Biochemical and Structural Characterization of (South)-Methanocarbathymidine That Specifically Inhibits Growth of Herpes Simplex Virus Type 1 Thymidine Kinase-transduced Osteosarcoma Cells*

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Two analogs of the natural nucleoside dT featuring a pseudosugar with fixed conformation in place of the deoxyribose residue (carbathymidine analogs) were biochemically and structurally characterized for their acceptance by both human cytosolic thymidine kinase isozyme 1 (hTK1) and herpes simplex virus type 1 thymidine kinase (HSV1 TK) and subsequently tested in cell proliferation assays. 3'-exo-Methanocarbathymidine ((South)-methanocarbathymidine (S-MCT)), which is a substrate for HSV1 TK, specifically inhibited growth of HSV1 TK-transduced human osteosarcoma cells with an IC50 value in the range of 15 μM without significant toxicity toward both hTK1-negative (TK-) and non-transduced cells. 2'-exo-Methanocarbathymidine ((North)-methanocarbathymidine (N-MCT)), which is a weak substrate for hTK1 and a substantial one for HSV1 TK, induced a specific growth inhibition in HSV1 TK-transfected cells comparable to that of (S)-MCT and ganciclovir. A growth inhibition activity was also observed with (N)-MCT and ganciclovir in non-transduced cells in a cell line-dependent manner, whereas TK- cells were not affected. The presented 1.95-Å crystal structure of the complex (S)-MCT-HSV1 TK explains both the more favorable binding affinity and catalytic turnover of (S)-MCT for HSV1 TK over the North analog. Additionally the plasticity of the active site of the enzyme is addressed by comparison the binding of (North)- and (South)-carbathymidine analogs. The presented study of these two potent candidate prodrugs for HSV1 TK gene-directed enzyme prodrug therapy suggests that (S)-MCT may be even safer to use than its North counterpart (N)-MCT.

The selective transduction of cells with herpes simplex virus type 1 thymidine kinase (HSV1 TK) appears to be a promising strategy for gene-directed enzyme prodrug therapy (GDEPT) (1, 2). Thereby the transduced cells acquire specific phosphorylation abilities for a wide range of non-natural nucleoside analogs that are substrates for HSV1 TK but not for the human cellular enzyme. Clinical trials of GDEPT applied to cancer based on HSV1 TK transduction into tumor cells and the systemic administration of the nucleoside analog ganciclovir (GCV) have given promising results in term of selective tumor regression (3–8). By analogy, selective transduction of hematopoietic stem cells with HSV1 TK is also foreseen to improve the efficacy of allogeneic bone marrow transplantation (9–11). Most recently the American Society of Gene Therapy ad hoc subcommittee on retroviral mediated gene transfer to hematopoietic stem cells has suggested the use of suicide genes for increasing safety of gene therapy (12). However, the approaches based on the paradigm HSV1 TK/GCV are plagued with certain myelosuppressive and hematological side effects related to the dosage of GCV needed for tumor regression (6, 13–15, 16). Additionally the transfection vector and the expression level of the transduced gene are also crucial factors for successful and safe GDEPT (17). Altogether these facts have contributed to the impairment of a wide use of GDEPT based on the HSV1 TK/GCV paradigm. In parallel to the development of new transduction vectors, several studies have led to the development of HSV1 TK mutants with higher specificity and catalytic activity for classic nucleoside analogs (13, 18–22).

