Potent Antiviral Activity of North-Methanocarbathymidine against Kaposi’s Sarcoma-Associated Herpesvirus

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Kaposi’s sarcoma (KS) is a multifocal malignant tumor of endothelial cell origin characterized by the proliferation of spindle-shaped cells with aberrant neovascularization and a large inflammatory cell infiltrate (14). KS usually manifests as pigmented nodular skin lesions, but can often spread to visceral organs in immunocompromised hosts, including AIDS patients (18, 34) and organ transplant recipients (15, 46, 54). This aggressive and disseminated form of KS was recognized as one of the first AIDS-defining conditions at the beginning of the human immunodeficiency virus (HIV) epidemic in the early 1980s (1, 29). Without effective therapy, visceral KS can be highly fatal unless the underlying causes of immune suppression are successfully treated (13, 21). Cytotoxic chemotherapeutic agents are commonly used in disseminated KS with tumor response to any chemotherapeutic regimen is only transient. There is no definitive cure for KS at the present time.

Kaposi’s sarcoma-associated herpesvirus (KSHV) infection is a prerequisite for the development of Kaposi’s sarcoma (KS). Blocking lytic KSHV replication may hinder KS tumorigenesis. Here, we report potent in vitro anti-KSHV activity of 2′-exo-methanocarbathymidine [North-methanocarbathymidine (N-MCT)], a thymidine analog with a pseudosugar ring locked in the northern conformation, which has previously been shown to block the replication of herpes simplex virus types 1 and 2. N-MCT inhibited KSHV virion production in lytically induced KSHV-infected BCBL-1 cells with a substantially lower 50% inhibitory concentration (IC50) than those of cidofovir (CDV) and ganciclovir (GCV) (IC50, mean ± standard deviation: 0.08 ± 0.03, 0.42 ± 0.07, and 0.96 ± 0.49 μM for N-MCT, CDV, and GCV, respectively). The reduction in KSHV virion production was accompanied by a corresponding decrease in KSHV DNA levels in the N-MCT-treated BCBL-1 cells, indicating that the compound blocked lytic KSHV DNA replication. A time- and dose-dependent accumulation of N-MCT-triphosphate (TP) was demonstrated in lytically induced BCBL-1 cells, while uninfected cells showed virtually no accumulation. The levels of N-MCT-TP were significantly decreased in the presence of 5′-ethynylthymidine, a potent inhibitor of herpesvirus thymidine kinase, resulting in the abrogation of anti-KSHV activity of N-MCT. N-MCT-TP more effectively blocked in vitro DNA synthesis by KSHV DNA polymerase with an IC50 of 6.24 ± 0.08 μM (mean ± standard deviation) compared to CDV-diphosphate (14.70 ± 2.47 μM) or GCV-TP (24.59 ± 5.60 μM). Taken together, N-MCT is a highly potent and target-specific anti-KSHV agent which inhibits lytic KSHV DNA synthesis through its triphosphate metabolite produced in KSHV-infected cells expressing a virally encoded thymidine kinase.

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(GCV) or foscarnet for cytomegalovirus infection (23, 38, 40). The use of antiviral agents might have deterred the development of KS, presumably by inhibiting KSHV lytic replication.

To exploit the involvement of KSHV in the tumorigenesis, KSHV-targeted molecular intervention has been proposed to treat KS and other KSHV-induced malignancies, including the use of GCV and foscarnet as antiherpetic DNA synthesis inhibitors (33). In the current study, we identified potent in vitro anti-KSHV activity of 2'-exo-methanocarba thymidine ([(North)-methanocarbanthymidine (N-MCT)], a thymidine analog with a pseudosugar moiety locked in the northern conformation, which has previously been shown to exert strong antiviral activity against herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) (37). Our data demonstrate that N-MCT effectively blocks KSHV DNA synthesis through its triphosphate (TP) metabolite, which is efficiently produced in KSHV-infected cells. N-MCT is 5- to 10-fold more potent than the previously identified inhibitors of KSHV DNA synthesis, cidofovir (CDV) and GCV. Higher potency and target specificity of N-MCT against KSHV may make it a more desirable anti-KS agent.

