ABSTRACT

Thymidine analogs containing o-carboranylalkyl groups at the 3-position were screened as potential substrates for human thymidine kinase 1 (TK1), an enzyme that is selectively expressed in a variety of rapidly proliferating cells, including tumor cells. On the basis of previous studies, 12 of these were identified as potential delivery agents for boron neutron capture therapy, a therapeutic method used for the treatment of high-grade brain tumors. Compound 4 with a pentylene spacer between the o-carborane cage and the thymidine scaffold and compound 10, which has an additional dihydroypropyl substituent at the o-carborane cage, were the best substrates for TK1 with Kea/Kma values of 27% and 36% relative to that of thymidine, respectively. These compounds showed partial competitive inhibition for thymidine phosphorylation by TK1. Neither compound was a substrate of recombinant human thymidine phosphorylase nor were their respective 5′-monophosphates substrates of 5′-deoxynucleotidase 1, thereby indicating potential in vivo stability. The octanol/water partition coefficient for compound 10 was 2.09, suggesting that it has excellent physicochemical properties for crossing the blood brain barrier and penetrating brain tissue. The in vitro cytotoxic effect of the 12 analogs was moderate to low in mammalian cell cultures with IC50 values between 10 and 160 μmol/L. Compounds 4 and 10 were taken up selectively and retained by the murine fibroblast L929 cell line, in contrast to its TK1-deficient variant. These findings suggest that compound 10 is a promising candidate for selective delivery of boron-10 to malignant cells, and additional in vivo studies are planned to evaluate it for boron neutron capture therapy of brain tumors.

INTRODUCTION

The treatment of high-grade gliomas and anaplastic astrocytomas remains refractory to conventional existing therapies, and new therapeutic modalities to improve the treatment are needed. Boron neutron capture therapy (BNCT) is a binary chemoradiotherapeutic system that has been used clinically to treat patients with primary brain tumors (1, 2) and metastatic melanoma (3). It is based on the nuclear capture and fission reactions that occur when boron-10 (10B) is irradiated with low-energy neutrons to produce high-linear energy-transfer α particles (4He2+) and lithium-7 (7Li3+) ions, which can lethally damage cells (4, 5). Two drugs, boronophenylalanine and mercapto-closo-decarborane, have been used clinically, but there is an urgent need for more effective 10B delivery agents. These should have low systemic cytotoxicity, selectively accumulate and be retained by malignant cells, and have sufficient bioavailability (6).

Boronated analogs of pyrimidine nucleosides such as thymidine (dTThd) and deoxyuridine potentially may be ideal vehicles for the selective delivery of 10B to malignant cells (6). They can be phosphorylated to their corresponding 5′-monophosphates by thymidine kinase 1 (TK1), a cytosolic enzyme that catalyzes the transfer of γ-phosphate group from ATP to the 5′-hydroxyl group of dThd and deoxyuridine. The resulting boronated 5′-monophosphates would then be retained intracellularly because of the negatively charged phosphate group (6). The expression of TK1 is cell cycle specific, and its enzymatic activity is absent in resting cells. Enzyme expression occurs in late G1 cells, increases in S phase, coinciding with increased DNA synthesis, and disappears during mitosis (7). Therefore, phosphate metabolites of carboranyl nucleosides should accumulate preferentially in tumor tissue.

Previously, we have reported the synthesis of several boronated dThd analogs as potential BNCT delivery agents (8, 9). These analogs are characterized by the attachment of a closo-carboranylalkyl group to the 3-position of dThd via a hydrocarbon tether of 2–7 methylene groups (Fig. 1). The rationale for choosing the carborane cage as the boron moiety is its remarkable chemical and hydrolytical stability, its high 10B content (10 atoms/molecule), and its lipophilicity, which facilitates cellular penetration of agents to which the cage is attached (10). Preliminary enzymatic studies have revealed that recombinant cytosolic human TK1, but not recombinant mitochondrial human thymidine kinase 2, tolerates bulky groups at 3-position of dThd (9, 11).

