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Metabolic pathways of *N*-methanocarbathymidine, a novel antiviral agent, in native and herpes simplex virus type 1 infected Vero cells

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Abstract

N-Methanocarbathymidine ((*N*)-MCT), a thymidine analog incorporating a pseudosugar with a fixed Northern conformation, exhibits potent antiherpetic activity against herpes simplex virus types 1 (HSV-1) and 2 (HSV-2). This study contrasts the metabolic pathway of (*N*)-MCT and the well-known antiherpetic agent ganciclovir (GCV) in HSV-1-infected and uninfected Vero cells. Treatment of HSV-1 infected Vero cells immediately after viral infection with (*N*)-MCT profoundly inhibited the development of HSV-1 infection. Using standard plaque reduction assay to measure viral infection, (*N*)-MCT showed a potency greater than that of ganciclovir (GCV), the IC_{50}s were 0.02 and 0.25 μM for (*N*)-MCT and GCV, respectively. (*N*)-MCT showed no cytotoxic effect on uninfected Vero cells ($CC_{50} > 100$ μM). Dose and time dependence studies showed high levels of (*N*)-MCT-triphosphate ((*N*)-MCT-TP), and GCV-triphosphate (GCV-TP) in HSV-1-infected cells incubated with (*N*)-MCT or GCV, respectively. In contrast, uninfected cells incubated with (*N*)-MCT showed elevated levels of (*N*)-MCT-monophosphate only, while low levels of mono, di- and triphosphates of GCV were found following incubation with GCV. Although the accumulation rate of (*N*)-MCT and GCV phosphates in HSV-1-infected cells were similar, the decay rate of (*N*)-MCT-TP was slower than that of GCV-TP. These results suggest that: (1) the antiviral activity of (*N*)-MCT against herpes viruses is mediated through its triphosphate metabolite; (2) in contrast to GCV, the diphosphorylation of (*N*)-MCT in HSV-1- infected cells is the rate limiting step; (3) (*N*)-MCT-TP accumulates rapidly and has a long half-life in HSV-1-infected cells; and (4) HSV-tk catalyzed the mono, and diphosphorylation of (*N*)-MCT while monophosphorylating GCV only. These results provide a biochemical rational for the highly selective and effective inhibition of HSV-1 by (*N*)-MCT.

Keywords: Herpes simplex virus; Herpes simplex thymidine kinase; *N*-Methanocarbathymidine; Ganciclovir; Phosphorylation; Plaque forming unit

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1. Introduction

Many studies have demonstrated that the antiherpetic activity of nucleosides analogues like ganciclovir (GCV) and acyclovir (ACV) involve their selective phosphorylation in HSV-infected cells by the herpes thymidine kinase (HSV-tk) (Elion et al., 1977; Fyfe et al., 1978; De Clercq et al., 1980; Keller et al., 1981; Field et al., 1983; Smee et al., 1983; Snoeck, 2000), and subsequent inhibition of herpes DNA polymerase by the drug's 5′-triphosphates (Derse et al., 1981; Germershausen et al., 1983; St. Clair et al., 1987; Frank et al., 1984). Thus, HSV-tk has proven to be an attractive target for development of antiherpetic chemotherapy.

We have synthesized a nucleoside analog with a pseudosugar rigidly fixed in the Northern conformation, *N*-methanocarbathymidine ((*N*)-MCT), (1*R*,2*S*,4*S*,5*S*)-1-(hydroxymethyl)-2-hydroxy-4-(5-methyl-2,4(1*H*,3*H*)-dioxopyrimidin-1-yl)bicyclo[3.1.0]hexane) (Fig. 1), which has been found to exhibit potent antiherpetic activity against herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) as confirmed by both the plaque reduction and the cytopathogenic effect assays with a potency greater than that of the reference standard ACV (Marquez et al., 1996). Furthermore, we have recently demonstrated that (*N*)-MCT displays antitumor effect against murine and human tumor cells transduced with the HSV-tk gene in vitro and in vivo without any cytotoxic effect on non-transduced tumor cells (Agbaria et al., submitted, Noy et al., submitted). In tumor cells expressing HSV-tk, we demonstrated that the tumoricidal property is effected through the phosphorylation of (*N*)-MCT to the 5′-triphosphate form and its subsequent incorporation into DNA, a mechanism that parallels that of GCV (Agbaria et al., submitted).

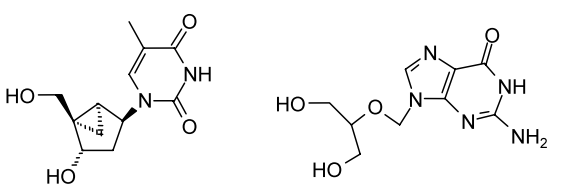


Fig. 1. Chemical structures of (*N*)-MCT and GCV.

The antiviral mechanism of action of (*N*)-MCT against herpes viruses is still unclear. In order to have a better understanding of mechanism of action of (*N*)-MCT, a study of its metabolism in virus-infected cells is required. Thus, we undertook the present work to gain some insight into kinetics and metabolic pathways of (*N*)-MCT, in comparison to GCV, in HSV-1-infected and uninfected Vero cells. This paper presents evidence of the selective phosphorylation of (*N*)-MCT to its 5-triphosphate in HSV-1-infected cells; the dose and time dependent nature of its cellular metabolism and how it might differ from the metabolism of GCV both in native and viral infected Vero cells.