Initially engineered to create a stable C–N bond resistant to chemical and enzymatic hydrolysis between the nucleobase and the pseudosugar ring while causing minimal structural changes, a novel class of nucleotide analogs featuring a conformationally fixed pseudosugar ring was developed (23). Indeed the sugar ring of nucleosides and nucleotides exists in solution in a rapid equilibrium between two extreme North (2'-exo/endo) and South (3'-exo/2'-endo) conformations (24). On the contrary, a pseudosugar ring based on a bicyclo[3.1.0]hexane thymidine kinase; HSV2, herpes simplex virus type 2; XTT, 2,3-bis(me-thoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide; S, South; N, North; (S)-MCT, 3'-exo-methanocarbathymidine, (South)-methanocarbathymidine; (N)-MCT, 2'-exo-methanocarbathymidine, (North)-methanocarbathymidine; (N)-MCTDP, 5'-<(North)-methanocarbathymidine diphosphate; (S)-MCTMP, 5'-<(South)-methanocarbathymidine monophosphate; (S)-MCTDP, 5'-<(South)-methanocarbathymidine diphosphate; (S)-MCTMP, 5'-<(South)-methanocarbathymidine monophosphate; GCV, ganciclovir; GDEPT, gene-directed enzyme prodrug therapy; hTK1, human cytosolic thymidine kinase isozyme 1; WT, wild type; NSCLC, non-small cell lung cancer; HPLC, high pressure liquid chromatography.
scaffold is either locked in South (S) or North (N) conformation. Using ligands featuring such restricted pseudosugar moiety, the conformational preferences for exogenous enzymes, such as human immunodeficiency virus reverse transcriptase (25, 26) and HSV1 TK (22), have been assessed. In the case of HSV1 TK, the flexible sugar ring of the natural substrate dT preferentially adopts a South conformation (27, 28). Over the past years, such nucleoside analogs featuring a conformationally restricted pseudosugar moiety have been tested for antiviral activity, and some of them have shown promising potency (23, 29–33). Among these, (N)-MCT (2′-exo-methanocarbamidine, Fig. 1A) demonstrated a potent antiviral activity against HSV1 and HSV2. Recently challenged in vivo and in vitro in a GDEPT trial with HSV1 TK suicide gene, (N)-MCT proved to be a promising alternative to GCV (34). Strikingly contrasting with its North counterpart, (S)-MCT (3′-exo-methanocarbamidine, Fig. 1B) appeared to be deficient of any antitherpetic activity when assessed by plaque reduction assay (23). A recent study has indeed suggested that kinases often prefer the S pseudosugar conformation, whereas DNA polymerases preferentially incorporate the N conformation of the 5′-triphosphate species, although both N and S triphosphate species may be present in the cell cytoplasm (35).

The present work features an extensive biochemical characterization of (S)-MCT as potent substrate for HSV1 TK in vitro. The presented crystal structure of the complex (S)-MCT-HSV1 TK uncovers the bioactive conformation of the ligand within the active site of the enzyme and offers a mechanistic understanding of its binding affinity and catalytic turnover constants. A comparison with the structure of the complex (N)-MCT-HSV1 TK shows how plastic the active site of HSV1 TK is to accommodate both North and South restricted pseudosugar ring conformations. Finally, the cell growth inhibition potency of (S)-MCT was assessed in HSV1 TK-transduced human osteosarcoma cells. Cell growth inhibition induced by (S)-MCT was close to that achieved by (N)-MCT and GCV within the same experimental conditions. Additionally, (S)-MCT was not significantly toxic in non-transfected osteosarcoma cells within the experimental range (0.2–500 μM), whereas a cell line-dependent nonspecific cytotoxicity of both (N)-MCT and GCV in non-transfected cells was observed. Thus, this study uncovers a remarkable plasticity of HSV1 TK active site and demonstrates a significant and specific cell growth inhibition induced by (S)-MCT in HSV1 TK-transfected human osteosarcoma cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—(South)-Methanocarba-[methyl-3H]-thymidine was obtained from Moravek (Brea, CA). The strain *Escherichia coli* BL21 that served as expression host for HSV1 TK strain F, the plasmid pGEX-6P-2, and PreScission™ protease were purchased from Amersham Biosciences. 5-BrdUrd calf intestinal phosphatase treatments were obtained from Sigma and Promega (Madison, WI). All other reagents including the components of the protease-specific cleavage buffer were obtained from Fluka (Buchs, Switzerland). The following cell lines and chemicals were used for the cell proliferation assays: JTKI-deficient 143B osteosarcoma (143B-TK−) (ATCC number CRL 8303), 143B-TK− cells stably transduced with the HSV1 TK wild type (143B-TK+/HSV1-WT cells) (ATCC number CRL-8304), MG-63 osteosarcoma cells (ATCC number CRL-1427), and H125 NSCLC adenocarcinoma cells (NCI, National Institutes of Health, Bethesda, MD). Dulbecco’s phosphate-buffered saline without Ca2+, Mg2+, and sodium bicarbonate; 1x trypsin-EDTA; 100x non-essential amino acids (minimum essential medium); sodium pyruvate; and 50x hypoxanthine, aminopterin, and thymidine supplement were obtained from Invitrogen. The minimum essential medium with Earle’s salts with 1-glutamine, HEPES buffer, and RPMI 1640 medium from Invitrogen was enriched with different supplements for each cell line. Both fetal calf serum (heat-inactivated) and penicillin/streptomycin/Penzone (10,000 units/ml, 10,000 μg/ml, and 25 μg/ml, respectively) mixture were purchased from Amimed Bioconcept (Allschwil, Switzerland). For the cell proliferation assay the antiviral agent GCV (Cytovene™) was bought from Roche Applied Science, and XTT (2,3-bis(methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolin-5-carboxanilide) as well as Menadione (vitamin K3) was from Sigma. (N)-MCT and (S)-MCT were synthesized as reported elsewhere (23, 36).

