±0.2	>1,000	>2,000	2.4
		TK^-	1.2 ±0.5	>1,000	>833	
	LLC-MK_2¶	WT	1.9 ±0.7	>1,000	>526	
		S-Var	4.1 ±1.2	>1,000	>244	21
		TK^-	85 ±14	>1,000	>12	
	A549#	WT	16 ±2.0	>1,000	>63	
		S-Var	13 ±6.0	>1,000	>77	3.2
		TK^-	42 ±16	>1,000	>24	
BrdU	C127I§	WT	1.2 ±0.5	>1,000	>833	
		S-Var	0.8 ±0.4	>1,000	>1,250	1.0
		TK^-	0.8 ±0.5	>1,000	>1,250	
	LLC-MK_2¶	WT	0.9 ±0.2	>1,000	>1,110	
		S-Var	3.5 ±1.3	>1,000	>286	16
		TK^-	55 ±13	>1,000	>18	
	A549#	WT	8.1 ±3.4	>1,000	>123	
		S-Var	7.6 ±4.4	>1,000	>132	4.6
		TK^-	35 ±13	>1,000	>29	
IdU	C127I§	WT	1.5 ±0.2	>1,000	>667	
		S-Var	0.6 ±0.5	>1,000	>1,667	1.7
		TK^-	1.0 ±0.5	>1,000	>1,000	
	LLC-MK_2¶	WT	2.7 ±1.0	>1,000	>370	
		S-Var	3.5 ±1.1	>1,000	>286	26
		TK^-	90 ±28	>1,000	>11	
	A549#	WT	14 ±1.5	>1,000	>71	
		S-Var	13 ±2.2	>1,000	>77	3.1
		TK^-	40 ±16	>1,000	>25	
(*N*)-MCT	C127I§**	WT	0.9 ±0.3	>1,000	>1,110	
		S-Var	0.5 ±0.1	>1,000	>2,000	0.8
		TK^-	0.6 ±0.1	>1,000	>1,667	
	LLC-MK_2¶	WT	253 ±85	>1,000	>4.0	
		S-Var	183 ±59	>1,000	>5.5	1.5
		TK^-	273 ±120	>1,000	>3.7	
	A549#	WT	29 ±3.5	>1,000	>34	
		S-Var	17 ±4.9	>1,000	>59	1.3
		TK^-	22 ±5.8	>1,000	>45	

The wild-type (WT) and S-variant (S-Var) viruses contain thymidine kinase (TK^+). The TK-deficient (TK^-) virus was derived from the S-Var virus. *Fifty percent effective (virus-inhibitory) concentration ±SD (three independent assays) determined by plaque reduction assay. †Fifty percent cell-inhibitory concentration ±SD (three independent assays) of actively dividing uninfected cells. ‡Selectivity index (IC_{50} divided by EC_{50}). Cells originated from §mouse, ¶monkey and #human. **EC_{50} values and activity ratios for (*N*)-MCT in this cell line were previously reported [9]. BrdU, 5-bromo-2′-deoxyuridine; CldU, 5-chloro-2′-deoxyuridine; FdU, 5-fluoro-2′-deoxuridine; IdU, 5-iodo-2′-deoxyuridine; (*N*)-MCT, *N*-methanocarbathymidine.

inhibiting the two TK+ viruses, indicating that the viral enzyme did not enhance antiviral potency as it did for BrdU and IdU in the LLC-MK_2 cell infections. To further validate this pattern of antiviral activity seen with (*N*)-MCT, we investigated its effects on vaccinia virus yield (Figure 1). Virus yields were inhibited by much lower concentrations of (*N*)-MCT in C127I cells than in A549 and LLC-MK_2 cells, confirming the plaque reduction data in Table 1. From the results in the figure, 90% (1 $\log_{10}$) and 99% (2 $\log_{10}$) inhibitory values were calculated (Table 2). EC_{90} and EC_{99} values for the TK- virus infections were within twofold of those of the S-Var virus and were similar to those of the WT virus. The shapes of the dose–response curves were similar for the three viruses in the three cell lines (Figure 1). The combined results indicate that the viral TK does not serve to markedly enhance the antiviral potency of (*N*)-MCT in cell culture.