2. Materials and methods

2.1. Materials

(*N*)-MCT was synthesized as previously described (Marquez et al., 1996). GCV (Cytovene-IV) was obtained from Hoffman La Roche Laboratories, Nutley, NJ. [Methyl-^{3}H]-(*N*)-MCT (1.7 Ci/mmol) and [8-^{3}H]-GCV (22 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). The radiochemical purity of these substances, as determined by HPLC, was $>99\%$. Other nucleoside and nucleotide standards were purchased from Sigma Chemical Co. (St. Louis, MO). The enzymes, phosphodiesterase I, type VII (*Crotalus atrox* venom), and alkaline phosphatase (*Escherichia coli*) were purchased from Sigma. All other reagents and chemicals were of the highest quality obtainable.

2.2. Cells and virus

African green monkey kidney (Vero) cells were purchased from the American Type Culture Col-

lection (ATCC), Rockville, MD, USA. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 1% glutamine, 50 U/ml penicillin, 50 ug/ml streptomycin and incubated at 37 °C in a humidified air containing 5% CO_2. HSV-1 was propagated to $> 10^8$ plaque forming units (PFU) per ml in Vero cells and its concentration was estimated by a standard plaque assay (Huleihel et al., 2001).

2.3. (N)-MCT and GCV cytotoxicity

For the determination of the cytotoxic effects of (*N*)-MCT and the reference standard GCV, exponentially growing Vero cells (10^5 cells) were cultured in 24-well plates overnight. Cells were then washed with fresh medium and varying concentrations (0–100 μM) of (*N*)-MCT and GCV were added. After an additional 48 h, cells were harvested by trypsinization, collected and counted in a Coulter counter. The cell growth rate was expressed as a percentage of the increase in cell number of the untreated control cultures.

2.4. In vitro antiviral assay

Plaque reduction assay was performed as described by Huleihel et al. (2001). Briefly, Vero cells were seeded at 0.25 million/well of a six-well culture plates, in DMEM with 10% FCS and antibiotics. Following overnight incubation, medium was removed and each well was infected at a multiplicity of infection (m.o.i.) of 1 PFU/cell, which was allowed to adsorb for 2 h at 37 °C. The unadsorbed virus was removed and cells were overlayered with DMEM medium supplemented with 2% FCS and 0.6% agar and increasing concentrations of (*N*)-MCT or GCV. Cells were allowed to grow for several days (4–7 days) until the cytopathic effect was obtained. Thereafter, the overlay was removed, cell monolayers were fixed with 10% formalin in saline, stained with crystal violet and plaques were counted. IC_{50}s were calculated by determining the drug concentration (μM) required to confer a 50% plaque inhibition on quadruplicate HSV-1-infected cell monolayers.

2.5. Preparation of cell extracts for (N)-MCT and GCV metabolites analysis

Vero cell cultures ($\sim 3–5 \times 10^6$ cells/25 cm^2 flask) were infected with 1 PFU/cell of HSV-1 for 2 h. After which medium was removed and replaced with fresh medium containing the appropriate radioactive drug. As a control, uninfected cells were treated with radioactive drugs only. In experiments where the HSV-tk inhibitor Ro-32-2313 was used (generous gift from Dr Joseph Martin from Roche Discovery Welwyn, UK), cells were incubated with 1 μM of the inhibitor for 30 min and thereafter (*N*)-MCT and GCV were added to medium. At the end of the appropriate incubations, cells were washed three times with phosphate buffer saline (PBS) to remove residual drug, then trypsinized and pelleted. The dry pellets were suspended in 250 μl of 60% methanol (HPLC grade), and heated at 95 °C for 3 min. After centrifugation at $12{,}000 \times g$ for 10 min, the clear supernatant fractions were evaporated under nitrogen and redissolved in 250 μl of water, and aliquots of the latter reconstituted samples were subjected to anion-exchange chromatography.

2.6. HPLC separation of (N)-MCT and GCV metabolites

Gradient anion-exchange HPLC: The separations of (*N*)-MCT and GCV and their phosphorylated metabolites were carried out using a Hewlett-Packard 1100 HPLC with a diode-array ultraviolet absorption detector. A Partisil-10 SAX column (250×4.6 mm) was used, with the following elution program: 0–5 min, 100% buffer A (0.01 M ammonium phosphate, native pH); 5–20 min, linear gradient to 25% buffer B (0.7 M ammonium phosphate with 10% methanol); 20–30 min linear gradient to 100% buffer B; 30–40 min 100% buffer B; 40–55 min, linear gradient to 100% buffer A and equilibration. The flow rate was 2 ml/min. One-minute fractions were collected and radioactivity was determined by scintillation spectrometry. The retention time of (*N*)-MCT and its phosphates were as follows: (*N*)-MCT, 3 min; (*N*)-MCT-monophosphate

((*N*)-MCT-MP), 6 min; (*N*)-MCT-diphosphate ((*N*)-MCT-DP), 20 min; (*N*)-MCT-triphosphate ((*N*)-MCT-TP), 30 min. For GCV and its phosphorylated products the retention times were: GCV, 3 min; GCV-monophosphate (GCV-MP), 12 min; GCV-diphosphate (GCV-DP), 24 min; GCV-triphosphate (GCV-TP), 32 min. Fractions containing radiolabeled (*N*)-MCT and GCV nucleotides were quantitated based on the known specific activity of the parent tritiated nucleoside.

