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Mini-review

The history of N-methanocarbathymidine: The investigation of a conformational concept leads to the discovery of a potent and selective nucleoside antiviral agent

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Dedicated to Prof. Erik De Clercq on the occasion of reaching the status of Emeritus-Professor at the Katholieke Universiteit Leuven in September 2006.

Abstract

Conformationally locked (North)-methanocarbathymidine (N-MCT) and (South)-methanocarbathymidine (S-MCT) have been used to investigate the conformational preferences of kinases and polymerases. The herpes kinases show a distinct bias for S-MCT, while DNA polymerases almost exclusively incorporate the North 5′-triphosphate (N-MCT-TP). Only N-MCT demonstrated potent antiviral activity against herpes simplex viruses (HSV-1 and 2) and Kaposi's sarcoma-associated herpesvirus (KSHV). The activity of N-MCT depends on its metabolic transformation to N-MCT-TP by the herpes kinases (HSV-tk or KSHV-tk), which catalyze the mono and diphosphorylation steps; cellular kinases generate the triphosphate. N-MCT at a dose of 5.6 mg/kg was totally protective for mice inoculated intranasally with HSV-1. Tumor cells that are not responsive to antiviral therapy became sensitive to N-MCT if the cells expressed HSV-tk. N-MCT given twice daily (100 mg/kg) for 7 days completely inhibited the growth of MC38 tumors derived from cells that express HSV-tk in mice while exhibiting no effect on tumors derived from non-transduced cells. After i.p. administration, N-MCT was rapidly absorbed and distributed in all organs examined with slow penetration into brain and testes. N-MCT-TP was also a potent inhibitor of HIV replication in human osteosarcoma (HOS) cells expressing HSV-tk.

Keywords: Herpes simplex virus; Kaposi's sarcoma-associated herpesvirus; Kinases; DNA polymerases; HIV reverse transcriptase; Delayed chain termination

Contents

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1. Introduction

For the most part, ribo- or 2′-deoxyribonucleoside drugs are essentially inactive until they are converted to 5′-nucleotides inside the cell. In the case of 2′-deoxyribonucleosides, where the desired biological response is principally derived from the incorporation of the drug into DNA, the ability of the molecule to interact effectively with three activating kinases and the target cellular or viral DNA polymerase is of vital importance. A clear understanding of these multifaceted structure-activity associations represents a formidable challenge if we wish to unravel the mode of action of nucleoside-based drugs.

2. Why conformationally locked nucleosides?

Although the sugar ring of nucleosides is flexible and can adopt several conformations, two identifiable and dynamically interconverting conformations have been observed in solution (Saenger, 1983). These have been designated North ($P = 0°$) and South ($P = 180°$) in the pseudorotational cycle (Fig. 1). When nucleosides bind to the active site of enzymes, one conformer interacts selectively with the enzyme. It is for that reason that locked nucleosides have been used to probe the conformational preferences of individual enzymatic steps.

3. Chemical and structural considerations of locked nucleosides

There are several ways of locking the conformation of the sugar in a nucleoside. In some systems, bridging the O2′ and C4′ atoms (Obika et al., 1997; Singh et al., 1998) can achieve a locked North conformation, whereas more elaborate and complex bridges—between C2′ and C3′, and between C3′ and C4′—have been utilized to achieve a locked South conformation (Nielsen et al., 1997; Obika et al., 2002; Thomasen et al., 2002). Our approach consists of using a bicyclo[3.1.0]hexane platform (Marquez et al., 1996; Ezzitouni and Marquez, 1997) in which the cyclopentane ring mimics the ribose ring and the fused cyclopropane ring locks the conformation of the cyclopentane in the vicinity (±18°) of either the North or the South conformation depending on the location of the cyclopropane ring relative to the nucleobase (Fig. 2).

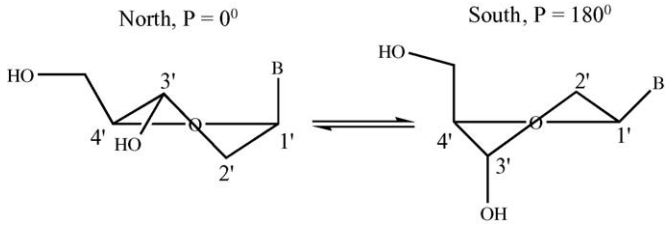

Fig. 1. Rapidly equilibrating North and South conformations of 2′-deoxynucleosides.