**Protein Expression and Purification**—Wild-type HSV1 TK was produced using the expression vector pGEX-6P-2-TK coding for a PreScission protease-cleavable glutathione S-transferase fusion protein. The protein was expressed in *E. coli* strain BL21 as glutathione S-transferase fusion protein and purified as described elsewhere (22). Human cytosolic thymidine kinase (hTK) was produced in *E. coli* strain BL21 using pET15b. The purification procedure was performed similarly to that of HSV1 TK on a glutathione-Sepharose column leading to active hTK.

**Assessment of Phosphorylation**—Phosphorylation of dT, (N)-MCT, and (S)-MCT was monitored by HPLC using a previously published protocol based on reverse-phase ion pair chromatography (38). Blank reactions without enzyme or without substrate were run concomitantly to account for background ATP hydrolysis. The detection limit for phosphorylated substrates is 20 nmol (38). The reactions were performed in a total volume of 70 μl during 60 min with 2 μg of HSV1 TK or 4 μg of hTK in the presence of 2 mM substrate, 5 mM ATP, and 5 mM Mg2+. A diode array detector was used to confirm the formation of monophosphate species of (N)-MCT and (S)-MCT. Calf intestinal phosphatase treatments were performed to recover the initial non-phosphorylated form of the substrate after HSV1 TK catalysis as follows: 19 μl of 10x phosphatase buffer, 0.05 units of calf intestinal phosphatase, 166 μl of diluted reaction incubated for 30 min at 37°C.

**Binding Affinity Assessment**—The kinetic constants of HSV1 TK for (S)-MCT were determined by measurement of initial velocities by monitoring the conversion of (South)-methanocarba-[methyl-3H]-thymidine to its monophosphate according to the DEAE-cellulose method described elsewhere (39). The kinetic study was performed with an enzyme-saturating ATP concentration (5 mM). Reactions were carried out in a final volume of 30 μl containing 50 mM Tris buffer, pH 7.5, 5 mM MgCl2, 5 mM ATP, 2.5 mg/ml bovine serum albumin. The amount of enzyme and concentrations of tritiated substrate were chosen in accordance with the Michaelis-Menten equation conditions for initial velocity measurements (Fig. 2). The compound was tested in the concentration range of 2–100 μM. The binding affinity (*Kd*) and the maximal velocity (*Vmax*) values were determined by a non-linear fit of the raw data (Fig. 2) to the Michaelis-Menten equation using Microcal Origin Software 6.0. The values summarized in Table 1 were measured based on six independent assays.