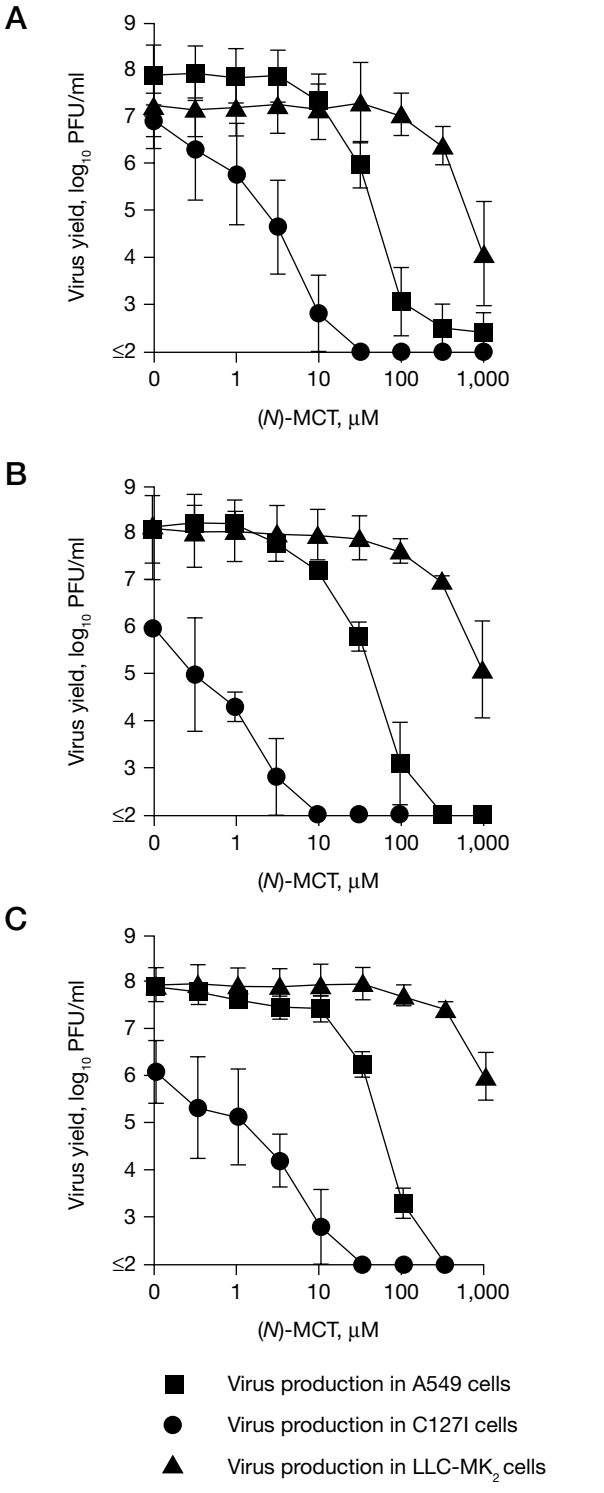
In untreated C127I cells, the S-Var and S-Var TK- viruses replicated to 100-fold lower titres than in the other cell lines. WT virus replicated equally well in untreated C127I and LLC-MK_2 cells, and to about a sixfold higher titre in A549 cells. These differences in viral growth did not seem to have a large impact on the antiviral potencies of (*N*)-MCT against the three viruses in a particular cell line.

Phosphorylation of thymidine, BrdU and IdU in C127I and LLC-MK_2 cells

Infected and uninfected cells were treated with ^{3}H-compounds and incubated for 12 h, followed by HPLC analysis of intracellular nucleotides (Figure 2). Thymidine was tested as a positive control because it is the normal substrate for viral and cellular TKs. In C127I cells, thymidine triphosphate (TTP) levels were slightly increased (1.4–1.7-fold) in TK+ virus-infected cells compared with uninfected cells. The level of TTP was depressed 46% in cells infected with the TK- virus. This same pattern of triphosphate levels was seen in IdU-treated C127I cells. IdUTP was enhanced 1.4-fold by TK+ virus infection and depressed 64% by TK- virus infection. In C127I cells treated with BrdU, similar levels of BrdUTP were present in uninfected and TK+ virus-infected cells. BrdUTP was suppressed 65% in TK- virus-infected cells. The lower levels of nucleotide triphosphates in TK- virus-infected C127I cells compared with uninfected cells might be due to host cell protein shutdown (as a result of infection) that would interfere with phosphorylating activity. This will require further experimentation to support this hypothesis.

In LLC-MK_2 cells, TTP, BrdUTP and IdUTP levels were increased in TK+ virus-infected cells 3.1–4.6-fold compared with uninfected and TK- virus-infected cells. The differences in TTP, BrdUTP or IdUTP levels between uninfected and TK- virus-infected cells were

Figure 1. Effect of (*N*)-MCT on virus yields from cells infected with TK+ (wild-type and S-Var) and S-Var TK- strains of vaccinia virus