4. Probing the role of conformational parameters in determining the substrate preference of cellular and viral kinases

When the locked analogues in Fig. 2 were examined, only the North (N-methanocarbathymidine, N-MCT) showed antiviral activity against herpes simplex 1 and 2 viruses (Marquez et al., 1996). Furthermore, N-MCT was more potent than the standard drug acyclovir (ACV) against HSV-1 and HSV-2 (Table 1).

4.1. Conformation of the pseudosugar

Although the antiviral activity of N-MCT is much greater than its antipode, S-MCT, many factors can contribute to this difference. As with ACV, the antiviral activities of N-MCT and S-MCT depend on the effective phosphorylation of the nucleoside analogues by the herpes thymidine kinase (HSV-tk). In the case of ACV, HSV-tk catalyzes only the monophosphorylation step, while for N- and S-MCT the enzyme catalyzes the first two phosphorylation steps; cellular kinases generate the triphosphate. The triphosphates then interact with viral or cellular DNA polymerases to interfere with DNA synthesis and/or DNA function.

When uninfected and HSV-1 infected Vero cells were separately incubated with [methyl-^{3}H]-N-MCT or [methyl-^{3}H]-S-MCT (10 μM; 5 μCi/ml) for 6 h, the results showed that in infected cells, phosphate levels of S-MCT nucleotides were

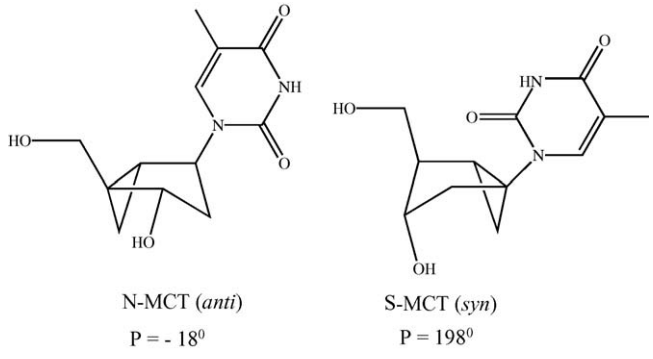

Fig. 2. Bicyclo[3.1.0]hexane nucleosides locked in two non-equilibrating North (N-MCT) and South (S-MCT) conformations.

Table 1
Antiviral activities of N- and S-MCT against herpes viruses in the plaque reduction assay

Compound	Virus (HFF cells)[a]	EC_{50} (μg/ml)[b]	CC_{50} (μg/ml)[c]	SI[d]	ACV control EC_{50} (μg/ml)
N-MCT	HSV-1	0.01	>20	>2000	0.30
N-MCT	HSV-2	0.12	>20	>250	0.80
S-MCT	HSV-1	>50	>50	1	0.15
S-MCT	HSV-2	>50	>50	1	0.60

[a] HFF, human foreskin fibroblasts.
[b] Inhibitory concentration required to reduce the number of virus plaques by 50%.
[c] Cytotoxic concentration that produces 50% of cell death.
[d] Selectivity index (CC_{50}/EC_{50}).

higher than those of N-MCT, particularly for the di- and triphosphates, with South/North phosphorylation ratios of 0.9, 4.6 and 2.5 for the mono-, di-, and triphosphates, respectively (Marquez et al., 2004). These results support the concept that in the last two kinase steps the South conformer is preferred.