2.7. Enzymatic identification of (N)-MCT metabolites

The identification of the (*N*)-MCT metabolites was carried out by selective enzymatic degradation of the extracts of cells exposed to (*N*)-MCT as previously described (Ford et al., 1991; Agbaria et al., submitted). Briefly, lyophilized methanolic cell extracts were redissolved in 100 μl of 0.01 M Tris–HCl, pH 9.0, containing 1 mM $MgCl_2$, and 1.5 units alkaline phosphatase or 0.03 units of venom phosphodiesterase were added to the appropriate aliquots. Samples were incubated for 4 h at 37 °C, enzymes were inactivated by heating at 95 °C for 2.5 min, and aliquots were then analyzed by anion-exchange and HPLC as described above. Greater than 95% of the total tritium coeluted with authentic standards for [^{3}H]-(*N*)-MCT.

3. Results

3.1. Antiviral effect of (N)-MCT and GCV

The antiviral activity of (*N*)-MCT against HSV-1 was tested using the plaque reduction assay method with GCV as positive control. Vero cell monolayers were treated with increasing concentrations of (*N*)-MCT or GCV for 1 h before infection with 1 m.o.i. of HSV-1. The treatment with the appropriate drug was continued after infection up to the end of the experiment. Under these conditions, (*N*)-MCT showed a significant and reproducible antiviral activity with a potency surpassing that of GCV. The IC_{50} values of (*N*)-MCT and GCV were 0.02 and 0.25 μM, respectively (Fig. 2B). Both drugs had no measurable cytotoxicity against uninfected Vero cells, the concentration found to cause 50% toxicity (CC_{50}) being >100 μM (Fig. 2A).

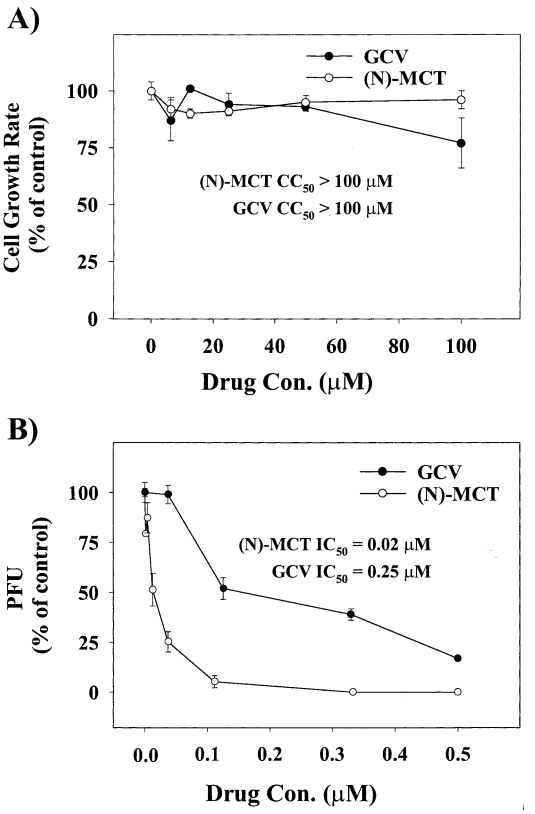


Fig. 2. Cytotoxicity and antiviral effects of (*N*)-MCT and GCV on HSV-1 virus. (A) Uninfected Vero cells were incubated with increasing concentration (0–100 μM) of (*N*)-MCT or GCV for 48 h. At the end of incubation, cells were harvested by trypsinization, counted and growth rate was calculated as described in Section 2. (B) Vero cells infected with 1 m.o.i. of HSV-1 were incubated with increasing concentrations of (*N*)-MCT or GCV. PFU were determined by the standard plaque assay as described in Section 2. Data are mean ± SD ($n = 4$).

3.2. Phosphorylation of (N)-MCT and GCV in HSV-1-infected cells

The intracellular phosphorylation of (*N*)-MCT and GCV to their triphosphate forms is thought to be a prerequisite for the drugs to be antivirally effective. Thus, as a first step, we characterized

the metabolic products of (*N*)-MCT in HSV-1-infected and uninfected Vero cells, using GCV as a reference compound. HSV-1-infected or uninfected Vero cells were incubated for 6 h at 37 °C with a medium containing [^{3}H]-(*N*)-MCT or [^{3}H]-GCV, 10 μM, 5 μCi/ml, 2 h post-virus adsorption. At the end of incubation, methanolic extracts were prepared and analyzed by HPLC using a Partisil 10 SAX column system.