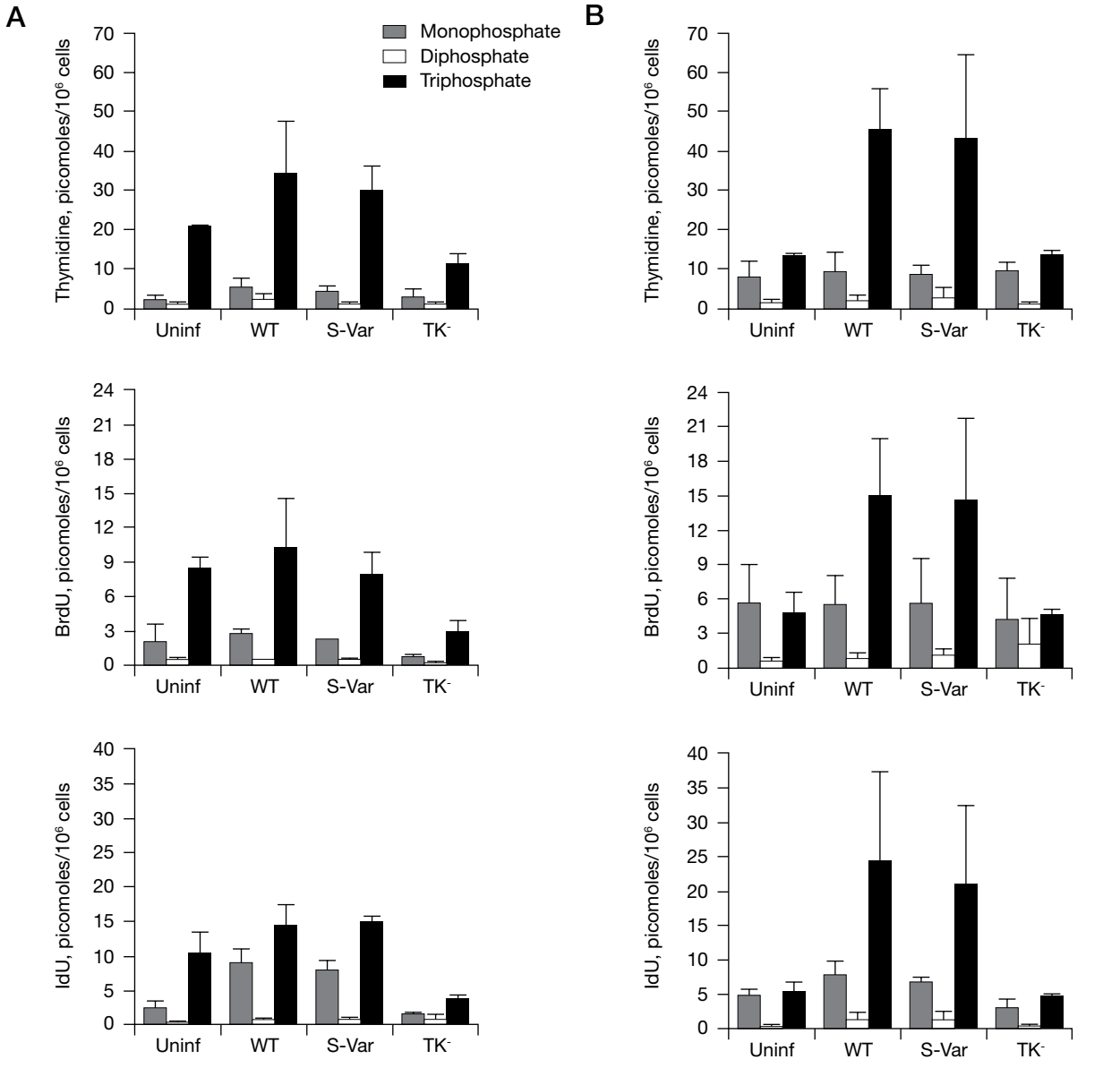
(**A**) Wild-type virus, (**B**) S-variant (S-Var) virus and (**C**) thymidine-kinase-deficient (TK-) virus. Virus replicated 3 days in the presence of the compound, then total virus yield was determined by plaque titration assays in Vero cells. Data represent means ±SD for two independent assays. (*N*)-MCT, *N*-methanocarbathymidine; PFU, plaque-forming units; TK+, thymidine-kinase-containing.

Table 2. Antiviral activity of (*N*)-MCT in various cell lines against vaccinia (WR strain) TK^+ and TK^- viruses as determined by virus yield

	Concentrations reducing virus yields in cells, μM					
	C127I (mouse cells)		LLC-MK2 (monkey cells)		A549 (human cells)	
Virus	EC_{90}*	EC_{99}*	EC_{90}*	EC_{99}*	EC_{90}*	EC_{99}*
Wild-type	1.0 ±0.8	3.0 ±1.5	365 ±191	765 ±474	13 ±4.2	33 ±1.4
S-Var	0.4 ±0.1	2.0 ±1.8	295 ±219	680 ±424	11 ±1.8	26 ±2.8
TK^-	0.8 ±0.7	3.3 ±0.2	465 ±120	1,000 ±425	16 ±4.9	36 ±4.2

The wild-type and S-variant (S-Var) viruses contain thymidine kinase (TK^+). The TK-deficient (TK^-) virus was derived from the S-Var virus. *Concentration of *N*-methanocarbathymidine [(*N*)-MCT] that reduced virus yield by 90 or 99%. Viruses were propagated in the respective cell line, with virus yields determined in Vero cells. Values are means ±SD for two independent assays.