4.2. Conformation of the nucleobase

Concerning the first phosphorylation step, the fact that N-MCT and S-MCT appear to be equally good substrates for HSV-tk was puzzling. It is known that the North or South conformation of the ribose also influences the conformation of the nucleobase (*syn* or *anti*) relative to the sugar moiety (Saenger, 1983). The isolated X-ray structures of N-MCT (Altmann et al., 1994a) and S-MCT (Altmann et al., 1994b) showed that the conformation of the nucleobase in N-MCT is *anti*, while the conformation of the nucleobase in S-MCT is *syn*, as depicted in Fig. 2. These crystallographic data are in perfect agreement with what has been observed in conventional nucleosides where the nucleobase tends to be *anti* when the sugar pucker is North and *syn* when the sugar pucker is South (Saenger, 1983). This propensity is accentuated in the locked bicyclo[3.1.0]hexane nucleosides, even in solution, because the energy barriers between *anti* and *syn* are larger (Marquez et al., 2004). Remarkably, the crystal structures of HSV-tk in complexes with N-MCT (Prota et al., 2000) or S-MCT (Schelling et al., 2004) show that in both complexes the nucleobase is *anti*. This means that when HSV-tk binds to S-MCT, the enzyme must overcome the *syn* conformational bias of S-MCT, explaining the lower S/N ratio of 0.9 observed for the first phosphorylation step. This was confirmed by testing an isomeric South-bicyclo[3.1.0]hexane analogue in which the thymine ring is in the *anti* conformation (S-MCdT/*anti*, Fig. 3). This structural change increased the S/N ratio for the first phosphorylation step catalyzed by HSV-tk from 0.9 to 2.3 (Marquez et al., 2005), which is in accord with the ratios observed for the subsequent kinase steps.

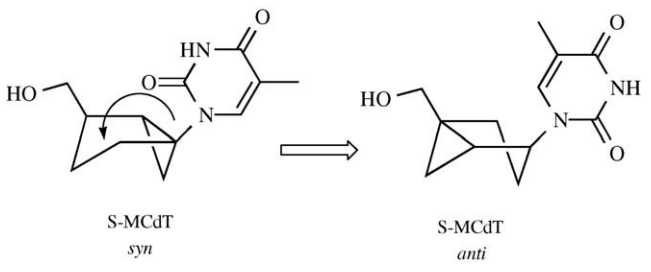

Fig. 3. The *syn*/*anti* barrier was lowered by repositioning the fused cyclopropane in S-MCdT allowing the thymine ring to become *anti*.

5. Probing the role of substrate conformation in determining the preference of viral and cellular DNA polymerases

Once it was established that S-MCT was more efficiently phosphorylated than N-MCT, the conundrum was why N-MCT is the biologically active compound? Examination of DNA extracted from 3-methylcholanthrene-induced colon adenocarcinoma tumor cells (MC38 wild-type) and MC38 cells transduced with *HSV-tk* 24 h after exposure to radiolabeled [methyl-^{3}H]-N-MCT and [methyl-^{3}H]-S-MCT (10 μM; 10 μCi/ml) showed that wild type MC38 cells incorporated negligible amounts of either N- or S-MCT into DNA, while MC38/*HSV-tk* cells incorporated significant quantities of N-MCT (Marquez et al., 2004). The negligible amounts of S-MCT that were incorporated implies that DNA polymerase(s) prefer incorporating nucleotide triphosphates with an N sugar pucker rather than the antipodal S conformer, despite the higher levels of the S triphosphate. Current models based on crystallographic analysis suggest that the intrinsic fidelity of a polymerase depends on its ability to impose an A-conformation (N sugar pucker) providing a structural buffer to conformational variability that may contribute to polymerase fidelity by minimizing mismatches (Timsit, 1999). These results explain why N-MCT is the biologically active conformer.

6. Antiviral activity of N-MCT against HSV-1

Because much of the mechanistic information for N-MCT and S-MCT was obtained from HSV-1 infected Vero cells, the activities of N-MCT and the well-known antiviral agent ganciclovir (GCV) were compared (Zalah et al., 2002). The antiviral activity was assessed using a plaque reduction assay in Vero cell monolayer cultures (0.25 million/well) infected with HSV-1 at a multiplicity of infection (m.o.i) of 1 PFU/cell. Treatment with N-MCT or GCV 1 h before infection resulted in significant and reproducible antiviral activity for both N-MCT and GCV with IC_{50} values of 0.02 and 0.25 μM, respectively. No measurable cytotoxicity was observed in uninfected cells for either agent; the CC_{50} values for both compounds are well above 100 μM.