The present report describes the biochemical and cell-biological evaluation of a group of carboranyl dThd, enzyme-kinetic studies with TK1, and a detailed determination of substrate and inhibitor characteristics. In addition, the capacities of compounds 4 and 10 (Fig. 1) as substrates for thymidine phosphorylase (TPase, ref. 12) were evaluated and the corresponding 5′-monophosphates were evaluated as substrates for 5′-deoxynucleotidase 1 (dNT-1, ref. 13). In contrast to TK1-deficient cells, there was selective accumulation and retention of the boronated nucleosides in TK1-positive cells, strongly suggesting that this class of drugs may have significant promise as delivery agents for BNCT.

MATERIALS AND METHODS

Chemicals and Cell Lines. Boronated dThd analogs (1-12) were synthesized as described previously (8, 9). Other natural and unnatural nucleosides and nucleotides were purchased from Sigma-Aldrich Corp (St. Louis, MO). The synthesis of compound 13 has been described previously (8) and that of compound 14 is described in Supplementary Data. The [methyl-3H]dThd (specific activity 25 Ci/mmol) was purchased from Amersham International (Arlington Heights, IL). L929 (ATCC-CCL-1) and LMTK− (L929 TK1−; ATCC-CCL-1; ref. 14) cell lines were purchased from The American Type Culture Collection (Manassas, VA). CCRF-CEM and CEM-TK− cells were kindly provided by Dr. Buddy Ullman (The Oregon Health Sciences University, Portland, Oregon; ref. 15). Culture media was purchased from Invitrogen (Carlsbad, CA).

Expression and Purification of the Recombinant Human Enzymes. TK1 (TK1 106Met sequence according to accession number NM_003258) was purified from bacterial expression system [BL 21 (DE3) pLys], as described previously (8). The human TPase (NM_001953) cDNA was a gift from Dr. Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden; ref. 16). Following the manufacturer’s protocol, human dNT-1 (NM_032526) was expressed in E.coli and purified by Ni-Sepharose and a Mono Q column (Amersham). The purity of the expressed enzymes was checked by SDS-PAGE and their activities were measured using ATP as substrate and deoxyuridine as inhibitor.

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was cloned from a human fetal brain cDNA library (Stratagene, La Jolla, CA) by PCR methods using Advanced-GC cDNA PCR kit (Clontech, Palo Alto, CA) and the specific primers (5'-gggccagctatgcccagcgactgcgg and 5'- gcacgcgctaccaggtgtagctag). The cDNAs of both TPase and dNT-1 were cloned into the NdeI and BamHI sites of the pET14b vector (Novagen, Madison, WI). The correct insertions were confirmed, and constructs were expressed in Escherichia coli, BL 21 (DH3) pLys host cells (8, 13), and the proteins were purified on Ni-NTA His bind resin column (Novagen).

TK Assays Using [γ-32P]ATP as a Phosphate Donor. The kinetic parameters for the boronated dThd analogs were determined using phosphoryl-transfer assays (9). These were carried out in reaction mixtures containing 50 mmol/L Tris-HCl (pH 7.6), 2 mmol/L DTT, 5 mmol/L MgCl2, 10 mmol/L KCl, 5 mmol/L ATP, 0.3 μmol/L [γ-32P]ATP (Amersham Pharmacia Biotech, Arlington Heights, IL), and various concentrations of boronated dThds ranging from 1 to 100 μmol/L. The DMSO concentration in all experiments was kept at 1%, and the reactions were initiated by the addition of pure recombinant TK1.

TK Assays Using Methyl-3H-Radiolabeled dThd. Competition studies were performed after the detection of [methyl-3H]dThd phosphorylation in the presence of various non-radio-labeled boronated dThd analogs. The standard reaction mixtures contained 50 mmol/L Tris-HCl (pH 7.6), 2 mmol/L DTT, 5 mmol/L MgCl2, 10 mmol/L NaF, 5 mmol/L ATP, 0.5 μg/ml BSA, 0.5 μmol/L or 5 μmol/L [methyl-3H]dThd, and purified recombinant TK1 (8). The samples were incubated at 37°C in the presence of various concentrations of boronated dThd analogs (1–80 μmol/L). The DMSO concentrations were kept at 4%. Aliquots of 10-μl each of the reaction mixtures were spotted onto DE-81 filter paper (Millipore, Billerica, MA) at time intervals of 0, 6, 12, and 18 minutes. The filters were washed three times with 5 mmol/L ammonium formate, 5 minutes for each wash, and the radioactivity was determined by γ-scintillation counting.