Figure 2. Formation of metabolites of thymidine, BrdU and IdU in cells infected with TK^+ (wild type and S-Var) and TK^- strains of vaccinia virus



(**A**) C127I and (**B**) LLC-MK_2 cells. Treatment of the infection (5 μM compound) was for 12 h, followed by nucleotide analysis. Data represent means ±SD for two independent assays. BrdU, 5-bromo-2′-deoxyuridine; IdU, 5-iodo-2′-deoxyuridine; S-Var, S-variant; TK^+, thymidine-kinase-containing; TK^-, thymidine-kinase-deficient; uninf, uninfected; WT, wild type.

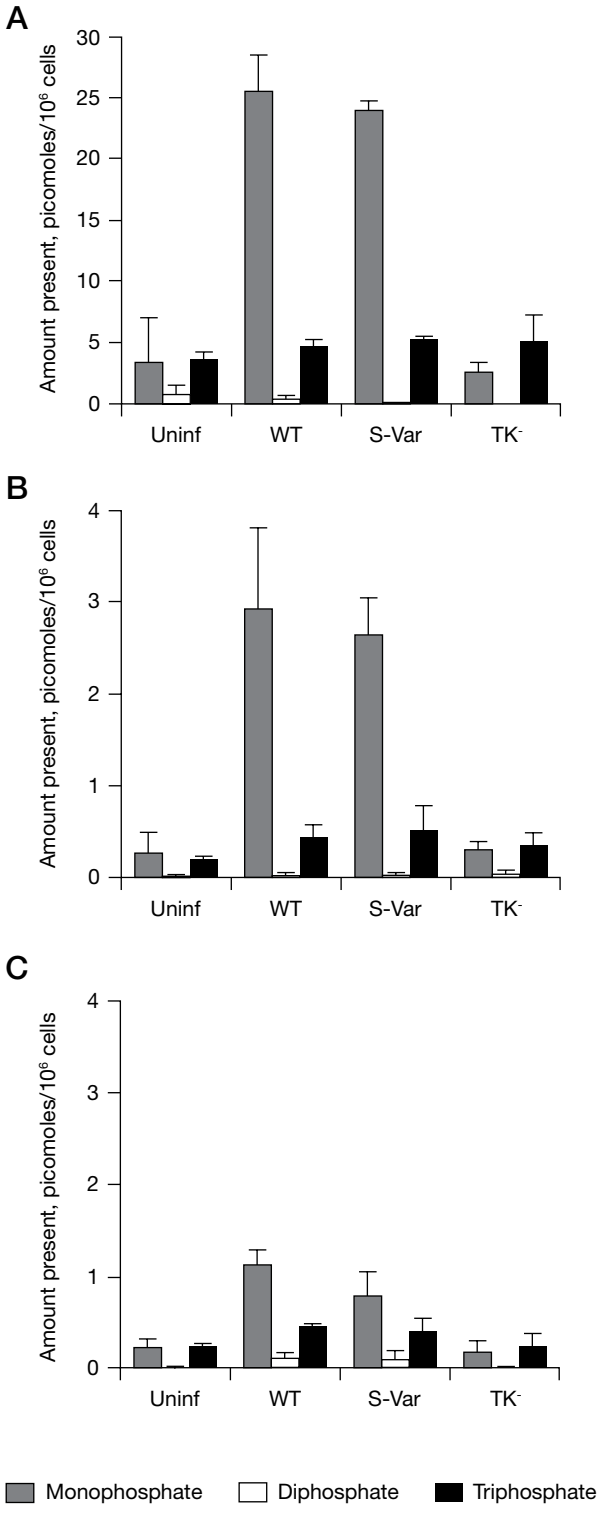
minimal, with levels in TK⁻ virus-infected cells being 100%, 97% and 87% of those found in uninfected cells, respectively. Overall, thymidine was phosphorylated to higher levels than were the two analogues in both cell lines. This was not surprising because thymidine is the natural substrate for the enzyme. IdU phosphorylation slightly exceeded (by 1.6-fold) that of BrdU (Figure 2, note that the Y-axis scales differ with each figure). Higher triphosphate levels were found in uninfected and TK⁺ virus-infected LLC-MK_2 cells than in comparable C127I cells.