Table 2
Levels of N-MCT and GCV phosphates (pmol/10^6 cells) in uninfected and HSV-1-infected Vero cells at 6 h post-virus infection

	N-MCT		GCV	
	Uninfected cells	HSV-1 infected	Uninfected cells	HSV-1 infected
Metabolite				
MP	25 ± 1.5	40 ± 12	0.86 ± 0.4	67 ± 4
DP	0.22 ± 0.1	65 ± 10	1.13 ± 0.5	112 ± 8
TP	1.11 ± 0.09	145 ± 17	2.10 ± 0.9	172 ± 12

MP, monophosphate; DP, diphosphate; TP, triphosphate.

The phosphorylation of radiolabeled N-MCT and GCV (10 μM; 5 μCi/ml) in Vero cells infected with 1 m.o.i. of HSV-1 was measured at different post-infection time intervals showing that levels of N-MCT-TP and GCV-TP peaked when the drugs were added at 6 h post-virus infection with the levels for the key metabolites, N-MCT-TP and GCV-TP, reaching concentrations at least two to three-fold higher than those for the mono- and diphosphates (Table 2).

After a 24 h incubation period, the decay of N-MCT-MP was monophasic ($t_{1/2} = 3.7$ h) whereas for N-MCT-TP ($t_{1/2}\alpha = 2$ h and $t_{1/2}\beta = 95$ h) and GCV-TP ($t_{1/2}\alpha = 1.3$ h and $t_{1/2}\beta = 43$ h) the decay appeared to be biphasic (Zalah et al., 2002). There are important differences between N-MCT and GCV. Uninfected cells are able to monophosphorylate N-MCT to adequate levels, yet unable to continue further phosphorylation. However, levels of N-MCT-MP are higher in infected cells. This means that N-MCT is a substrate for both cellular tk and HSV-tk, but that N-MCT-MP is a substrate only for HSV-tk and not for the cellular tk. Thus, the diphosphorylation of N-MCT, which requires HSV-tk, is the rate-limiting step. GCV, on the other hand, requires HSV-tk only for monophosphorylation (Kokoris and Black, 2002); the cellular kinases efficiently convert GCV-MP to the di- and triphosphate metabolites. Thus, for GCV, the rate-limiting step is the formation of GCV-MP. The ability of HSV-tk to phosphorylate N-MCT-MP and not GCV-MP reflects the capacity of the enzyme to discriminate against the purine nucleobase of GCV-MP in the second phosphorylation step. Although the activation rates for both drugs are comparable, the decay rate of N-MCT-TP was significantly lower than that of GCV-TP and the persistence of N-MCT-TP in virus-infected cells may contribute to the 10-fold higher potency of N-MCT.

The pharmacodynamics of N-MCT has also been investigated (Huleihel et al., 2005). Vero cells infected with 1 m.o.i. of HSV-1 were fully protected when 1 μM N-MCT was added immediately and even 8 h post-infection. When N-MCT was added 12 h post-infection, only a partial inhibition was observed, and antiviral activity waned when the drug was added 18 or 24 h post-infection. Because the majority of HSV-DNA synthesis occurs between 8 and 12 h post-infection, administration of the drug beyond this window may not be effective. However, when the infectivity was reduced to 0.01 m.o.i., N-MCT provided full protection even when the drug was added 24 h post-infection. This means that the initial size of viral inoculum is an important factor. Another important parameter is the length of treatment. The inhibitory activity of N-MCT was reversed when treatment was terminated between 10 and 24 h post-infection, depending on the titer of the virus. Conversely, the inhibitory effect of N-MCT was not reversed when treatment was terminated at either 24 or 48 h post-infection.

All the dynamic factors discussed above, plus the pharmacokinetics of absorption, distribution and elimination are important factors for in vivo activity. N-MCT showed good antiviral activity in BALB/c mice inoculated intranasally with HSV-1 (Table 3). The intranasal inoculation is a model for herpes encephalitis in humans, which involves the nasal route of infection. Animals were treated i.p. twice daily for 7 days at the doses indicated and monitored for 21 days (Prichard et al., 2006). Significant efficacy was demonstrated by N-MCT at all concentrations compared to a placebo. The ACV control also demonstrated significant efficacy at all concentrations. No overt signs of toxicity were observed with either drug.