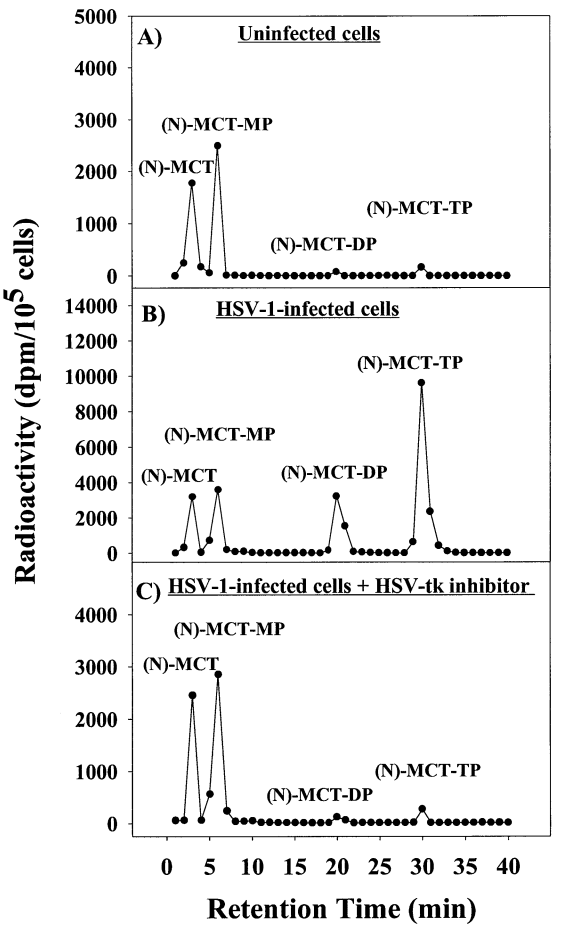


Fig. 3. HPLC radiochromatograms of [^{3}H]-metabolites arising from [^{3}H]-(*N*)-MCT in HSV-1-infected and uninfected Vero cells. [^{3}H]-(*N*)-MCT, 10 μM, 5 μCi/ml, was added to the uninfected (A) or HSV-1-infected (B) and to HSV-1-infected cells incubated for 30 min with 1 μM of the HSV-tk inhibitor Ro-32-2313 (C) and incubated for 6 h. Thereafter, cells were harvested and methanolic extracts were prepared and analyzed by HPLC as described in Section 2.

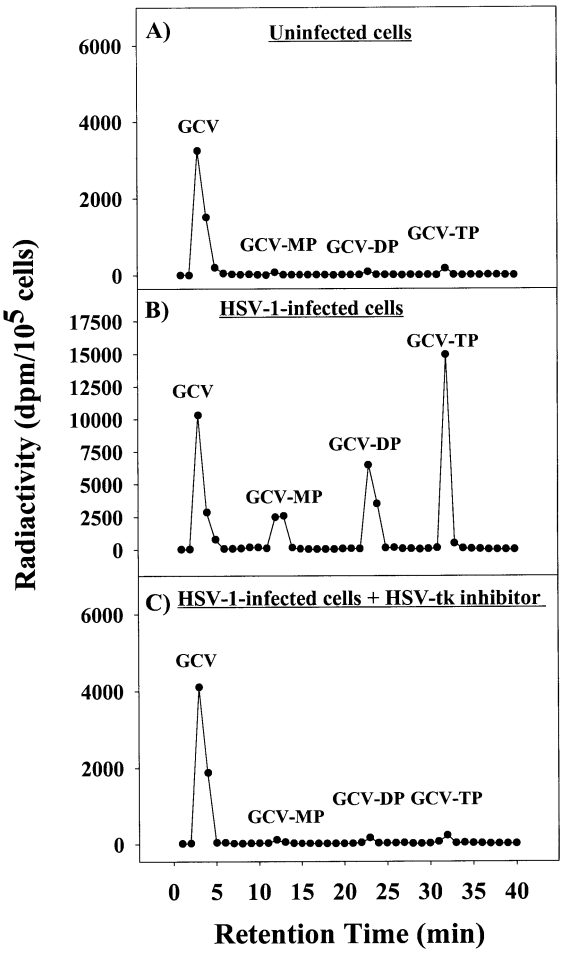


Fig. 4. HPLC radiochromatograms of [^{3}H]-metabolites arising from [^{3}H]-GCV in HSV-1-infected and uninfected Vero cells. [^{3}H]-GCV, 10 μM, 5 μCi/ml, was added to the uninfected (A), HSV-1-infected (B) and to HSV-1-infected cells incubated for 30 min with 1 μM of the HSV-tk inhibitor Ro-32-2313 (C) cultures for 6 h. Thereafter, cells were harvested and methanolic extracts were prepared and analyzed by HPLC as described in Section 2.

HPLC profiles of uninfected cell extracts incubated with (*N*)-MCT showed that in addition to the radioactivity associated with (*N*)-MCT (first peak), an elevated radioactive peak in the monophosphate regions was detected along with two small peaks in the di- and triphosphate regions (Fig. 3A and Table 1). In contrast to (*N*)-MCT, uninfected cell extract analysis of Vero cells incubated with GCV showed only trace lev-

Table 1
Levels of (*N*)-MCT and GCV phosphates in uninfected and HSV-1-infected Vero cells

Metabolite (pmol/10^6 cells)	(*N*)-MCT			GCV		
	Uninfected cells	HSV-1 infected cells[a]	HSV-1 infected cells + Ro-32-2313[b]	Uninfected cells	HSV-1 infected cells[a]	HSV-1 infected cells + Ro-32-2313
Monophosphate	25 ± 1.5	40 ± 12 (1.6)[c]	32 ± 4.3 (1.3)	0.86 ± 0.4	67 ± 4 (78)	1.42 ± 0.4 (1.7)
Diphosphate	0.22 ± 0.1	65 ± 10 (295)	0.41 ± 0.2 (1.9)	1.13 ± 0.5	112 ± 8 (100)	2.02 ± 0.3 (1.8)
Triphosphate	1.11 ± 0.9	145 ± 17 (131)	2.89 ± 0.4 (2.6)	2.10 ± 0.9	172 ± 12 (82)	4.21 ± 0.4 (2)

Data are mean $\pm$ SD ($n = 3$).

[a] Vero cells were infected with HSV-1 for 2 h and incubated with 10 μM, 5 μCi/ml GCV for 6 h.

[b] Ro-32-2313, HSV-tk inhibitor, was added 30 min prior to the addition of (*N*)-MCT or GCV.

[c] Values in parenthesis are the ratio between HSV-1-infected and uninfected cells.

els of radioactive materials eluting in the mono-, di- and triphosphate regions (Fig. 4A and Table 1). However, the nucleotide profile of HSV-1-infected cell extracts incubated with (*N*)-MCT or GCV showed that in addition to (*N*)-MCT and GCV, three radioactive materials were detected in large amounts in the mono-, di-, and triphosphate regions and that the triphosphate peaks were predominant (Fig. 3B, Fig. 4B and Table 1). In HSV-1-infected cells treated with the HSV-tk inhibitor Ro-32-2313, only (*N*)-MCT metabolite was measured in high levels while the levels of the metabolites in the di- and triphosphate regions were very low and similar to the levels measured in uninfected cells (Fig. 3C). When GCV metabolites were measured in HSV-1-infected cells in the presence of the HSV-tk inhibitor, trace levels of GCV metabolites were found in the mono-, di- and triphosphate regions (Fig. 4C).