Phosphorylation of (*N*)-MCT in C127I and LLC-MK_2 cells

Infected and uninfected cells were treated with ^{3}H-(*N*)-MCT and incubated for 12 h, followed by HPLC analysis of intracellular nucleotides (Figure 3). Based upon the antiviral studies reported in Table 1 and Figure 1, the 5 μM concentration of (*N*)-MCT was expected to be highly inhibitory to virus production in C127I cells, but not in LLC-MK_2 cells. The 100 μM concentration of (*N*)-MCT was expected to exert a moderately inhibitory effect on virus replication in LLC-MK_2 cells. Using a 5 μM (*N*)-MCT treatment, infection with the two TK⁺ virus strains caused increases in (*N*)-MCT monophosphate levels as compared with uninfected and TK⁻ virus-infected cells, but more so in LLC-MK_2 cells (10.7-fold) than in C127I cells (4.2-fold; Figure 3). A similar effect (7.3-fold increase) was observed in LLC-MK_2 cells with the 100 μM treatment. The increase in intracellular (*N*)-MCT monophosphate by infection with the TK⁺ viruses did not result in a corresponding increase in the amount of (*N*)-MCT triphosphate in these cells, where only 2.4–2.8-fold increases in triphosphate levels were seen with the 5 μM treatment and up to 1.5-fold increase at 100 μM. These differences are minimal, considering that the TK⁻ virus infection resulted in similar triphosphate levels to those found in both uninfected and TK⁺ virus-infected cells. Importantly, the increases in (*N*)-MCT triphosphate levels in TK⁺ cells did not translate into an increase in antiviral potency (Tables 1 and 2). The phosphorylation results suggest that the cells poorly converted (*N*)-MCT monophosphate to diphosphate, even though the viral enzyme was able to phosphorylate the compound to much higher monophosphate levels in TK⁺ virus-infected cells.

The amounts of (*N*)-MCT triphosphate in C127I cells were comparable to those in LLC-MK_2 cells at the 5 μM concentration (Figure 3). The average amount of triphosphate (mean value derived from a summation of triphosphate present in uninfected and virus-infected cells) was 0.33 picomoles in C127I cells compared with 0.36 picomoles in LLC-MK_2 cells. In LLC-MK_2 cells exposed to 100 μM (*N*)-MCT, 12.7-fold more (*N*)-MCT triphosphate was present than at the 5 μM concentration (note that the Y-axis scales differ between

Figure 3. Formation of metabolites of (*N*)-MCT in C127I and LLC-MK_2 cells infected with TK⁺ (wild type and S-Var) and TK⁻ strains of vaccinia virus



(**A**) LLC-MK_2 cells treated with 100 μM *N*-methanocarbathymidine ([*N*]-MCT). (**B**) LLC-MK_2 cells treated with 5 μM *N*-MCT. (**C**) C127I cells treated with 5 μM *N*-MCT. Treatment of the infection was for 12 h followed by nucleotide analysis. Data represent means ±SD for two independent assays. TK⁺, thymidine-kinase-containing; TK⁻, thymidine-kinase-deficient; S-var, S-variant; uninf, uninfected; WT, wild type.

the two figures). There was an approximately 14-fold difference in the amount of triphosphate between LLC-MK_2 cells (100 μM compound) versus C127I cells (5 μM compound).

These data show that a much higher concentration of intracellular (*N*)-MCT triphosphate must be present in LLC-MK_2 cells than in C127I cells to cause a comparable degree of inhibition of vaccinia virus replication.

Phosphorylation of (*N*)-MCT in L929, MA-104, Vero and A549 cells

The high antiviral potency of (*N*)-MCT in C127I mouse cells and low potency in LLC-MK_2 monkey cells is not unique to these two cell types. The same effect was reported in L929 mouse cells and in other monkey cell lines such as MA-104 and Vero, where plaque reduction 50% effective concentration values for the compound against WT vaccinia virus (WR strain) were 0.8, >1,000 and 620 μM, respectively [9]. (*N*)-MCT was active against WT virus in A549 cells at 29 μM (Table 1). Treatment of these cell lines with 5 μM (*N*)-MCT resulted in the following amounts of (*N*)-MCT triphosphate at 12 h, comparing uninfected to WT virus-infected (uninfected/WT virus-infected): 0.44/0.4, 0.17/0.2, 0.17/0.36, and 0.1/0.34 picomoles/10^6 cells for L929, MA-104, Vero, and A549 cells, respectively. Thus, (*N*)-MCT inhibited vaccinia virus in L929 cells at very low (<1 μM) concentrations, even though the amount of (*N*)-MCT triphosphate in those cells at 5 μM was only twofold greater than the level in MA-104 cells, where no antiviral activity at 1,000 μM occurs.

Discussion

FdU, CldU, BrdU and IdU were found to be inhibitory to TK^+ and TK^- strains of vaccinia virus in cell culture. The antiviral potencies of BrdU and IdU compared favourably with results obtained against cowpox virus in human foreskin fibroblasts (HFF cells) [7]. However, the reported potency of FdU was much less against cowpox virus than against vaccinia virus. FdU was cytotoxic to actively replicating C127I and LLC-MK_2 cells, whereas the other three compounds were not toxic at 1,000 μM. Prichard *et al.* [7] indicated that FdU was not toxic to HFF cells, although it was not clear whether they performed their assays using actively dividing cells as opposed to stationary monolayers. We previously reported some toxicity by IdU in actively dividing C127I cells [18], but we were unable to confirm those results with multiple experiments using the current lot of cells.