7. Antiviral activity of N-MCT against Kaposi sarcoma

Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi sarcoma (KS). KSHV is a gamma-2 herpesvirus (*Rhadinovirus*) closely related to other oncogenic gamma herpesviruses, including Epstein-Barr virus. The anti-KSHV activities of N-MCT, GCV and cidofovir (CDV) were assessed by measuring the levels of newly synthesized KSHV virion-associated DNA in the supernatant of lytically-induced KSHV-infected BCBL-1 cells compared to untreated controls (Zhu et al., 2005). The compounds were added to the BCBL-1 culture after the lytic cycle was fully induced by phorbol myris-

Table 3
Effect of N-MCT treatment on the mortality of BALB/c mice inoculated intranasally with HSV-1 (reproduced with permission)

	Placebo[a]	N-MCT (mg/kg)			ACV (mg/kg)		
		50	16.7	5.6	50	16.7	5.6
Mortality	7/14	0/15	0/15	1/15[b]	0/15	0/15	0/15
% Mortality	50	0	0	7	0	0	0
p-Value	–	<0.01	<0.01	<0.06	<0.01	<0.01	<0.01
MDD[c]	0.80	–	–	20	–	–	–
p-Value	–	–	–	NS[d]	–	–	–

[a] 0.4% CMC.
[b] In a duplicate experiment there were no deaths.
[c] MDD, mean day of death.
[d] Not statistically significant when compared to placebo.

Table 4
Anti-KSHV activity of N-MCT, cidofovir, and ganciclovir in PMA-induced BCBL-1 cells (mean from three independent experiments)

Compound	Mean IC_{50} (μM) ± S.D.[a]	Mean IC_{90} (μM) ± S.D.[b]	SI[c]
N-MCT	0.08 ± 0.03	0.68 ± 0.10	>2500
CDV	0.42 ± 0.07	4.01 ± 2.05	>476
GCV	0.96 ± 0.49	7.11 ± 0.28	>208

[a] Inhibitory concentration required to reduce KSHV virion-associated DNA by 50%.
[b] Inhibitory concentration required to reduce KSHV virion-associated DNA by 90%.
[c] Calculated on the basis of a cytotoxic concentration of >200 μM and the IC_{50} values.

tate (PMA) for 24 h. After 3 days, dose-dependent decreases in KSHV virion-associated DNA copies determined by quantitative PCR were readily observed for N-MCT, CDV and GCV (Table 4). None of the compounds showed any significant cytotoxicity in BCBL-1 cells up to a concentration of 200 μM.

KSHV-infected BCBL-1 cells incubated with [methyl-^{3}H]-N-MCT (10 μM; 5 μCi/ml) produced the monophosphate (N-MCT-MP) with or without PMA stimulation. However, sharp increases in the levels of N-MCT-DP and N-MCT-TP were observed only in PMA-stimulated cells, which contained five to eight-fold higher levels of N-MCT-DP and N-MCT-TP than unstimulated cells. In contrast, there was no appreciable accumulation of N-MCT-DP and N-MCT-TP in uninfected T lymphocyte cells (CEM-SS) with or without PMA stimulation. The reduction in KSHV virion production by N-MCT was accompanied by a corresponding decrease in intracellular KSHV DNA levels, indicating that the compound probably blocked lytic KSHV DNA replication either directly or indirectly. Synthetic N-MCT-TP strongly inhibited in vitro DNA synthesis mediated by baculovirally-expressed recombinant KHSV polymerase and polymerase processivity factor. In this assay N-MCT-TP proved to be a more potent inhibitor of DNA synthesis by the KHSV polymerase than CDV-DP or GCV-TP (Table 5).

The open reading frame (ORF21) of KSHV has been reported to encode a functionally active thymidine kinase. Therefore, it was not surprising that the anti-KSHV activity of N-MCT was abrogated in a dose-dependent manner by 5′-ethynylthymidine (5′-ET), a known inhibitor or HSV-tk. A dose of 50 μM 5′-ET completely neutralized the effect of 1 μM N-MCT; however, the activity of CDV [(S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]-cytosine], which is metabolically equivalent to a nucleoside monophosphate, was not affected by 5′-ET administration (Zhu et al., 2005).

Table 5
Inhibitory activity of N-MCT-TP, CDV-DP and GCV-TP on in vitro DNA synthesis mediated by KSHV polymerase (mean ± S.D. of triplicate experiments)

Compound	IC_{50} (μM)[a]
N-MCT-TP	6.24 ± 0.08
CDV-DP	14.70 ± 2.47
GCV-TP	24.59 ± 5.60

[a] Inhibitory concentration required to reduce DNA synthesis by 50%.