MATERIALS AND METHODS

Cells and compounds. BCBL-1, a latently KSHV-infected B-cell line established from a primary effusion lymphoma, was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institutes of Health, contributed by Michael McGraith and Don Gunem (48). Toledo cells (a human B-cell line) and CEM-SS cells (a human T-cell line) were employed to examine the toxicity of the test compounds. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone, Research and Reference Reagent Program, Division of AIDS, National Institutes of Health) at 37°C in 5% CO2-containing humidified air in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone, Research and Reference Reagent Program, Division of AIDS, National Institutes of Health) at 37°C in 5% CO2-containing humidified air in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone, Research and Reference Reagent Program, Division of AIDS, National Institutes of Health). The 20-μl reaction mixture consisted of the LightCycler FastStart DNA Master SYBR Green I reagent mix (Roche Applied Science), 2.5 mM MgCl2, and 500 nM each of the KSHV ORF65 primer (5'-AGCGTGTTGTTCAAGATCGT-3') and MTC/R (5'-ATG GGCCGGGTTGTATGAGT-3') was used as an internal control for each LMW DNA PCR sample (63). The amplified products were visualized by electrophoresis on a 1.8% agarose gel.

KSHV virions were pelleted from 300 μl of BCBL-1 culture supernatants by a microcentrifugation at 37°C for 10 min at 4°C (53). The pelleted virions were resuspended in 150 μl PBS and treated with 20 units of DNase I (Promega, Madison, WI) at 37°C for 30 min to remove cellular DNA from the samples, followed by incubation with stop solution (20 mM EDTA) at 70°C for 5 min. Virion-associated KSHV DNA was then extracted by QIAamp Viral RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions.

We subjected 1 μl of DNA eluted in 100 μl of elution buffer to real-time quantitative PCR using a LightCycler instrument (Roche Applied Science, Indianapolis, IN). The 20-μl reaction mixture consisted of the LightCycler FastStart DNA Master SYBR Green I reagent mix (Roche Applied Science), 2.5 mM MgCl2, and 500 nM each of the KSHV ORF65 primer (5'-AGCGGGAAGGATCCCCATTT-3' and 5'-CCCGGTGTTGTATGAGT-3'), 10 mM Tris-HCl pH 8.4, 500 μM each dNTP, 1 unit rTaq Platinum DNA polymerase (Invitrogen), and 200 nM of each primer and template DNA, was subjected to 25 cycles of PCR amplification at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 5 min. In addition, the mitochondrial DNA primer pair MTCF (5'-TGGAGCGGGAACCCCTATGTG-3') and MTC/R (5'-ATGGGCCGGGTTGTATGAGT-3') was used as an internal control for each LMW DNA PCR sample (63). The amplified products were visualized by electrophoresis on a 1.8% agarose gel.

Measurements of cell- and virion-associated KSHV DNA by PCR. Low-molecular-weight (LMW) DNA was extracted from the pelleted cells according to the manufacturer’s instructions (27). The 20-μl reaction mixture consisted of the LightCycler FastStart DNA Master SYBR Green I reagent mix (Roche Applied Science), 2.5 mM MgCl2, and 500 nM each of the KSHV ORF65 primer (5'-AGCGGGAAGGATCCCCATTT-3' and 5'-CCCGGTGTTGTATGAGT-3'), 10 mM Tris-HCl pH 8.4, 500 μM each dNTP, 1 unit rTaq Platinum DNA polymerase (Invitrogen), and 200 nM of each primer and template DNA, was subjected to 25 cycles of PCR amplification at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 5 min. In addition, the mitochondrial DNA primer pair MTCF (5'-TGGAGCGGGAACCCCTATGTG-3') and MTC/R (5'-ATGGGCCGGGTTGTATGAGT-3') was used as an internal control for each LMW DNA PCR sample (63). The amplified products were visualized by electrophoresis on a 1.8% agarose gel.