**Catalytic Turnover Constant Assessment**—Catalytic turnover const-
kits (k_{cat}) were assessed using a continuous spectrophotometric assay described elsewhere (40). The data presented are the results of five independent series of measurements performed in triplicate. Control experiments were performed to take into account the spontaneous hydrolysis of ATP under the experimental conditions.

Tissue Culture and Cell Lines—All three osteosarcoma cell lines were cultivated in minimum essential medium, whereas adenocarcinoma H125 NSCLC cells were grown in RPMI 1640 medium. Both media were supplemented with 10% fetal calf serum and 1% penicillin/streptomycin/Fungizone. To maintain a TK negative phenotype 0.1 mg/ml 5-BrdUrd was added to the culture medium of the adherent 143B-TK human osteosarcoma cells, which feature neither human cytosolic nor viral TK activity. For MG-63 human osteosarcoma cells featuring human TK, enriched minimum essential medium contained 1 mM sodium pyruvate and 1× minimum essential medium supplements, whereas the same supplementation plus 1× hypoxanthine, aminopterin, and thymidine was used for 143B-TK-HSV1-WT. All cell lines were subcultured by trypsinization and resuspended in fresh medium every 4th day (41). All cells were incubated at 37 °C in an atmosphere with 5% CO₂.

Cell Proliferation Assay—The cytotoxicity of each compound, namely (S)-MCT, (N)-MCT, and GCV, was determined using the tetrazolium-based XTT colorimetric assay (42, 43). Single cell suspensions obtained by trypsinization were seeded in 96-well tissue culture plates in a total volume of 200 μl/well and incubated at 37 °C with 5% CO₂. After 4.5 days, shortly before cells reached confluence, 50 μl of prewarmed (37 °C) XTT in RPMI 1640 medium (1.0 mg/ml) supplemented with 100 μM Menadione and 25 mM HEPES, pH 7.5, were added to each well. The plates were incubated for 2–8 h depending on the metabolic activity of the corresponding cell line. The absorbance was read on a microplate reader (Versamax, Amersham Biosciences) at 450 nm with a reference at 750 nm. The percentage of cell growth was standardized on untreated cells and on medium alone with XTT according to the following equation: 100 × (A drug-treated cells/A untreated control cells – A medium-alone control cells). Three series of independent tests were performed in triplicates and mean values were calculated. The correlation between the number of cells and XTT metabolism was strictly linear, as had been demonstrated before (42).

Crystalization and Structure Determination—Pure HSV1 TK protein was concentrated to 25 mg/ml for crystallization. Crystals grew to 400 × 350 × 100 μm³ after 2 weeks at 23 °C by mixing equal volumes of protein solution and precipitant (0.15 M LiSO₄, 2 mM dithiothreitol, and 0.1 M HEPES at pH 7.5) in hanging drops. The (S)-MCT-HSV1 TK complex was produced by soaking the crystals with 4 mM (S)-MCT for 2 h. Subsequently the crystals were cryoprotected with 30% glycerol and quick-frozen at 100 K. The (S)-MCT-HSV1 TK crystals belong to the space group C222₁, (a = 113.7 Å, b = 118.0 Å, c = 108.2 Å) and contain two subunits in the asymmetric unit. A native data set collected to 1.95 Å was used for structure determination. A native data set to 1.95 Å produced yielding a model with good refinement statistics (Table II). Alternating rounds of manual adjustments in program O (45) and re-starting model (22). Water and ligand molecules were removed prior to refinement. The structure was determined by using the coordinates of HSV1 TK crystals belong to the space group R32 and 1× minimum essential medium supplements, whereas the same supplementation plus 1× hypoxanthine, aminopterin, and thymidine was used for 143B-TK-HSV1-WT. All cell lines were subcultured by trypsinization and resuspended in fresh medium every 4th day (41). All cells were incubated at 37 °C in an atmosphere with 5% CO₂.