Table 6
Intracellular levels of N-MCT phosphates (pmol/10^6 cells) in MC38 and MC38/*HSV-tk* tumor cells (mean ± S.D. from three experiments)

Cell type	N-MCT-MP	N-MCT-DP	N-MCT-TP
MC38	9.5 ± 2.0	0.5 ± 0.01	0.9 ± 0.02
MC38/*HSV-tk*	22.3 ± 1.2	9.8 ± 2.0	61.3 ± 3.6

8. Antitumor activity of N-MCT

As a tool, *HSV-tk* transfection has been very useful in allowing a number of antiherpetic agents, such as GCV, to be used for tumor suppression using suicide gene therapy (Moolten, 1986). In tumor cells that express HSV-tk, GCV can be activated and lethal amounts of GCV-TP are produced. In view of the clinical interest generated by the GCV/*HSV-tk* protocol in cancer gene therapy, the activity of N-MCT was compared using MC38 cells that do, and do not, express the *HSV-tk* gene (Noy et al., 2002a). Wild-type and *HSV-tk*-transduced cells were exposed to various concentrations of N-MCT and GCV (0.2–100 μM). Both compounds showed a strong antiproliferative effect in cells that express HSV-tk. In these cells, both compounds had IC_{50} values of nearly 3 μM after a 48 h incubation (N-MCT, IC_{50} = 2.9 μM and GCV, IC_{50} = 3.0 μM). All three metabolites were effectively generated in MC38/*HSV-tk* cells incubated with [methyl-^{3}H]-N-MCT (10 μM; 5 μCi/ml) for 6 h (Table 6). N-MCT-MP was present in wild type cells; however, as with HSV-1-infected Vero cells, more N-MCT-MP was formed in the MC38/*HSV-tk* cells. Also, consistent with the data obtained in HSV-1-infected Vero cells, only trace amounts of N-MCT-DP and N-MCT-TP were detected in non-transduced MC38 cells.

Examination of DNA and RNA extracted from both wild type MC38 and MC38/*HSV-tk* cells after a 24 h exposure to [methyl-^{3}H]-N-MCT (10 μM; 10 μCi/ml) showed little or no incorporation in the DNA of wild-type MC38 DNA. On the other hand, significant quantities of radiolabeled drug (4.7 pmol/μg DNA) were detected in DNA isolated from MC38/*HSV-tk* cells. The levels of incorporation into RNA were <10% of the levels found in DNA.

Encouraged by the fact that the antiproliferative properties of N-MCT appear to depend on tumor cells expressing HSV-tk, and because the potency of the drug was equivalent to that of GCV, the antitumor effect was assessed in vivo. Two groups of six C57/BL6 male mice, 6–8-weeks old, were inoculated with 0.25×10^6 wild-type MC38 cells in the left flank and the same number of MC38/*HSV-tk* cells in the right flank. Tumors were allowed to grow for 7 days, reaching a size of 50–100 mm^3 before treatment was initiated with 100 mg/kg i.p. of N-MCT or GCV twice daily for 7 days. Tumor sizes measured at 1, 3, 5, and 7 days demonstrated that both N-MCT and GCV completely inhibited tumor growth in the HSV-tk-expressing tumors, while no inhibitory effect was observed in the control tumors. The phosphorylation profile of N-MCT in MC38 and MC38/*HSV-tk* transduced tumors was the same as in the in vitro experiments, confirming the critical role of the triphosphate.