Because of the minimal potency differences of FdU against TK^+ and TK^- viruses, we conclude that the viral TK enzyme did not contribute to metabolism of the compound, and that phosphorylation by a cellular enzyme was sufficient for both antiviral and cytotoxic effects. This is supported by the fact that the compound exhibited no antiviral activity in A549 cells, even when infected with TK^+ virus. It appears that A549 cells lack a necessary phosphorylating enzyme for FdU.

CldU, BrdU and IdU were much more potent in TK^+ virus-infected LLC-MK_2 cells than in TK^- virus-infected cells. Prichard *et al.* [7] demonstrated a higher degree of antiviral potency of BrdU and IdU against TK^+ cowpox virus compared with TK^- virus in HFF cells. Later they demonstrated that purified vaccinia virus TK phosphorylated BrdU, IdU and other compounds [8]. The phosphorylation studies we conducted using vaccinia-virus-infected cells treated with thymidine, BrdU and IdU confirmed that higher levels of nucleoside triphosphate were produced in TK^+ virus-infected LLC-MK_2 cells. Less of a viral TK stimulatory effect on triphosphate levels was seen in C127I cells. The antiviral potencies of BrdU and IdU in C127I cells infected with TK^+ viruses were similar to or greater than those in LLC-MK_2 cells, although at lower triphosphate levels in the C127I cell line. Since higher levels of triphosphate should equate to greater inhibition of virus, the reason why the compounds should be more active in C127I cells is unclear. The same effect was also detected with (*N*)-MCT in C127I-infected cells (see below).

(*N*)-MCT was shown to be inhibitory to vaccinia plaque formation and virus yields from cell culture, and the potency of inhibition was highly cell-line-dependent, as was first reported [9]. Potency was greatest in C127I cells, less in A549 cells and least in LLC-MK_2 cells, confirming the published data. We saw greater potency of inhibition of virus in A549 cells than was previously reported, which may be attributable to using a different lot of cells. The virus yield reduction data were in agreement with the plaque reduction results for the three cell lines.

(*N*)-MCT phosphorylation studies were conducted to better understand the cell line dependency effect that greatly favoured antiviral potency in mouse cells. We hypothesized that intracellular levels of (*N*)-MCT triphosphate should be much greater in C127I cells than in monkey or human cells treated with an equal concentration of the inhibitor, and this would explain antiviral potency differences *in vitro*. For example, the greater potencies of ribavirin and cidofovir in mouse cells compared with monkey cells were due to greater phosphorylation ([10] and unpublished data). Our hypothesis proved to be incorrect because similar amounts of (*N*)-MCT triphosphate were found in C127I and LLC-MK_2 cells when treated with (*N*)-MCT at 5 μM. Similar amounts of triphosphate were also found in L929, MA-104 and Vero cells, yet (*N*)-MCT was considerably more potent in L929 cells. The reason why (*N*)-MCT should be considerably more active in C127I and L929 cells than in the other cell lines is unclear.

Viral replication efficiency in mouse cells is less than in the monkey cell lines, perhaps making them more sensitive to antiviral therapy. Considerable effort may be required to study viral replication kinetics to understand why (*N*)-MCT is more efficient as an antiviral agent in mouse cells than in monkey cells.

The purified vaccinia virus TK is able to phosphorylate (*N*)-MCT to its monophosphate form (Mark Prichard, University of Alabama at Birmingham, personal communication). Infection of cells with TK^+ virus led to increases in (*N*)-MCT monophosphate levels in C127I and LLC-MK_2 cells, but this did not translate into comparable increases in intracellular triphosphate levels. The data suggest that the monophosphate is converted poorly to the diphosphate, thus inhibiting the conversion to triphosphate. Because of this effect, the viral TK is not able to contribute to an enhancement of the potency of (*N*)-MCT in cell culture, as measured by plaque and virus yield reduction assays.

This article did not address the ability of triphosphate forms of each compound to interact with the viral DNA polymerase complex and possibly be incorporated into viral DNA, thus stopping viral DNA replication. This was beyond the scope of the project and would require considerably more research effort. The data for BrdU and IdU in LLC-MK_2 cells suggest that the dose–response curve for inhibition of viral DNA synthesis is steep. This is based upon the observation that increase in triphosphate in TK^+ virus-infected cells compared with TK^- virus-infected cells was moderate (Figure 3), whereas the realized gain in antiviral potency was much greater (Table 1). For example, the difference in the antiviral potency of IdU against the S-Var (TK^+) and TK^- viruses was 26-fold, yet the difference in IdUTP in cells infected with these viruses was only 4.6-fold. Apparently, the amount of IdUTP in cells infected with the S-Var (and WT) viruses reached a threshold to cause profound inhibition of viral replication. (*N*)-MCT triphosphate must be an extremely potent inhibitor of viral DNA synthesis, because only very small quantities of it were found intracellularly.