Phosphorylation Pattern of (S)-MCT—The phosphorylation levels were assessed by monitoring the decrease of ATP and the formation of ADP peaks due to the phosphorylation of (S)-MCT and (N)-MCT by HPLC. A significant formation of monophosphate and diphosphate species of (S)-MCT, (S)-MCTMP and (S)-MCTDP, respectively, could already be monitored after 60 min of incubation with HSV1 TK (Fig. 3, A and D). Furthermore the identity of (S)-MCTMP and (S)-MCTDP could be verified by recording the UV spectra of the newly formed peaks using the diode array detector. The spectra revealed for both peaks at T_{max} of 22 and 44 min an absorption maximum at 273.3 nm (λ_{max} 273.3 nm) that is characteristic for thymine derivatives. Thus, (S)-MCT appeared to undergo a second phosphorylation step catalyzed by the HSV1 TK, which can function as a diphosphorylating enzyme in the presence of certain substrates as shown previously (49, 50). By comparing the area under peaks, HSV1 TK transformed 63 ± 4.5% of (S)-MCT into monophosphate and 1.2 ± 0.0% into diphosphate. On the contrary, no significant phosphorylated species of (S)-MCT could be detected after 60 min of incubation with hTK1 (Fig. 3, B and D). The peak of the (N)-MCT monophosphate form, namely (N)-MCTMP, was coincident with the ATP peak so that the ratio ADP/ATP could not be directly assessed (Fig. 3C). By lowering ATP concentrations (in the range of 0.5–2 mM) we could efficiently distinguish both (N)-MCTMP and ATP peaks. Additionally we assessed the presence of (N)-MCTMP under the ATP peak using a diode array detector. As a result, the entire UV spectra as well as the maxima of absorbance could be precisely determined for each compound. The maxima of absorbance of ATP peak (λ_{max} 256.7 nm) was thus shifted toward that of (N)-MCT/(N)-MCTMP (λ_{max} 273.3 nm). To evaluate the (N)-MCTMP area under the ATP peak, we injected each sample twice by treating the second with calf intestinal phosphatase that led to a complete recovery of the (N)-MCT initial form and the dephosphorylation of both ADP and ATP into adenosine (Fig. 3C). Thereafter, the total area of (N)-MCT for each sample could be assessed, and an evaluation of ATP area occupied by the (N)-MCTMP peak was made possible.

Although HSV1 TK primarily has a thymidine kinase activity that releases monophosphate species as products, we noted that a low accumulation of both (N)-MCT and (S)-MCT diphosphate species occurred over time. Eventually as monophosphate species kept accumulating in the environment, HSV1 TK started using these as secondary substrates as previously reported (50, 51). As summarized in Fig. 3D, (S)-MCT is a good substrate for HSV1 TK, whereas hTK1 does not accept it as substrate (Fig. 3C). On the other hand, (N)-MCT is a good substrate for HSV1 TK and a weak substrate for hTK1.

Binding Affinity and k_{cat} Assessment—The binding affinity of (S)-MCT toward HSV1 TK was evaluated on at least six independent measurements using (South)-methanocarbamyl-thymidine as substrate under enzyme-saturating ATP concentration (Fig. 2), whereas the k_{cat} was determined in five independent experiments using a continuous spectrophotometric assay (40). The results clearly show that the conformationally restricted (S)-MCT and (N)-MCT are both substrates of HSV1 TK with lower K_m and higher k_{cat} values than those of GCV (Table I). The determined K_m value for (S)-MCT (0.25 s⁻¹) is close to that of dT (0.35 s⁻¹) (38), whereas the value of the binding affinity expressed as the Michaelis-Menten constant K_m of (S)-MCT (4.1 μM) is 1 order of magnitude higher than that of dT (0.2 μM) (Table I).