Kinetic Analysis. The kinetic parameters were determined by nonlinear regression analysis using the Michaelis-Menten and Hill equations and were analyzed primarily by Eadie-Hofstee plots, as described previously (17). The compound concentrations resulting in 50% inhibition of enzyme activity (IC50) were determined by the equation v1 = v0/(1 + [I]/IC50), as described previously (17). Data were analyzed by the Sigma Plot Enzyme Kinetic Module version 1.1 (SPSS Inc.).

5'-Deoxynucleotidase-1 Assay. The dephosphorylation of compounds 13 and 14, as well as TNP, dGMP, UMP, 3'-azido-2', 3'-dideoxyuridine-mono-phosphate (AZT-MP) as controls, was measured by high performance liquid chromatography (HPLC) analysis. The reaction mixture containing 0.25 mmol/L sodium acetate (pH 5.5), 30 mmol/L KCl, 10 mmol/L MgCl2, 5 mmol/L DTT, 0.2 mg/ml BSA, 10 μmol/L nucleoside-MP and 20 ng of purified recombinant dNT-1, was incubated at 37°C (13). Nucleosides were separated from their monophosphates using a gradient system, 98% buffer E1 [50 mmol/L ammonium phosphate (pH 5.5) and 5 mmol/L tetrabutylammonium hydrogen sulfate] and 2% buffer E2 (5 mmol/L tetrabutylammonium hydrogen sulfate in 100% methanol) over 15 minutes. Subsequently, buffer E2 was increased over a period of 15 minutes from 2 to 20% and then from 20 to 50% over a 20-minute period. UV-based detection was performed at 267 and 280 nm.

Thymidine Phosphorylase Assay. The phosphorylisis of boronated dThds (compounds 4 and 10) as well as dThd, AZT, and 5-(2-bromovinyl)-2'-deoxyuridine (BVDU) as controls by the bacterial E. coli TPase (Sigma-Aldrich Corp, St. Louis, MO) and the human TPase, was measured by HPLC analysis, as described previously (12). Nucleosides were separated from their nucleoside bases and quantified by reversed-phase-C18 column chromatography (Re pro Sil-Pur, C18-AQ, Bischoff, Leonberg, Germany), using a linear gradient of 98% buffer A [1 mmol/L potassium phosphate buffer (pH 5.5) and 2% buffer B [1 mmol/L potassium phosphate buffer (pH 5.5) in 80% methanol]) to 20% buffer A and 80% buffer B. After injection of the samples, 98% buffer A and 2% buffer B were run for 10 minutes before the start of the gradient (5 minutes from 2-80% buffer B). UV-based detection was performed at 267 and 280 nm, as described previously (18). Evaluation of the inhibitory effects of the compounds was performed as described previously (12), and the conversion of dThd to thymine was quantified by HPLC.

Log P Values. The physiochemical properties of the compounds were determined by HPLC experiments, as described by Teijeiro et al. (19). The chromatographic analyses were performed on a reversed phase-18 (5 μm), LiChrosphere 100Å (250 mm/4 mm) column, using a Rainin HPLC instrument with UV detection at λ = 254 nm. Samples were dissolved in 20 μl of methanol for injection, and retention times were measured in different solvent systems (methanol dissolved in water at concentrations of 30, 60, 70, and 80%) at flow rate of 1 ml/minute. A plot of the retention time against the composition of mobile phase was generated for each compound to obtain the intercept of the plot, which corresponded to the retention time of the compound in 100% water (t0). All measurements were carried out at ambient temperature. The log P was calculated using the following equation: Log P = 1.882 log k'w - 1.346, where k'w = t0/t00 (t0 is the retention time of methanol (1.78 minutes); t00 is the extrapolated “k” value at 0% methanol.