The cytotoxicity of the compounds was determined in unstimulated and PMA-stimulated BCBL-1 cells harvested, washed once with PBS, and resuspended in serum-free AIM-V medium with bovine serum albumin (Invitrogen) at 1 × 106 cells/ml without PMA in the absence or presence of 10 μM N-MCT, CDV, or GCV and 5 μCi/ml of the corresponding radiolabeled compound. Control cultures containing the same concentration of the test compounds but without the radiolabeled formulations were simultaneously set up in 96-well microplates, using the XTT (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-3-[phenylamino]carbonyl]-2H-tetrazolium) assay (58). In selected experiments, the anti-KSHV activity of N-MCT was compared in the presence and absence of 5'-ET, a potent inhibitor of herpesvirus thymidine kinase (TK) (43), in order to investigate whether virally encoded TK played a role in the intracellular production of an active triphosphate metabolite of N-MCT, as has been demonstrated with other nucleoside analogs, such as GCV, in KSHV-infected cells (7).

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Upon harvest, the cells were centrifuged at 1,500 rpm for 10 min and washed once with cold PBS. The cell pellets were resuspended in 250 μl of 60% methanol and heated at 95°C for 3 min, followed by a microcentrifugation at 12,000 × g for 10 min at 4°C. The clarified supernatant fractions were evaporated under nitrogen, redissolved in 250 μl of water, and subjected to high-pressure liquid chromatography (HPLC) separation of the phosphorylated metabolites as described in detail elsewhere (64). Fractions containing radiolabeled nucleotides were quantitated based on the known specific activity of the parent tritiated nucleoside (64). The phosphorylated metabolites of CDV were identified as CDV-phosphate, CDV-DP (active metabolite), and a phosphate ester adduct of CDV as described above (38).

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In vitro DNA synthesis inhibition assay. To investigate whether the triphosphate metabolite of N-MCT could directly block KSHV DNA polymerase-mediated DNA synthesis, a rapid microplate-based DNA synthesis assay (50) was carried out in the absence or presence of increasing concentrations of N-MCT-TP, using recombinant KSHV DNA polymerase (rPol) and polymerase processivity factor (rPPF). KSHV rPol and rPPF were expressed and purified from the recombinant baculovirus vector-infected Sf9 cells (12). The DNA synthesis reaction was carried out in a 96-well microplate coated with a 5'H1P1-biotinylated 100-mer oligonucleotide template with a 20-mer primer annealed to its 3'H1P1-end (primed template, 0.2 pmol/well) with 10 ng each of KSHV rPol and rPPF in a 50-μl reaction mixture, containing 50 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT), 2% glycerol, 40 μg/ml bovine serum albumin, 0.625 μM deoxynucleoside triphosphates, and 0.125 μM digoxigenin-11-2'-deoxyuridine-5'-triphosphate (DIG-dUTP) (Roche Applied Science), at 37°C for 60 min in the absence or presence of increasing concentrations of N-MCT-TP, CDV-DP, or GCV-TP. The amounts of newly synthesized DNA which incorporated DIG-dUTP were determined by the DIG detection kit (Roche Applied Science) according to the manufacturer’s instructions.

RESULTS

Anti-KSHV activity of N-MCT. In the BCBL-1 cell-based assay developed for the current study, the number of newly released KSHV virion-associated DNA copies determined by quantitative PCR was consistently 10- to 50-fold increased (median, 16.5-fold) in PMA-induced cells over the uninduced control, with a corresponding increase in the amount of KSHV DNA in the Hirt LMW DNA (Fig. 1B). To determine the biological effects of the test compounds specifically on lytic KSHV DNA replication, the compounds were added to the BCBL-1 culture after the lytic cycle was fully induced by PMA for 24 h. After 3 days, dose-dependent decreases in KSHV vDNA and KSHV DNA in the Hirt DNA were readily observed for N-MCT, CDV, and GCV at the concentrations tested, from 0.03 to 10 μM (Fig. 1B). No significant cytotoxicity was observed with any of the three compounds, although at a much higher concentration (200 μM), mild cytotoxicity was detected with N-MCT or GCV (mean ± standard deviation: 71.9% ± 3.0%, 88.4% ± 11.4% and 65.2% ± 6.0% cell growth for N-MCT, CDV, and GCV, respectively, at 200 μM) (Fig. 1C) (50% cytotoxic concentration >200 μM for all three compounds).
These compounds did not show significant cytotoxicity in unstimulated BCBL-1 cells or in uninfected cell lines, Toledo cells (B-cell line, also EBV negative), and CEM-SS cells (T-cell line), at the concentrations tested up to 200 μM (data not shown). N-MCT exhibited the highest anti-KSHV activity with a 50% inhibitory concentration (IC₅₀) of 0.08 ± 0.03 μM (mean ± standard deviation) (therapeutic index, >2500), compared to 0.42 ± 0.07 (therapeutic index, >476) and 0.96 ± 0.49 (therapeutic index, >208) for CDV and GCV, respectively (Table 1). In contrast, there were no appreciable accumulations of N-MCT-DP and N-MCT-TP in uninfected CEM-SS cells with or without PMA stimulation (Fig. 2B). These data suggested that the intracellular phosphorylation of N-MCT to its monophosphate form could take place in both KSHV-infected and uninfected cells, but the conversion to the di- and triphosphorylated metabolites was significantly more efficient in KSHV-infected cells, especially during the lytic replication cycle.