Cell Proliferation Assay—The cell growth inhibition activity of (S)-MCT, (N)-MCT, and GCV toward osteosarcoma cells featuring different TK profiles was examined in three independent experiments performed in triplicates (Fig. 4). The first cell line is stably transduced with HSV1 TK wild type (143B-TK⁻-HSV1-WT), the second does not feature any cytosolic TK activity (143B-TK⁻), and the third naturally features human TK activity (MG-63). The two latter cell lines represent the negative control for assessing the intrinsic cytotoxicity of the analyzed compounds. Under the experimental conditions, already starting below 0.5 μM (S)-MCT, a dose-dependent cell growth...
inhibition was monitored in 143B-TK-HSV1-WT cells reaching an IC₅₀ value of 14.6 ± 3.3 μM (defined as the dose required for 50% cell growth inhibition ±S.D.). On the contrary, (S)-MCT induced no significant cell growth inhibition in 143B-TK and MG-63 cells within the experimental range. Therefore (S)-MCT appeared to be nontoxic in its native form and has to undergo a specific phosphorylation by HSV1 TK to be active. In comparison, IC₅₀ values for GCV were measured at 3.9 ± 4.1 μM in the HSV1 TK-transduced cells, 263 ± 29 μM in the non-transduced MG-63 cells, and 119 ± 39 μM in the 143B-TK cells. (Fig. 4). Finally the IC₅₀ values for (N)-MCT were 11.4 ± 4.5 μM in 143B-TK-HSV1-WT cells, whereas 143B-TK cells were not affected even at 500 μM. Contrasting with the poor phosphorylation level of (N)-MCT achieved in vitro by hTK1, the very same compound triggered a significant cytotoxicity in MG-63 cells (IC₅₀ 61 ± 30 μM). Therefore, we tested (N)-MCT and GCV cytotoxicity toward H125 NSCLC non-transduced adenocarcinoma cells. These cells were less affected than MG-63 cells by (N)-MCT and GCV, which both exhibited an IC₅₀ value higher than 500 μM.

Crystal Structure—The complex (S)-MCT:HSV1 TK crystalized with one homodimer (subunits A and B) per crystallographic asymmetric unit (22). The structure was refined to 1.95-A resolution (Table II). The final R-factor was 18.4% (R_free = 21.8%) with data collected from one single crystal. According to a Ramachandran analysis the model features 93.5% residues in
most favored and 6.5% in additionally allowed regions (47). Only residue Arg-163 is in a disallowed region in both subunits probably because it participates in catalysis (27, 52–55). Due to a lack of defined density presumably caused by high mobility, some parts of the protein could not be modeled. These comprise residues 1–45, 72–75, 148–152, 266–278, and 375–376 of subunit A and residues 1–45, 148–152, 221–223, 266–273, and 375–376 of subunit B. The overall α/β fold of the enzyme is conserved as compared with the previously published structures (53, 55). The structure contains 340 water molecules and two sulfate ions that occupy the position of the β-phosphate of ATP/ADP at the P-loops of the two subunits (56). Each subunit contains one (S)-MCT molecule clearly defined in its electron density (Fig. 5).

The nucleobase moiety is stacked between Met-128 and Tyr-172 and firmly fixed by a complex hydrogen bonding network (22, 53, 55) (Fig. 5). Direct hydrogen bonds between N-3 and O-4 of the nucleobase and the side chain of Gln-125 and two water-mediated hydrogen bonds from O-2 of the nucleobase to the side chain of Arg-163 are tightly fixing the nucleobase within the active site as in the dT-HSV1 TK (53, 55) and in the (N)-MCT-HSV1 TK (22) structures. The fixed ring pucker clearly defined in the electron density is showing a South conformation, which corresponds to the dT conformation within the HSV1 TK active site (53, 55). Therefore, all the aspects of dT binding are kept (Figs. 5 and 6): the 3′-OH is fixed by two hydrogen bonds to Arg-163 and to Glu-83 (2.9 Å in subunit B and 3.7 Å in subunit A). The South conformation of (S)-MCT superimposes well with the bioactive conformation of dT adopted in the HSV1 TK (weighted root mean square value, 0.14 Å). However, when aligning on the nucleobases, shifts of 0.8 Å for the 5′-OH and 0.9 Å for the 3′-OH group occur due to the slight geometric differences between (S)-MCT and dT. The cyclopropyl moiety within the bicyclo[3.1.0] ring contacts Ile-97 at the ribosyl site. The distance between the C-7′ atom of (S)-MCT and the C-γ1 atom of Ile-97 is 3.7 Å. To accommodate the bulkier bicyclo[3.1.0] ring, Ile-97 moves about 0.3 Å away as compared with the x-ray structure of HSV1 TK in complex with dT (Fig. 6). No other side chains interacting with the compounds show any displacement. Finally a sulfate ion bound to the P-loop is present in the structure and accompanied by a water molecule (water 164). This water molecule is likely to occupy the place of the γ-phosphate of ATP.