Cell Culture and Cytotoxicity Assay. Cells were cultured in DMEM with glutamax-1 medium containing 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2. Cytotoxicity assays were performed as described previously (20, 21). Cells were incubated in media containing the test compounds at final concentrations of 10–160 μmol/L in 0.5% DMSO. After 72 hours of incubation, 1 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (Sigma-Aldrich Corp.) was added. Formazan crystals were allowed to develop and were subsequently dissolved in 10% SDS/0.04 eq/L HCl solution for 1 hour at 37°C. Absorbance was measured at 540 nm using an ELISA plate reader (Labsystems Multiscan PLUS, software Delta Soft 3). Cell survival was expressed as the percentage of control cells [CS = (Mean Arelated well/Mean Acontrol well) x 100%].
Cellular Uptake and Retention of Boronated dThd Analogos. L929 wild-type and TK1− cells (1 × 10⁴) were cultured in DMEM (14), as described above. Upon reaching semi-confluence, the cells were incubated in a fresh medium containing 17.5 μmol/L of compounds 4, 6, 10, or 12. After 24 hours of incubation, the boron-containing medium was discarded, and the cells were washed with PBS and counted, and their boron content was determined by direct current plasma-atomic emission spectroscopy. Values were normalized to micrograms of boron per gram of cells (× 10⁷ cells; ref. 22). For retention studies, after 24 hours of incubation with the boron-containing medium, the cells were washed with PBS and incubated for 12 hours with fresh boron-free medium. Then the cells were collected and counted, and their boron content was determined by direct current plasma-atomic emission spectroscopy (22).

RESULTS AND DISCUSSION

We have had a longstanding interest in boron-containing pyrimidine nucleosides as substrates for TK1 for application in BNCT (6–11). These compounds acquire a negative charge by phosphorylation and are primarily entrapped in rapidly proliferating malignant cells in which TK1 is highly expressed. Closely-Carboranylalkyl dThd analogs (compounds 1–12, Fig. 1) are characterized by the attachment of the carborane cage to the 3-position at dThd via a hydrocarbon tether (2–7 methylene groups), which presumably enhances binding of the carboranyl dThds to the enzyme by reducing steric interference of the bulky carborane cluster with the active site of TK1 (8, 23).

The rational for choosing the 3-position to link the carborane cage was based on substrate activity studies with TK1 providing that 3-substituted dThd derivatives have TK1 substrate/inhibitor characteristics (24, 25). Previously, we had observed that TK1 tolerated substituents at the 5- and 3’-positions of deoxyuridine and dThd, respectively, which were comparable in size and physicochemical properties to a methyl and a hydroxyl group, respectively (26–28), whereas the introduction of bulky carboranyl groups at these positions resulted in poor substrate characteristics (8, 29). In contrast, 3-methyln-1, 3-ethyl-, and 3-isopropyl- dThd were reported to have high relative phosphorylation rates with TK1 (30), prompting the synthesis and evaluation of 3-carboranylalkyl-substituted dThds in our laboratories. Previously, we have reported that recombinant mitochondrial thymidine kinase 2 can tolerate dThd analogs with small substituents at the 3-position, such as 3-methyl dThd, with moderate activity (11, 30). However, there was no indication for substantial phosphorylation of compounds 1–12 with thymidine kinase 2 (<0.3% relative to dThd), even when high substrate concentrations of 100 μmol/L were used (9).

Kinetic Properties of Boronated dThds. In general, the enzyme kinetics of boronated dThds (1-12) followed the Michaelis-Menten equation. TK1 phosphorylation of compounds 1–12 had 1.5- to 15-fold higher apparent $K_m$ values compared with those of dThd (Table 1). In the first series of compounds (1-6), lacking the dihydroxypropyl group, there was no strong correlation between tether lengths and kinetic parameters. In this series, compound 4 with a pentylene linker had the lowest $K_m$ value (3.7 μmol/L), whereas compounds with propylene and heptylene spacers showed higher $K_m$ values (26.1 and 36.5 μmol/L, respectively). The relative efficiencies ($k_{cat}/K_m$), expressed as percentage relative to that of dThd are shown in Table 1. The relative catalytic efficiencies for this set of compounds decreased with increasing tether lengths except for compound 4 with 26.7% that of dThd.