We next compared the levels of phosphorylated metabolites of N-MCT, CDV, and GCV in PMA-induced and uninduced BCBL-1 cells after 24 and 72 h of incubation with 10 μM each of unlabeled and 5 μCi/ml of ³H-labeled compound. As shown in Fig. 3, PMA-stimulated BCBL-1 cells generally contained higher levels of phosphorylated metabolites of all three compounds compared to unstimulated BCBL-1 cells. Notably, the levels of N-MCT-TP were significantly higher than those of CDV-DP and GCV-TP throughout the 72-hour incubation period, especially in PMA-stimulated BCBL-1 cells (Fig. 3).

Herpesvirus TK inhibitor blocks anti-KSHV activity of N-MCT and formation of N-MCT-TP. KSHV ORF21 has been reported to encode a functionally active TK (7, 26). To further elucidate whether N-MCT-TP formation was directly linked to the anti-KSHV activity of N-MCT, and whether its synthesis was mediated through the virally encoded TK as has been shown in HSV-1-infected cells (64), we evaluated the effects of analyzed by gradient anion-exchange HPLC (64). The HPLC profiles clearly showed the presence of N-MCT-monophosphate (MP) in both cell lines regardless of PMA stimulation as early as 6 h of incubation (Fig. 2A and B). Sharp increases in N-MCT-DP and N-MCT-TP levels were also observed in BCBL-1 cells in 24 h, especially in PMA-stimulated BCBL-1 cells, which contained five- to eightfold higher levels of N-MCT-DP and N-MCT-TP than unstimulated BCBL-1 cells (Fig. 2A). The levels of N-MCT-TP were consistently higher than N-MCT-DP in PMA-induced as well as uninduced BCBL-1 cells (Fig. 2A). In contrast, there were no appreciable accumulations of N-MCT-DP and N-MCT-TP in uninfected CEM-SS cells with or without PMA stimulation (Fig. 2B).

### Table 1. Anti-KSHV activity of N-MCT, cidofovir, and ganciclovir in PMA-induced BCBL-1 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean IC₅₀ (μM) ± SD⁺</th>
<th>Mean IC₉₀ (μM) ± SD⁺</th>
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<tbody>
<tr>
<td>N-MCT</td>
<td>0.08 ± 0.03</td>
<td>0.68 ± 0.10</td>
</tr>
<tr>
<td>CDV</td>
<td>0.42 ± 0.07</td>
<td>4.01 ± 2.05</td>
</tr>
<tr>
<td>GCV</td>
<td>0.96 ± 0.49</td>
<td>7.11 ± 0.28</td>
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⁺ Means from three independent experiments.

![FIG. 2. Intracellular phosphorylation profiles of N-MCT in KSHV-infected BCBL-1 cells (A) and uninfected CEM-SS cells (B) with (solid lines) and without (dotted lines) PMA stimulation. Shown are the levels of mono-, di-, and triphosphorylated N-MCT metabolites. The data shown are representative of two independent experiments. Compounds: (□) N-MCT-MP; (▲) N-MCT-DP; (●) N-MCT-TP in PMA-stimulated and unstimulated cells, respectively.](http://aac.asm.org/ on October 14, 2017 by guest)
5'-ET in PMA-stimulated BCBL-1 cells treated with N-MCT. The thymidine analog 5'-ET has been shown to exert a strong inhibitory activity against HSV-1 TK (43) as well as EBV TK (31), but not against human cellular TK (43).