The various phosphorylated (*N*)-MCT metabolites found in HSV-1 infected cell extracts were identified by two methods. The first one was based on the characteristic retention time of the metabolites, which eluted in the mono-, di- and triphosphate regions of the chromatogram. Second, by peak-shift experiments which showed that the radioactive triphosphate could be converted to the corresponding monophosphate derivative by the use of snake venom phosphodiesterase, and to the parent compound by alkaline phosphatase (Fig. 5).

3.3. Phosphorylation rate of (N)-MCT and GCV at various times

We examined the phosphorylation rate of (*N*)-MCT and GCV as a function of time in the infected cell monolayers. Vero cells were infected with 1 m.o.i. of HSV-1 and at different times post-infection, the cells were incubated for 6 h with fresh medium containing (*N*)-MCT or GCV, 10 μM, 5 μCi/ml. As shown in Fig. 6, the levels of (*N*)-MCT and GCV triphosphates peaked when drug was added 6 h post-virus infection; after this post-infection time, a lower levels of triphosphate formation were observed.

3.4. Effect of extracellular drug concentration on (N)-MCT and GCV phosphorylation

The extent of formation of (*N*)-MCT and GCV phosphates was dependent upon the concentration of the drugs in the medium (Fig. 7). Similar phosphate levels of (*N*)-MCT and GCV were measured in HSV-1-infected cell extracts

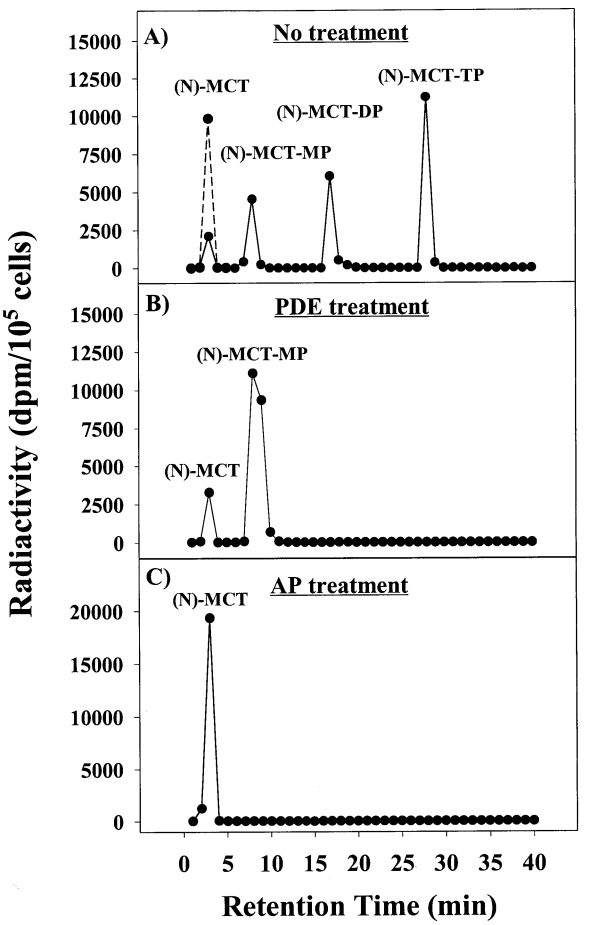


Fig. 5. Anion-exchange HPLC chromatography for enzymatic identification of (*N*)-MCT metabolites in HSV-1-infected Vero cells. Aliquots of methanolic extracts of HSV-1-infected Vero cells exposed to [^{3}H]-(*N*)-MCT for 6 h, were analyzed by ion-exchange HPLC (A) and following 4 h treatment with phosphodiesterase (PDE) (B) or alkaline phosphatase (AP) (C) in Tris–HCl, pH 9. Dotted line represents authentic [^{3}H]-(*N*)-MCT standard.

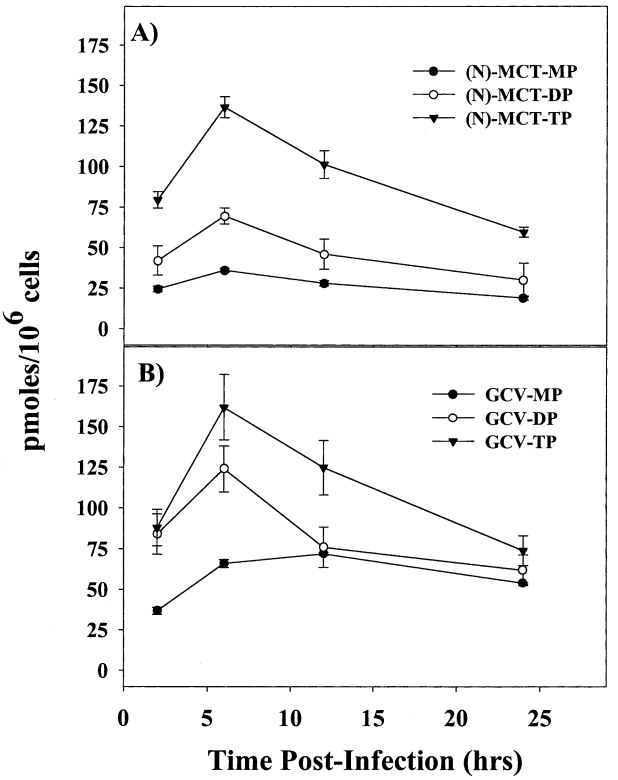


Fig. 6. Phosphorylation rate of (*N*)-MCT and GCV at various post-infection time. Vero cells were infected with HSV-1 and at the indicated times points cells were washed and incubated with fresh medium contains [^{3}H]-(*N*)-MCT (A) or [^{3}H]-GCV (B), 10 μM, 5 μCi/ml, for 6 h. Levels of drug phosphates were determined by HPLC as described in Section 2. Data are mean ± SD, ($n = 3$).