In the second series of compounds (7-12), containing a dihydroxypropyl group at the second carbon atom of the carborane cage, the kinetic parameters correlated with the tether length. The compounds could be classified in two groups: compounds with short tether length (7-9) and compounds with long tether length (10-12). In both groups, the $K_m$ values increased in accordance with the length of the tether (Table 1). The $K_m$ values were 1.5- to 11-fold higher than that of thd. Compounds 7 and 10 had the lowest $K_m$ values of 6.1 and 3.4 μmol/L, respectively. Furthermore, the catalytic efficiency ($k_{cat}/K_m$) of these compounds was 31.9 and 35.8% relative to that of dThd. The phosphorylation of compounds 9 and 12 demonstrated biphasic saturation curves with two detectable apparent $K_m$ and $k_{cat}$, values (Table 1). However, in both concentration ranges, the catalytic efficiency did not exceed 2% that of dThd.

Overall, the kinetic results were in accordance with relative phosphorylation rates reported previously (9). In both cases, TK1 phosphorylation capacity decreased with increasing tether length. However, this tendency was not observed for all compounds. TK1 showed the best overall kinetic properties for compounds having a pentylene spacer (compounds 4 and 10). The results further indicate that the active site of TK1 has also sufficient space to accommodate a bulky carborane cage in closer proximity to the dThd scaffold as indicated by favorable relative $k_{cat}/K_m$ values for compounds 1 and 7 (27.4 and 31.9%, respectively) having short ethylene spacers. Whereas the tether length increased, the carborane cluster appeared to interfere with the enzymatic activity. In the case of compounds with a pentylene linker, the carborane cluster presumably is located outside of the active site, which results in significantly improved activity. In the case of compounds with longer tether arms (5, 6, 11, and 12), the carborane cluster could interfere with substrate binding at the same active site by folding on itself or at active sites of other TK1 units of this tetrameric holoenzyme (9, 11).

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m$ (μmol/L)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ % to dThd</th>
<th>IC₅₀ (μmol/L)</th>
<th>IC₅₀ (μmol/L)</th>
<th>IC₅₀ (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.1 ± 1.2</td>
<td>0.32 ± 0.11</td>
<td>27.4 ± 3.8</td>
<td>21.1 ± 3.2</td>
<td>35.2 ± 4.9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>26.1 ± 4.2</td>
<td>0.72 ± 0.08</td>
<td>13.1 ± 3.8</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8.5 ± 1.8</td>
<td>0.16 ± 0.02</td>
<td>5.2 ± 2.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.7 ± 0.5</td>
<td>0.22 ± 0.03</td>
<td>14.0 ± 6.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>16.7 ± 0.1</td>
<td>0.18 ± 0.02</td>
<td>5.2 ± 1.9</td>
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<tr>
<td>6</td>
<td>36.5 ± 8.4</td>
<td>0.12 ± 0.01</td>
<td>18.8 ± 0.9</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>6.1 ± 2.7</td>
<td>0.27 ± 0.00</td>
<td>31.9 ± 14.1</td>
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<tr>
<td>8</td>
<td>10.3 ± 2.0</td>
<td>0.29 ± 0.06</td>
<td>16.4 ± 0.2</td>
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</tr>
<tr>
<td>9</td>
<td>15.5 ± 7.2</td>
<td>0.31 ± 0.01</td>
<td>14.4 ± 6.0</td>
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<tr>
<td>10</td>
<td>3.4 ± 0.6</td>
<td>0.21 ± 0.02</td>
<td>35.8 ± 2.6</td>
<td></td>
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</tr>
<tr>
<td>11</td>
<td>7.6 ± 0.5</td>
<td>0.33 ± 0.02</td>
<td>24.9 ± 1.5</td>
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<tr>
<td>12</td>
<td>28.4 ± 7.4</td>
<td>0.08 ± 0.00</td>
<td>1.7 ± 0.5</td>
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</table>