The anti-KSHV activity of N-MCT was first compared in PMA-induced BCBL-1 cells treated with N-MCT alone or in combination with various concentrations of 5'-ET. CDV, which is converted to its active metabolite, CDV-DP, by cellular kinases (11, 28), was used as a reference compound. Compared to cells treated with 1 μM N-MCT alone, marked increases in the level of KSHV DNA in the Hirt DNA were identified as CDV-monophosphate, CDV-DP (active metabolite), and a phosphate ester adduct of CDV (CDV-adduct) as previously described (28). The data shown are means ± standard deviations of two separate assays.

The inhibitory effect of 50 μM 5'-ET on the anti-KSHV activity of N-MCT was clearly demonstrated even at higher concentrations of N-MCT tested up to 10 μM. PMA-induced BCBL-1 cells were treated with 1, 3, or 10 μM N-MCT alone or in combination with 50 μM 5'-ET. The amounts of virion- and cell-associated KSHV DNA were significantly higher in the cells treated with both N-MCT and 5'-ET compared to that of the cells treated with N-MCT alone at all three concentrations (Fig. 4B). The intracellular levels of phosphorylated N-MCT metabolites N-MCT-MP, N-MCT-DP, and N-MCT-TP were dose-dependently increased in the cells treated with 1, 3, or 10 μM N-MCT and 5 μCi/ml [3H]N-MCT (Fig. 4C, top panel). In the presence of 50 μM 5'-ET, which significantly diminished the anti-KSHV effect of N-MCT in PMA-induced BCBL-1 cells (Fig. 4B), the levels of N-MCT-DP and N-MCT-TP in the methanolic cell extracts were substantially decreased, while there appeared to be an accumulation of N-MCT-MP (Fig. 4C, bottom). These data suggested that anti-KSHV activity of N-MCT was most likely mediated through its triphosphate metabolite, N-MCT-TP, which was converted from its precursor, N-MCT-MP, through N-MCT-DP more efficiently in KSHV-infected cells expressing the viral TK.

N-MCT-TP inhibits DNA synthesis in vitro. We have previously shown that inhibitors of KSHV Pol-mediated processive DNA synthesis could be screened by a rapid microplate-based in vitro DNA synthesis assay (50). In order to further ascertain that N-MCT-TP was indeed an active metabolite of N-MCT, which blocked lytic KSHV DNA replication, we evaluated the inhibitory effect of N-MCT-TP on processive DNA synthesis in vitro, using baculovirus-expressed recombinant rPol and rPPF (12). The KSHV Pol-specific accessory protein KSHV PPF, which specifically associates with Pol and tethers it onto extending DNA to facilitate processive DNA polymerization (50), was added to the rPol DNA synthesis reaction mixture in order to emulate specific KSHV DNA replication. Active forms of phospho metabolites of CDV and GCV, CDV-DP and GCV-TP, respectively, were included as a reference.

All three phosphorylated compounds blocked KSHV rPol and rPPF-mediated DNA synthesis (Fig. 5) with IC90 values (mean ± standard deviation from three independent experiments) of 6.24 ± 0.08 μM, 14.70 ± 2.47 μM, and 24.59 ± 5.60 μM for N-MCT-TP, CDV-DP, and GCV-TP, respectively (Fig. 5, inset). Within the concentrations tested up to 500 μM, N-MCT-TP was the only compound that achieved greater than 90% inhibition (IC90: 76.47 ± 13.95 μM) (Fig. 5). Although CDV-DP inhibited in vitro DNA synthesis more effectively than GCV-TP at lower concentrations, its inhibitory activity appeared to level off around 60 to 70%, whereas GCV-TP dose-dependently blocked DNA synthesis (Fig. 5).