As was discussed previously [9], we do not believe that (*N*)-MCT monophosphate exhibits antivaccinia virus activity. There are several reasons for this position. First, nucleoside monophosphates are not known to directly inhibit viral DNA polymerases. The nucleoside monophosphates that are antivirally active do so by inhibiting cellular enzymes. For example, the inhibition of cellular thymidylate synthetase results in antipoxvirus activity [19]. Inhibition of cellular thymidylate synthetase also causes cytotoxicity. Because (*N*)-MCT is not cytotoxic at 1,000 μM, this suggests no inhibitory effect on this cellular enzyme. In addition, the data in Figure 3 indicate an enhancement of (*N*)-MCT monophosphate in TK^+ virus-infected cells, without any enhancement in antiviral potency. Thus, the presence of additional (*N*)-MCT monophosphate inside the cells is inconsequential to virus inhibition.

The TK^- virus that was used for these studies has not been, to our knowledge, genetically characterized. It is clear that the virus displays the correct phenotype as a drug-resistant virus, particularly in LLC-MK_2 cells treated with BrdU and IdU. It also displays the correct phenotype with regard to the lack of stimulation of phosphorylation of thymidine, BrdU and IdU in cells infected with the virus. Because the principal substrate, thymidine, was not stimulated in its conversion to nucleotides in infected cells (Figure 2), this suggests that the enzyme is non-functional rather than merely being altered in terms of substrate specificity. Because the virus was propagated in cells treated with BrdU, the possibility exists that it contains an altered DNA polymerase that is less sensitive to inhibition by the compounds. As was discussed previously [9], this seems unlikely based upon the fact that the TK^- virus and WT viruses were nearly equally sensitive to BrdU in C127I cells as well as were the other 5-halogenated 2′-deoxyuridines and (*N*)-MCT (as presented in this report).

It is concluded from these studies that in certain cell lines the vaccinia virus TK plays a role in increasing the phosphorylation of these analogues ([*N*]-MCT, CldU, BrdU and IdU) of thymidine to their respective monophosphate form. This may lead to increased antiviral potency, as was exemplified by the activities of CldU, BrdU and IdU in LLC-MK_2 cells. However, in other cell lines such as C127I and L929, the presence or absence of the viral TK does not appreciably affect the potencies of these compounds. Because of the poor conversion of (*N*)-MCT monophosphate to diphosphate in cells, viral TK does not contribute to increased antiviral potency of this compound. The differences in metabolism of BrdU, IdU and (*N*)-MCT in uninfected, TK^+ virus-infected and TK^- virus-infected cells could not explain why these compounds are more active in mouse cells than in monkey cells, because nucleoside triphosphate levels were nearly equal to or even higher in LLC-MK_2 monkey cells. In the case of (*N*)-MCT, its antiviral potency in mouse cells, as determined by plaque reduction assay, was 100–1,000-fold greater than in monkey cells [9]. This difference might be related to less robust replication of the virus in the mouse cells.

Cellular TK was presumably the enzyme responsible for phosphorylation of BrdU and IdU in uninfected cells, based upon the action of this enzyme in other cell lines [20]. Cellular TK may also have phosphorylated (*N*)-MCT, although no definitive metabolism studies have been published with this compound and cellular enzymes. In the cell lines used in the research, viral TK made an additional contribution toward enhancing phosphorylation and increasing antiviral potency

of the 2′-deoxyuridines. Other investigators have indicated that poxvirus TK is responsible for activation (monophosphorylation) of certain nucleoside analogues related to thymidine [7,8]. Some of those compounds were highly dependent upon viral TK for antiviral activity, suggesting that phosphorylation by cell TK was minimally contributory. This effect confers a great degree of selectivity to the antiviral agent. The results presented here indicate that the particular cell line in which the virus infects influences how much of the compound reaches the triphosphate form. Thus, one could surmise that the antiviral efficacies of these types of selective compounds might be effected (either favourably or negatively) based upon the particular cells in which the viral infection was taking place. When considering the treatment of animals or humans, this might depend upon the animal species or even within the same species where there are innumerable cell types. It will be important to evaluate these newer antipoxvirus compounds in a variety of cells from various animal species to determine how cell-line-dependent they are.

Acknowledgements

This work was supported in part by Contract NO1-AI-30048 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.

Disclosure statement

The authors declare no competing interests.