9. Pharmacokinetics and distribution of N-MCT

In C57/BL6 mice inoculated with MC38 and MC38/*HSV-tk* cells, the pharmacokinetic profile was studied following the i.p. administration of 100 mg/kg of N-MCT containing 400 μCi of labeled drug (Noy et al., 2002b). The levels of N-MCT in plasma and other tissues were determined by measuring total radioactivity. The plasma pharmacokinetic profile for N-MCT fits a two-compartment model similar to that reported for GCV. The time-concentration in plasma showed a biexponential decline ($t_{1/2}\alpha = 0.20 \pm 0.01$ h and $t_{1/2}\beta = 4.71 \pm 0.62$ h) reaching a peak concentration (C_{max}) of 120 μg/ml 30 min after drug administration. The AUC (area under concentration versus time curve) was 147 μg h/ml and systemic clearance (CL) was 0.69 l/h/kg. HPLC analysis of methanolic extracts of plasma and urine obtained at various intervals revealed no N-MCT metabolites in plasma, and the compound was secreted unchanged in the urine. In all organs studied, the concentration of N-MCT reached peak levels (C_{max}) between 15 and 30 min (T_{max}) after administration, and then rapidly declined within the first 2 h, followed by a slower decline over 24 h. The highest concentrations of N-MCT were found in kidney, spleen and liver, while the lowest concentrations were found in testes and brain. As expected, the levels of N-MCT between 4 and 24 h after drug administration were significantly higher in HSV-tk-expressing tumors than in wild type tumors. This difference may be attributed to cellular trapping of the phosphorylated metabolites, which are preferentially formed in HSV-tk-expressing tumors. On the other hand, the C_{max} (55 ± 10 and 52 ± 10 μg/g of tissue) and T_{max} (0.25 h) values for total radioactivity in both types of cells were similar. The AUC in contrast, was nearly two-fold higher in the *HSV-tk* tumors (219 ± 39 μg h/g) with respect to wild type (134 ± 15 μg h/g). In summary, N-MCT is rapidly absorbed and distributed in normal murine tissue after a single i.p. administration. As expected, the drug is efficiently phosphorylated only in tumor cells transduced with the *HSV-tk* gene.

10. Mechanism of action of N-MCT

From all of the data discussed above, the decisive step is the incorporation of N-MCT-TP by either the viral or the host DNA polymerase; however, the exact mechanism of action remains unexplained. The compound has an OH group on the pseudosugar cyclopentane ring in a position equivalent to the 3′-OH group of a normal ribose ring, which should allow continuation of DNA synthesis. Neither N-MCT nor S-MCT displayed any activity against HIV-1 in cells that do not express HSV-tk; however, the interaction of N-MCT-TP with HIV-1 reverse transcriptase (HIV-1 RT) suggests that N-MCT-TP is a delayed DNA chain terminator (Boyer et al., 2005). As expected from the data obtained with cells expressing HSV-tk and with the herpes polymerase, the polymerase activity of HIV-1 RT showed a similar preference for N-MCT-TP. The ability of N-MCT-TP to be incorporated by HIV-1 RT and inhibit DNA synthesis was examined with a 5′-labeled 18-mer DNA primer annealed to a 43-mer template in the presence of 10 μM each dCTP, dGTP and dATP and 10 μM of (1) dTTP, (2) ddTTP or (3) N-MCT-TP

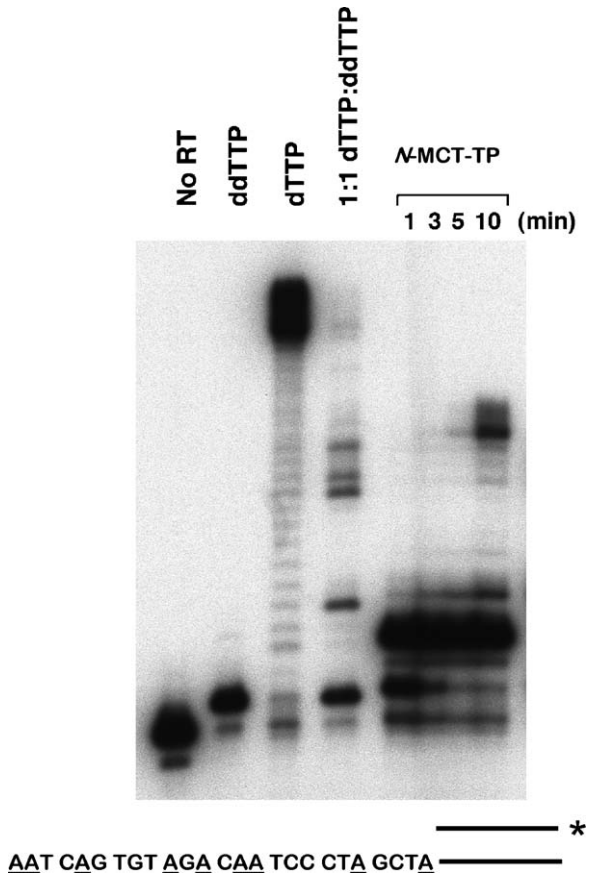

Fig. 4. Inhibition of DNA synthesis by N-MCT-TP. The template is shown with the sites of incorporation underscored and the primer was 5′-end labeled (*).