DISCUSSION

The unique aspects of intracellular phosphorylation of N-MCT were first discovered in HSV-1-infected cells (64). Unlike acyclovir and GCV, which are selectively monophosphorylated in herpesvirus-infected cells because they are better substrates for virally encoded kinases than for cellular nucleoside kinases (17, 19), N-MCT was found to be efficiently monophosphorylated in HSV-1-infected as well as in uninfected cells, indicating that the compound was a suitable substrate for cellular TK for monophosphorylation (64). However, the successive conversion of N-MCT-MP to N-MCT-DP and N-MCT-TP could only be detected in the HSV-1-infected cells, and the use of an HSV-1 TK inhibitor resulted in the accumulation of N-MCT-MP in the infected cells (64). HSV-1 TK is a multifunctional enzyme with diverse substrate specificity, known to exhibit TK and thymidylate kinase activities (10). Findings by Zalah et al. suggested that N-MCT-MP was not recognizable by cellular thymidylate kinase, and that the rate-limiting step for N-MCT activation was the conversion of N-MCT-MP to N-MCT-DP presumably catalyzed by HSV-1-en-
coded TK/thymidylate kinase (10), as N-MCT-DP was thought to be readily phosphorylated to N-MCT-TP by cytosolic nucleoside diphosphate kinase (64). The discovery also suggested that N-MCT could be specifically activated (tri-phosphorylated) in cells infected with herpesviruses, which encoded TK/thymidylate kinases capable of recognizing N-MCT-MP as an optimal substrate.

Inhibitory activities of various nucleoside analogs against KSHV replication have previously been evaluated in KSHV-infected cell lines (such as BCBL-1) lytically induced by PMA (30, 39). Of the compounds examined to date, CDV has been identified as one of the most potent anti-KSHV agents, while GCV was found to be moderately active against KSHV (30, 39). In the current study, we found that N-MCT blocked KSHV lytic replication in BCBL-1 cells at a 5- to 10-fold lower IC₅₀ than those of CDV and GCV without notable cytotoxicity (the 50% cytotoxic concentration of N-MCT was >200 μM). As has been shown in HSV-1-infected cells exposed to N-MCT (64), we observed a time- and dose-dependent accumulation of N-MCT-TP almost exclusively in KSHV-infected cells, while both uninfected and infected cell lines contained abundant levels of N-MCT-MP.

Our data suggested that the intracellular conversion of N-MCT-MP to N-MCT-DP was most likely mediated by KSHV ORF21-encoded TK, which has been shown to exhibit thymidylate kinase activity (26). Indeed, in the presence of a potent herpesvirus TK inhibitor, 5'-ET (31, 43), the levels of N-MCT-DP and N-MCT-TP were significantly reduced, resulting in the abrogation of anti-KSHV activity of N-MCT. These findings further supported our notion that KSHV TK catalyzed phosphorylation of N-MCT-MP to N-MCT-DP, which was then converted to N-MCT-TP by cellular nucleoside diphosphate kinase, and that the triphosphate form of N-MCT was directly responsible for the anti-KSHV activity. Interestingly, we found that the intracellular accumulation of N-MCT-TP was significantly greater than those of CDV-DP and GCV-TP, the active metabolites of CDV and GCV, respectively, in BCBL-1 cells treated with each compound at the same concentration. These data may, at least in part, account for the superior anti-KSHV activity of N-MCT identified in our study.

Compared to HSV-1 TK, which is known to possess a broad range of substrate specificities, KSHV TK has more restricted substrate specificity. It has been reported that KSHV TK pref-
erentially phosphorylated thymidine derivatives, while GCV, a guanine analog, was a poor substrate for the enzyme (26). Although it is still possible that GCV may be phosphorylated by a KSHV ORF36-encoded phosphotransferase as has previously been suggested (7), we found that the intracellular level of GCV-TP was, nonetheless, significantly lower than that of N-MCT-TP in KSHV-infected BCBL-1 cells, corresponding to the lower anti-KSHV efficacy of GCV than of N-MCT. Our data further support future efforts to explore and develop thymidine-based analogs as anti-KSHV agents. In addition to KSHV, N-MCT may also exert antiviral activity against another gammaherpesvirus, EBV, which has been shown to encode a TK similar to KSHV TK, exhibiting thymidylate kinase activity and a substrate preference for thymidine analogs (25). Considering the lack of well-established, effective anti-EBV agents available at the present time, further studies are warranted to explore the inhibitory activity of N-MCT against EBV replication and its possible utility in EBV-induced malignancies.