References

1. Fyfe JA, Keller PM, Furman PA, Miller RL, Elion GB. Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl) guanine. *J Biol Chem* 1978; **253:**8721–8727.
2. Cheng YC, Dutschman G, Fox JJ, Watanabe KA, Machida H. Differential activity of potential antiviral nucleoside analogs on herpes simplex virus-induced and human cellular thymidine kinases. *Antimicrob Agents Chemother* 1981; **20:**420–423.
3. De Clercq E, Brancale A, Hodge AV, Field HJ. *Antiviral Chemistry and Chemotherapy*'s current antiviral agents FactFile 2006 (1st edition). *Antivir Chem Chemother* 2006; **17:**113–166.
4. Black ME, Hruby DE. Site-directed mutagenesis of a conserved domain in vaccinia virus thymidine kinase. Evidence of a potential role in magnesium binding. *J Biol Chem* 1992; **267:**6801–6806.
5. Black ME, Hruby DE. A single amino acid substitution abolishes feedback inhibition of vaccinia virus thymidine kinase. *J Biol Chem* 1992; **267:**9743–9748.
6. Prichard MN, Keith KA, Quenelle DC, Kern ER. Activity and mechanism of action of N-methanocarbathymidine against herpesvirus and orthopoxvirus infections. *Antimicrob Agents Chemotherapy* 2006; **50:**1336–1341.
7. Prichard MN, Williams AD, Keith KA, Harden EA, Kern ER. Distinct thymidine kinases encoded by cowpox virus and herpes simplex virus contribute significantly to the differential antiviral activity of nucleoside analogs. *Antiviral Res* 2006; **71:**1–6.
8. Prichard MN, Keith KA, Johnson MP, *et al*. Selective phosphorylation of antiviral drugs by vaccinia virus thymidine kinase. *Antimicrob Agents Chemother* 2007; **51:**1795–1803.
9. Smee DF, Wandersee MK, Bailey KW, *et al*. Cell line dependency for antiviral activity and *in vivo* efficacy of *N*-methanocarbathymidine against orthopoxvirus infections in mice. *Antiviral Res* 2007; **73:**69–77.
10. Smee DF, Bray M, Huggins JW. Antiviral activity and mode of action studies of ribavirin and mycophenolic acid against orthopoxviruses *in vitro*. *Antivir Chem Chemother* 2001; **12:**327–335.
11. Schwartz SA. Preferential utilization of bromodeoxyuridine and iododeoxyuridine triphosphates by DNA polymerase gamma *in vitro*. *Biosci Rep* 1981; **1:**387–398.
12. Ruth JL, Cheng YC. Nucleoside analogues with clinical potential in antivirus chemotherapy. The effect of several thymidine and 2′-deoxycytidine analogue 5′-triphosphates on purified human (α, β) and herpes simplex virus (types 1, 2) DNA polymerases. *Mol Pharmacol* 1981; **20:**415–422.
13. Marquez VE, Ben-Kasus T, Barchi JJ Jr, Green KM, Nicklaus MC, Agbaria R. Experimental and structural evidence that herpes 1 kinase and cellular DNA polymerase(s) discriminate on the basis of sugar pucker. *J Am Chem Soc* 2004; **26:**543–549.
14. Zalah, L, Huleihel M, Manor E, *et al*. Metabolic pathways of *N*-methanocarbathymidine, a novel antiviral agent, in native and herpes simplex virus type 1 infected Vero cells. *Antiviral Res* 2002; **55:**63–75.
15. Russ P, Schelling P, Scapozza L, Folkers G, De Clercq E, Marquez VE. Synthesis and biological evaluation of 5-substituted derivatives of the potent antiherpes agent (north)-methanocarbathymine. *J Med Chem* 2003; **46:**5045–5054.
16. Marquez VE, Siddiqui MA, Ezzitouni A, *et al*. Nucleosides with a twist. Can fixed forms of sugar ring pucker influence biological activity in nucleosides and oligonucleotides? *J Med Chem* 1996; **39:**3739–3747.
17. Smee DF, Matthews TR. Metabolism of ribavirin in respiratory syncytial virus-infected and uninfected cells. *Antimicrob Agents Chemother* 1986; **30:**117–121.
18. Smee DF, Sidwell RW. Anti-cowpox virus activities of certain adenosine analogs, arabinofuranosyl nucleosides, and 2′-fluoro-arabinofuranosyl nucleosides in cell culture and in mice. *Nucleosides Nucleotides Nucleic Acids* 2004; **25:**375–383.
19. De Clercq E. Vaccinia virus inhibitors as a paradigm for the chemotherapy of poxvirus infections. *Clin Microbiol Rev* 2001; **14:**382–397.
20. Balzarini J, De Clercq E, Torrence PF, *et al*. Role of thymidine kinase in the inhibitory activity of 5-substituted 2′-deoxyuridines on the growth of human and murine tumor cell lines. *Biochem Pharmacol* 1982; **31:**1089–1095.

Received 3 October 2007, accepted 13 February 2008