over a wide range of drug concentrations. Although the triphosphate metabolites measured in HSV-1-infected cells at all concentrations were the highest, a difference in (*N*)-MCT-MP levels between the uninfected and HSV-1-infected cells was found at all drug concentrations. For instance, at 10 μM, there was an approximately twofold greater amount of (*N*)-MCT-MP in the infected than in uninfected cells (Table 1). The formation of the triphosphate of (*N*)-MCT and GCV in the HSV-1-infected cells appeared to be saturable, reaching concentrations at least 2–3-fold higher than the levels of mono- and diphosphates (Fig. 7).

3.5. Time-course of (N)-MCT and GCV phosphate metabolites accumulation and decay

The time course of accumulation and decay of (*N*)-MCT and GCV phosphates in HSV-1-infected Vero cells was investigated. Cells were infected with 1 m.o.i. of HSV-1 for 2 h, washed and incubated with a medium containing [^{3}H]-(*N*)-MCT and [^{3}H]-GCV, 10 μM, 5 μCi/ml, for various times. To assess the decay rate of drug phosphates, at the end of the 24-h incubation

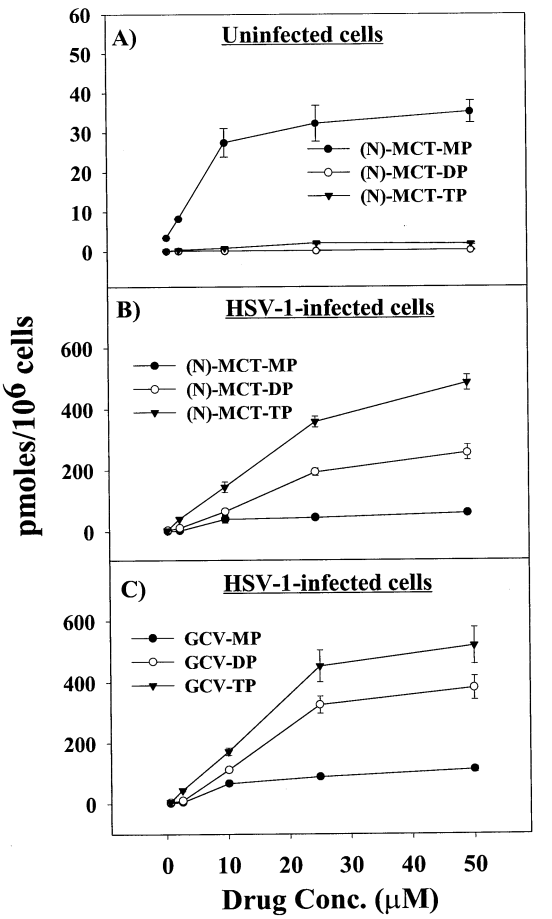


Fig. 7. Dose-dependent accumulation of (*N*)-MCT and GCV phosphates in uninfected and HSV-1-infected Vero cells. Methanolic extracts of uninfected (A) and HSV-1-infected (B) Vero cells were prepared after 6 h incubation with increasing concentrations of [^{3}H]-(*N*)-MCT or GCV (C) as indicated. Extracts were prepared and analyzed by ion-exchange HPLC as described in Section 2. Data are mean ± SD, ($n = 4$).

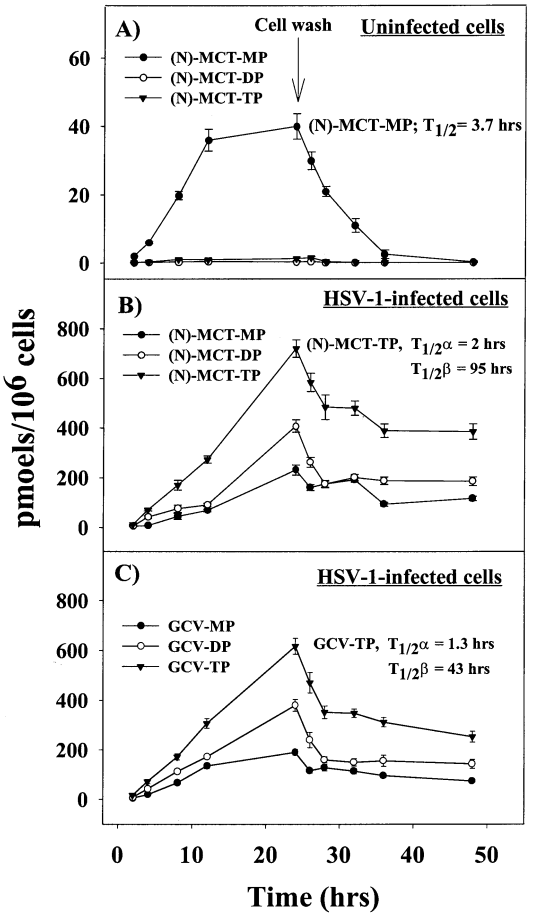


Fig. 8. Time-course of accumulation and decay of (*N*)-MCT and GCV metabolites in HSV-1-infected Vero cells. Uninfected Vero cells (A) or HSV-1-infected (B and C) were incubated with [^{3}H]-(*N*)-MCT (A and B) or [^{3}H]-GCV (C), 10 μM, 5 μCi/ml, and collected at the times indicated for measurements of (*N*)-MCT and GCV phosphates by ion-exchange HPLC as described in Section 2. At 24 h (arrows), cells were washed and reincubated in drug-free medium, and harvesting continued at the times indicated for the measurement of (*N*)-MCT or GCV phosphates. Half life's of drug metabolites were calculated by analyzing the data using Multifit Pharmacokinetic software (University of Groningen, the Netherlands) using a 2-compartment open and the r^2 calculated for data fitting was >0.97. Data are mean $\pm$ SD, ($n=4$).