The intracellular accumulation of monophosphorylated (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU-MP) or (E)-5-(2-iodovinyl)-2'-deoxyuridine (IVDU-MP) has been linked to cytostatic effects in TK-deficient tumor cells expressing HSV TK (3). BVDU-MP and IVDU-MP were suspected to target host thymidylate synthase, thereby hindering cellular DNA synthesis (3). While both KSHV-infected and uninfected cells exposed to 10 μM N-MCT-TP were found to contain abundant levels of N-MCT-TP in our study, there was no significant cytotoxicity noted in either cell group until the test concentration reached 200 μM. Therefore, it is unlikely that N-MCT-TP interferes with host thymidylate synthase in cells exposed to KSHV-inhibitory concentrations of N-MCT. KSHV also encodes a functional thymidylate synthase (20). Although it has yet to be determined whether N-MCT-MP can interfere with virally encoded thymidylate synthase, the role of N-MCT-TP in KSHV inhibition is probably minimal, since the KSHV core lytic DNA replication machinery does not include KSHV thymidylate synthase (51, 61).

Another critical determinant of antitherpetic activity of nucleoside-based agents is the efficiency with which the active metabolites are “misin incorporation” into viral DNA. For example, S-MCT has not been associated with significant inhibitory activity against HSV-1 (37) or KSHV, as observed in the current study, despite evidence to suggest that it is an excellent substrate for virally encoded TK (36, 52). This is probably because S-MCT-TP is not a preferred substrate for DNA polymerases compared to N-MCT-TP (36), clearly illustrating the two distinct factors involved in attaining antiviral activity. It has also been shown that herpesvirus polymerases possess an inherent 3' to 5' exonuclease activity (35, 57), as with other well-characterized DNA polymerases (5, 24). Therefore, the antiviral potency of nucleoside analogs can be greatly influenced by the sensitivity or insensitivity (resistance) of phosphorylated metabolites to the exonuclease activity of viral polymerases. Furthermore, the processivity factors of HSV-1 and EBV polymerases, UL42 and BMRF1, respectively, have been shown to enhance the exonuclease activity of the viral polymerases, substantially reducing the extent of nucleotide misincorporation into DNA (55, 57).

It is highly plausible that KSHV Pol exhibits a similar exonuclease activity, and in the presence of KSHV PPF, the enzyme can efficiently remove mismatched nucleotides from the DNA chain during processive DNA synthesis. In the current study, N-MCT-TP was shown to block in vitro DNA synthesis...
mediated by KSHV rPol and rPPF more efficiently than CDV-DP and GCV-TP. The data not only indicate that N-MCT-TP is efficiently incorporated into DNA, ultimately terminating the processive DNA synthesis, but also imply that N-MCT-MP may be more resistant to excision than two other reference compounds examined. In contrast to deoxyribonucleoside compounds known as immediate DNA chain terminators (2, 32), the active metabolites of N-MCT, CDV, and GCV do not block DNA chain elongation at the site of incorporation (4, 47, 62). Although the mechanisms are unclear, this mode of delayed chain termination may confer relative resistance to excision (4, 62). It will be of great interest to investigate whether the rigid conformation of the pseudosugar moiety of N-MCT plays a critical role in excision resistance.

In summary, we discovered the potent anti-KSHV activity of N-MCT, which is specifically triphosphorylated in KSHV-infected cells undergoing lytic replication and efficiently blocks KSHV DNA replication. The compound may represent a new option for the prevention and treatment of KSHV-induced malignancies.

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REFERENCES

11. Dorjsuren, D., Y. Badralmee, J. Mikovits, A. Li, R. Fisher, R. Ricciardi, R.
from recombinant baculovirus-infected insect cells. Nucleic Acids Res. 18: 1207–1215.