NOTE: The parameters were calculated using Michaelis-Menten equation. The concentration of compounds 1-12 ranged from 1 to 100 μmol/L. The $k_{cat}$ values were calculated using the equation $V_{max} = k_{cat} \times [E]$, where [E] is the total enzyme concentration based on one active site/monomer. Under the same experimental conditions, the kinetic parameters for dThd were $K_m = 2.4 ± 0.8 \mu$mol/L and $k_{cat} = 0.6 ± 0.2 \text{s}^{-1}$, data represent the mean ± SDs of three independent experiments. The IC₅₀ is defined as the concentrations of a compound required to inhibit the TK1 phosphorylation of dThd by 50%. Data represent the mean ± SDs of at least three independent experiments, each performed in duplicate.

* Kinetic parameter values at high concentrations of the compounds.
Inhibition of dThd Phosphorylation by Boronated dThds. 

We hypothesize that the substrate-binding pocket for pyrimidine nucleosides in TK1 is close to the enzyme surface and that the 3-position of dThd is directed toward the active site of the enzyme. This hypothesis is supported by the fact that carboranyl dThds with even longer linkers than in compounds 6 and 12 also were sufficiently phosphorylated by TK1. Several other kinases are known to have substrate-binding pockets near the surface of the protein, e.g., the crystal structure of the bacterial pantothenate kinase shows that CoA competitively binds to the ATP site as a feedback inhibitor at the enzymes surface. Furthermore, 5'-adenylmido-diphosphate, a nonhydrolyzable analog of ATP, was also found to bind to the enzyme surface of pantothenate kinase (31).

Although both compounds 4 and 10 have similar biochemical properties, differences in inhibition of dThd phosphorylation were observed. The IC₅₀ values for compound 10 at both dThd concentrations were 2-fold lower than those for compound 4. This demonstrates the importance of the additional hydrophilic dihydroxypropyl group attached to the second carbon atom of the carboxen moiety of compound 10. The increase of TK1 inhibitor capacity attributable to the dihydroxypropyl group was only observed in case of compounds 4 and 10 having pentylene tethers, indicating that only compound 10 had favorable TK1 inhibitor and substrate characteristics. Interestingly, compounds 1 and 7, both with ethylene spacer, had kinetic properties similar to those of compounds with pentylene spacers (4 and 10). The IC₅₀ values for both compounds, however, were 2- to 3-fold higher than those of compound 10, indicating a decreased capacity to compete with dThd for the active site of TK1. Kinetic studies outlined here show that compounds 4 and 10 have favorable kinetic properties, and both compounds are apparently partially effective competitive inhibitors of dThd phosphorylation. Therefore, both were chosen for an in depth biological evaluation.

Boronated dThd Monophosphates as Substrates for Human Cytosolic dNT-1. Human dNT-1 presumably is the principle-degrading enzyme of deoxynucleoside monophosphates in mammalian cells (13). Figure 2 summarizes the substrate specificity of pure recombinant dNT-1 (containing a His-tag attached to the NH₂-terminal) with various nucleoside 5'-monophosphates, including compounds 13 and 14 (the 5'-monophosphates of compounds 4 and 10, respectively). Similar to previous observation (33), dNT-1 preferentially catalyzes the dephosphorylation of 5'-deoxyribonucleotides (dGMP and dTMP) rather than 5'-ribonucleotides (UMP). dGMP and dTMP were rapidly converted to 2'-deoxyguanosine (90%) and dThd (60%) after 30 minutes incubation with dNT-1. Almost 60% of the monophosphates of the antiviral dThd analog AZT were degraded to AZT under these conditions, whereas only a small amount (10%) of UMP was degraded to uridine (Fig. 2). In contrast, no dephosphorylation of the 5'-monophosphates 13 and 14 was observed even after 120 minutes of incubation. Compounds 13 and 14 were not hydrolyzed by dNT-1 in vitro, which leads us to conclude that the same may be the case in vivo. However, in vivo, several additional types of 5'-dNTs may be operational (13).