(Fig. 4). The reference lane shows no DNA synthesis because HIV-1 RT was omitted, and in the following lane, incorporation of ddTTP immediately terminated DNA synthesis. Natural substrate dTTP allows the extension to go to completion (third lane) giving full-length double stranded DNA. The 1:1 dTTP:ddTTP lane reveals the positions at which dTTP or the other T analogues can be incorporated, and the final four lanes show the effects of incorporation of N-MCT-TP for the indicated length of time (1, 3, 5, and 10 min). When N-MCT-TP was the first nucleotide added to the primer strand, DNA synthesis was not blocked immediately by incorporation of N-MCT-TP. Comparing the N-MCT-TP lanes with the 1:1 control lane, there are strong bands that corresponded to a size of two nucleotides beyond the site where N-MCT-TP was incorporated. This suggested that after the N-MCT-TP was incorporated HIV-1 RT extended the primer by the addition of two nucleotides before DNA synthesis was blocked. The blockage of DNA synthesis was not absolute; when the HIV-1 RT reaction mixtures were incubated for longer periods of time, larger products were detected, although full-length DNA was not synthesized. All the other pauses showing larger bands occurred approximately two nucleotides after sites where N-MCT-TP was incorporated. However, not all sites where N-MCT-TP was incorporated produced a block two nucleotides beyond the site of incorporation of N-MCT-TP.

Additional experiments showed that the sequence of the template and the number of consecutive N-MCT-TP units incorpo-

rated have a major role in determining where DNA synthesis will be blocked; in some cases, blocks were seen at positions corresponding to five nucleotides beyond the site of incorporation of the first N-MCT-TP, or even at 17 nucleotides beyond the site of incorporation. A likely mechanism of the delayed chain termination that occurs two to three nucleotides beyond the polymerase active site is that the added N-MCT analogue makes an unfavorable steric contact with the amino acids in the thumb subdomain, thus blocking continued DNA synthesis. The delayed chain termination five nucleotides beyond might occur as the nucleic acid makes the transition from A form to B form DNA, and the 17 nucleotide block could involve contacts with the RNase H domain.

11. An interesting window into HIV resistance

Because N-MCT is poorly phosphorylated beyond the monophosphate level by cellular kinases, it does not have anti-HIV activity. However, N-MCT effectively blocked HIV-1 replication of an HIV-1 vector containing HIV-RT in human osteosarcoma (HOS) cells modified to express the *HSV-tk* gene (Boyer et al., 2005). Because strict chain terminators, like AZT, inhibit DNA synthesis at the point of insertion, they can potentially be removed by pyrophosphorolysis. This is a major mechanism of resistance of HIV-1 to certain nucleoside analogues. The excision mechanism is related to the reverse reaction of incorporation (Nuc-TP $\leftrightarrows$ DNA–Nuc-MP + PP_i); however, in the excision reaction that causes resistance ATP is the pyrophosphate donor. Since such excision requires that the analogue be at the 3′-end of the primer, and because DNA synthesis following the incorporation of N-MCT-TP is able to continue at least two nucleotides beyond, N-MCT-MP was relatively resistant to removal by proficient RT mutants. The replication of drug-resistant HIV-1 variants efficient at ATP-dependent excision was blocked by N-MCT in HOS cells expressing *HSV-tk*. These combined results provide proof-of-principle that delayed chain terminators can effectively overcome HIV-1 resistance to nucleoside analogues that depend on the excision mechanism.

12. Summary

N-MCT is a potent and target-specific antiviral agent capable of inhibiting DNA synthesis through its 5′-triphosphate metabolite produced in cells expressing a virally encoded thymidine kinase. The compound is stable and active in vivo and has a favorable pharmacokinetic profile that warrants further investigations to assess its clinical potential. The mechanism of inhibition of DNA synthesis by N-MCT is different from that of a direct chain terminator.

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