### Table I

**Comparison of the binding affinity and k_{cat} values of several substrates of HSV1 TK**

|          | (S)-MCT TK | (N)-MCT TK | (S)-MCT TK |
|----------|------------|------------|------------|
| K_{m} (µM) | 0.2 ± 0.05 | 16.1 ± 7.6 | 4.1 ± 2.4' |
| k_{cat} (s^{-1}) | 0.35 ± 0.01 | 0.16 ± 0.04 | 0.25 ± 0.03' |

*(Reported by Pilger et al. (38)).

### Table II

**Data collection and refinement statistics**

| Data set | (S)-MCT-HSV1 TK |
|----------|----------------|
| Data set | (S)-MCT-HSV1 TK |
| X-ray source | BW7B (DESY, EMBL, Hamburg, Germany)|
| Unit cell dimensions (Å) | a = 113.7, b = 118.0, c = 108.2 |
| Resolution range (Å) | 40.0–1.95 (2.06–1.95) |
| Completeness (%) | 99.2 (95.0) |
| Multiplicity | 5.2 (4.9) |
| Unique reflections | 52,523 (7,440) |
| R_{merge} (%) | 4.9 (16.6) |
| I/σ | 9.7 (4.2) |
| Refinement and final model | R_{free}/R_{training} (%) | 18.4 (21.8) |
| Average B-factors (Å²) | 33 |
| Polypeptide atoms | 4,737 |
| Substrate atoms | 36 |
| Water molecules | 340 |
| Sulfate ions | 2 |

*(Crystals belong to space group C222₁. All data were collected at 100 K. Values in parentheses refer to the last shell. DESY, Deutsches Elektronen Synchrotron; EMBL, European Molecular Biology Laboratory.)*

**DISCUSSION**

Within the development of potent antiviral prodrugs for the treatment of herpes infections, a range of specific substrates for the exogenous HSV1 TK has emerged over the years. Among these, nucleoside analogs that feature a pseudosugar ring with a fixed conformation have been reported to lead to different acceptance between the cellular enzymes and exogenous enzymes such as viral kinases and DNA polymerases or even reverse transcriptase (25, 26, 35). Partial explanation of this differential activity between a pseudosugar locked in either S or N conformation may be found in the reduction of entropy due to the conformationally locked ring and in the plasticity of the active site of the enzyme. So far, the active site of HSV1 TK has
The conformation of the compound and the position of the sulfate group are well defined by the electron density contoured at 1.3 σ. The hydrogen bonding patterns for the thymine moiety as well as for both 3'-OH and 5'-OH groups are the same as in the HSV1 TK-dT structure. (S)-MCT and HSV1 TK carbon atoms are represented in orange and black; nitrogen, oxygen, and sulfur atoms are in blue, red, and yellow, respectively; and water molecules are represented as green balls. H-bonds are depicted by dashed lines between donor (D) and acceptor (A) and defined as follows: distance D−A, 2.8–3.2 Å, angle D−H−A, 140–180°.