period, cells were washed, reincubated in drug-free medium, and incubations terminated at different time points. Levels of drug phosphates were determined by HPLC as described in Section 2. As can be seen in Fig. 8, in the uninfected cells, high levels of (*N*)-MCT-MP were detected which seem to reach saturation after 8 h of incubation, achieving an intracellular concentration of 35 pmol/10^6 cells. After drug removal from medium, the (*N*)-MCT-MP decay was monophasic with a half-life of 3.7 h (Fig. 8A). The levels of (*N*)-MCT-DP and -TP in uninfected cells were very low and no increase in their levels was found during this incubation period. In HSV-1-infected Vero cells, all three phosphorylated metabolites of (*N*)-MCT, like GCV, increased with time without reaching saturation (Fig. 8B and C). However, the triphosphate metabolite of (*N*)-MCT and GCV showed the most rapid rate of increase. The respective levels of triphosphate metabolites of (*N*)-MCT and GCV at 24 h of incubation were 720 ± 35 and 617 ± 32 pmol/10^6 cells. The decay of the triphosphate of (*N*)-MCT and GCV appeared to be biphasic, for (*N*)-MCT-TP the half-life's were $T_{1/2}\alpha = 2$ and $T_{1/2}\beta = 95$ h and for GCV-TP were $T_{1/2}\alpha = 1.3$ and $T_{1/2}\beta = 43$ h (Fig. 8).

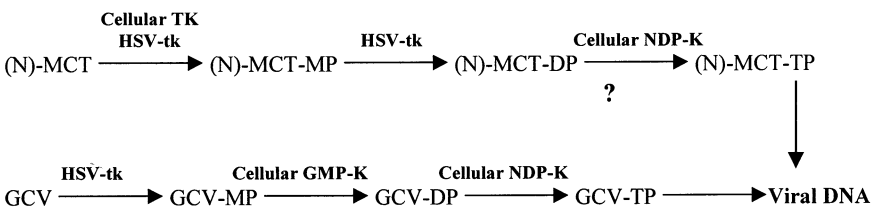
4. Discussion

We have demonstrated that (*N*)-MCT significantly inhibits the infection of Vero cells by HSV-1 with a potency much higher than that of GCV, used as positive control. Our previous studies (Agbaria et al., submitted) showed that formation of the triphosphate metabolite of (*N*)-MCT was a prerequisite for the compound to show antitumor activity in HSV-tk transduced cancer cells. It can be suggested that the antiviral activity of (*N*)-MCT against herpes viruses is also mediated through the formation of its triphosphate metabolite. Additionally, our crystallographic examination of (*N*)-MCT with purified HSV-tk demonstrates favorable kinetic and binding properties for this nucleoside analogue with the viral kinase and limited activity with cytoplasmic thymidine kinase (Prota et al., 2000). This would suggest that pharmacological activity, whether antiviral or antitumor, necessitates viral-induced thymidine kinase activation of (*N*)-MCT. However, unlike HSV-tk gene transduced tumor cells where HSV-tk is persistently expressed, in HSV

infected cells the metabolic rate of cellular and viral enzymes activity is dependent upon many factors such as, infection time, viral load, drug concentration and others. In this study, the phosphorylation rate and pathway of (*N*)-MCT in HSV-1-infected and uninfected Vero cells compared with GCV is described to better understand the mechanism of its antiviral activity.

To assess the antiviral effect of (*N*)-MCT on the HSV-1, we have investigated the phosphorylation rate of (*N*)-MCT in HSV-1-infected and uninfected cells using ^{3}H labeled (*N*)-MCT and GCV. The phosphorylation pattern of (*N*)-MCT is very similar to that of GCV showing simple but successive phosphorylation to the triphosphate in agreement with many previous studies examining GCV and ACV phosphorylation in HSV-infected or HSV-tk transfected cell models (Smee et al., 1983; Field et al., 1983; Elion et al., 1977; Fyfe et al., 1978; Balzarini et al., 1994; Gentry, 1992; St. Clair et al., 1987; Agbaria et al., 1994; Ishii-Morita et al., 1997). As shown in Scheme 1, GCV, like ACV, is monophosphorylated by HSV-tk and then converted to the corresponding diphosphates by cellular guanylate kinase (Boehme, 1984). The triphosphates are ultimately generated by non-specific cellular enzymes, (Miller and Miller, 1980). GCV monophosphorylation requiring HSV-tk is the rate-limiting step in HSV-infected cells, and the further phosphorylations are catalyzed by cellular kinases. In contrast to GCV, uninfected cells are able to monophosphorylate (*N*)-MCT to adequate levels, yet are incapable of further phosphorylation. This suggest that diphosphorylation of (*N*)-MCT requires a viral enzyme and becomes the rate-limiting step in infected cells. The ability of HSV-tk inhibitor, Ro-32-2313 (Martin et al., 1998; Watkins et al., 1998) to inhibit the formation of (*N*)-MCT-DP indicates that HSV-tk responsible for the diphosphorylation of (*N*)-MCT-MP. These findings suggest that: (1) (*N*)-MCT behaves as a substrate for both HSV-tk and cellular thymidine kinase; (2) (*N*)-MCT-MP is a substrate only for HSV-tk but not for the cellular kinases (Scheme 1). Balzarini et al. (1987) and others (Chen et al., 1979; Herdewijn et al., 1985), reported that HSV-tk-1 specific dTMP kinase is able to further phosphorylate 5′-monophosphate of thymidine analogues such as (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) and (*E*)-5-(2-iodovinyl)-2′-deoxyuridine (IVDU) to their 5′-diphosphates. The ability of HSV-tk to diphosphorylate these monophosphates, but not GCV-MP, may be explained by the common pyrimidine-type base shared by each active substrate, whereas GCV is a purine derivative.

Although the present study focused on the metabolic activation of (*N*)-MCT in HSV-1-infected cells, the metabolism of the drug in uninfected cells has importance in understanding the safety and selectivity of the drug. Since the phosphorylation of (*N*)-MCT is limited to formation of (*N*)-MCT-MP with little apparent cytotoxicity in uninfected cells, this might suggest that (*N*)-MCT is a more selective drug for HSV treatment than GCV. For instance, the administration of GCV results not only in the death of the recipient tumor cell expressing HSV-tk, but also in the death of surrounding non-transfected cells primarily because GCV-MP can diffuse via gap junctions into neighboring non-transfected cells and be further phosphorylated to the triphosphate (the 'bystander' effect) (Bi et al., 1993; Ishii-Morita et al., 1997). It is unlikely that this phenomenon can occur with (*N*)-MCT because uninfected neighboring cells to HSV-infected cells



Scheme 1. Phosphorylation of (*N*)-MCT and GCV in HSV-1 infected Vero cells. GMP-K, guanylate monophosphate kinase; NDP-K, nucleoside 5′-diphosphate kinase.

lack the machinery to convert (*N*)-MCT-MP to (*N*)-MCT-TP. Thus, (*N*)-MCT may have an advantage in only acting at the local infection site. However, in virus-infected cells (*N*)-MCT-TP, like GCV-TP, is undoubtedly the most important metabolite of (*N*)-MCT phosphates found in this study. We therefore examined the virus-infection time, the dose response for the formation of this nucleoside, and the rates of its appearance and decay over time. Although (*N*)-MCT was found to be 10-fold more potent against HSV-1 virus than GCV, the levels of the triphosphate of both drugs, the active metabolites, measured in HSV-1-infected cells were similar. This finding suggests that the superior (*N*)-MCT potency is beyond the phosphorylation rate of the drug.

Although the metabolic activation rate of (*N*)-MCT to its 5′-triphosphate under various conditions appeared similar to that for GCV, the decay rate of (*N*)-MCT-TP was significantly slower than that of GCV-TP, $T_{1/2}\beta$, 95 vs. 45 h, respectively (Fig. 8). Long persistence of the active metabolite in virus infected cells after drug removal may contribute significantly to the potency of the drugs in vivo. Cheng et al. (1983) showed that GCV could be removed at 8 h from infected cells without a subsequent increase in virus replication. Thus, the persistence of (*N*)-MCT triphosphate in infected cells may involve more efficient turnover by phosphorylating enzymes.

The exact mechanism of the antiviral effect of (*N*)-MCT against HSV-1 remains unexplained. Based on the well-known mechanism of action of GCV and ACV, it can be suggested that the (*N*)-MCT triphosphate metabolite plays an important role in its antiviral activity. Previous studies showed that ACV-TP is an obligate DNA chain terminator, lacking a 3′C–OH moiety necessary for DNA chain extension (Reardon and Spector, 1989). By contrast, GCV-TP possesses a 3′-hydroxyl group that can be incorporated internally into the DNA of dividing cells expressing functional HSV-tk, although the rate of DNA elongation appears to be slower (St. Clair et al., 1987). The incorporation of this false guanosine nucleotide results in base-pair mismatches, DNA fragmentation, sister chromatid exchange, and lethal genomic instability (Hamzeh and Lietman, 1991; Haynes et al., 1996; Thurst et al., 1996; Ilsley et al., 1995). Template incorporation and extension studies suggested that the DNA polymerase of HSV encounters translocation difficulties after incorporation of GCV-TP (Reid et al., 1988). Since a 3′-hydroxyl group is common to both (*N*)-MCT and GCV but not ACV, incorporation of (*N*)-MCT-TP into viral DNA can, potentially allow elongation. Although further studies are needed to explore the end result of (*N*)-MCT-TP incorporation into viral DNA, this may provide a plausible explanation for viral DNA damage which may be similar to that caused by GCV. A comprehensive study involving the synthesis of (*N*)-MCT-TP and testing its inhibitory effects on HSV–DNA polymerases and/or viral DNA synthesis is underway to clarify the exact antiviral mechanism of action of (*N*)-MCT.

In conclusion, the major observations emerging from these studies are the highly potent and specific antiviral effect of (*N*)-MCT, with IC_{50} of 0.01 μM, and its rapid conversion to the triphosphate form in HSV-1-infected Vero cells. While the exact antiviral mechanism of action of (*N*)-MCT is subject to further study, the selective phosphorylation and potent antiviral activity of (*N*)-MCT observed in HSV-1-infected Vero cells indicate that (*N*)-MCT has considerable promise as a useful anti-HSV agent.

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