The comparison of the x-ray structures of (S)-MCT and (N)-MCT in complex with HSV1 TK reveals a difference in the hydrogen bond pattern comparable to that described for dT and (N)-MCT. The more favorable hydrogen pattern of (S)-MCT is in agreement with the kinetic data showing a better $K_m$ value of (S)-MCT compared with the North analog (Table I). These structural data as well as the phosphorylation pattern analysis corroborate the recently published data on phosphorylation in whole cells (35). Moreover the pseudosugar moiety of both (S)-MCT and (N)-MCT maintains a favorable dipole moment effect toward the residue Glu-225 (52). By contrast, the narrow substrate specificity of the cellular thymidine kinase speaks for a lower plasticity of hTK1 in adapting non-natural substrates. However, lacking structural information on hTK1, the rationale behind the absolute refusal of (S)-MCT and the weak acceptance of (N)-MCT by this enzyme remains partially unclear. Nevertheless a recent study performed on the nucleosides suggests that nucleoside/nucleotide kinases prefer nucleosides having the thymine ring in the anti disposition relative to the sugar moiety (35). Because the thymine ring of (S)-MCT is in the syn conformation in solid state as well as in solution (35), one may hypothesize that the lower plasticity of hTK1 does not allow it to accommodate (S)-MCT in its favored syn conformation, whereas HSV1 TK does.

More striking is the high efficiency and specific growth inhibition of HSV1 TK-transduced cells achieved by (S)-MCT in osteosarcoma cells. Indeed this compound is efficiently processed to the 5'-triphosphate in HSV1 TK-transduced cells (35) and appears to be as efficient as GCV and (N)-MCT. Since it is not a substrate for hTK1, its activation exclusively relies on the presence of HSV1 TK activity, thus explaining the observed absence of toxicity toward non-transduced cells within the experimental range. Subsequent to the initial phosphorylation, the formation of the 5'-diphosphate of (S)-MCT facilitated by the diphosphorylating activity of HSV1 TK (50) may also account for the exclusive cytotoxicity observed in HSV1 TK-transduced cells. So far, the presented data on (N)-MCT and GCV cytotoxicity in HSV1 TK-transduced osteosarcoma cells are in complete agreement with values reported in another cell line (34). By contrast, our data underline a significant dose-dependent effect of (N)-MCT on the MG-63 cells that does not corroborate the in vitro phosphorylation assays. The strong decrease of cell proliferation observed beyond 5 μM (N)-MCT may be the consequence of a higher division rate of MG-63 cells. The division rate is crucial in both GCV- and (N)-MCT-mediated cytotoxicity (34, 58). A lower concentration of dTTP, the natural substrate of the DNA polymerase, competes less with the phosphorylated species of (N)-MCT and may account for the higher sensitivity toward (N)-MCT observed in MG-63 cells. The lower cytotoxicity of (N)-MCT obtained within the H125 NSCLC cell line indeed suggests that the MG-63 cell line is especially...
sensitive to such thymidine analogs.

The cell growth inhibition profiles induced by (N)-MCT and (S)-MCT demonstrate that both conformations significantly decrease cell proliferation in HSV1 TK-transfected cells. Interestingly, the activity of (S)-MCT appeared to be largely related to the source of the DNA polymerase used. Plaque reduction assays concluded that (N)-MCT, but not (S)-MCT, has antiviral activity against HSV1 and HSV2 (25), suggesting that the viral DNA polymerase discriminates between N- and S-conformation.

Another study showed that (S)-MCT had only weak antiviral activity of HSV1 TK before it can exhibit cell growth inhibitory activity. This is involved in cell proliferation, is also a target for the activated species of (S)-MCT.

The conformationally restricted (S)-MCT seems to implicate a specific HSV1 TK-mediated phosphorylation and eventually requires a second phosphorylation step efficiently catalyzed by the deoxyphosphorylating activity of HSV1 TK before it can exhibit cell growth inhibition activity. These two HSV1 TK-dependent activation mechanisms confer high selectivity and low intrinsic toxicity to (S)-MCT revealing it as a highly selective prodrug candidate for safer gene-directed enzyme prodrug therapy approaches.

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Biochemical and Structural Characterization of (South)-Methanocarbathymidine That Specifically Inhibits Growth of Herpes Simplex Virus Type 1 Thymidine Kinase-transduced Osteosarcoma Cells

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