Boronated dThds as Substrates or Inhibitors for Thymidine Phosphorylase. Another cellular catabolic enzyme of pyrimidine nucleosides is TPase, which catalyzes the cleavage of dThd into 2'-deoxyribose and thymine. Therefore, we evaluated the activity of commercially available E. coli TPase using dThd, BVDU, AZT, and carboranyl dThds 4 and 10 (Fig. 3A). No hydrolysis of compounds 4 and 10 was detected after 120 minutes of incubation with the bacterial TPase. In contrast, dThd and BVDU were efficiently converted to thymine and (E)-5-(2-bromovinyl)uracil base (75% and 65%, respectively). As reported earlier and confirmed by us, AZT was resistant to hydrolysis by bacterial TPase (34). In a second set of experiments, the inhibitory effects of deoxynucleoside analogs on cleavage of dThd by TPase were studied. The amount of TPase was adjusted to hydrolyze ~50% of the dThd within 30 minutes. As shown in Fig. 3B, BVDU, AZT, and compound 10 did not inhibit the bacterial TPase. Surprisingly, compound 4, which was not a substrate for TPase (Fig. 3A), inhibited dThd degradation significantly (40%). Further investigation of the mechanism of this inhibitory activity is currently in progress in our laboratory.

Recombinant human TPase was also prepared and it appeared to have similar substrate specificity for compounds 4 and 10 to that observed for the bacterial TPase. The human enzyme did not degrade AZT and the boronated dThds, whereas dThd and BVDU were better substrates for the human TPase than for the E. coli TPase (data not

\*J. Johnsamuel, unpublished results.
TK1 substrates for BNCT

TK1 activity, were determined and are summarized in Table 2. Both cell lines have been well characterized (20, 21). The TK activities in the cell lines were determined using a [methyl-3H]dThd assay. The specific activity of TK in exponentially growing L929 and CCRF-CEM wild-type cells were 25- and 40-fold higher than that of cells lacking TK1. The minor activity observed in TK− cells most likely was because of thymidine kinase 2 (Table 2, footnote §).

Cell growth inhibition was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium assay (21) and is expressed as the concentration of a compound that produces a 50% inhibition of cell growth over a 72-hour incubation period (IC50). In case of L929 and L929 TK− cells, the cytotoxic effect of the boronated dThds was generally low. However, compounds 1-6 showed 2- to 3-fold more growth inhibition than compounds 7-12. Furthermore, the IC50 values decreased with increasing tether lengths of the compounds. There were no obvious differences in growth inhibition of L929 as compared with L929 TK− cells, with the exception of compounds 4 and 10, in which cases the TK− cells were ~2-fold more resistant than the wild-type cells.

For CCRF-CEM cells, the IC50 values of all compounds were ~2- to 3-fold lower than those observed for L929. Similarly, the cytotoxic effect of the compounds appeared to be dependent on the tether lengths, but this effect was not as pronounced as for L929 cells. The cytotoxic effects of the compounds were similar both in wild-type and TK− CCRF-CEM cells except for compounds 7-9, which had a lower growth-inhibitory effect. This probably was related to the higher hydrophilicity of these hydroxylated analogs with a shorter methylene spacer compared with compounds with a longer tether length.

Compounds with shorter tether lengths (1, 2, 7, and 8) had lower cytotoxicity and log P values (range 1.27-1.87) than those with long tethers (compounds 5, 6, 11, and 12), which showed some cytotoxicity and higher log P values (range 2.41-3.23). The compounds with intermediate tether length (compounds 3, 4, 9, and 10) had intermediate to low toxicity and their log P values ranged between 1.52 and 2.45. Thus, carborane-dependent lipophilicity most likely was involved in the growth inhibition that was observed. This conclusion shown). Similar results have been reported in previous studies (12). In addition, human TPase was efficiently inhibited by BVDU, and this was not observed with the bacterial TPase (data not shown). Compounds 4 and 10 appeared to be resistant to degradation by both the human and the E. coli TPases, which indicates possible stability of 4 and 10 to these types of enzymes in vivo.

Log P Values of the Boronated dThd Analogs. The physicochemical properties of a drug are critical for passing the blood brain barrier and penetrating the brain to reach intracerebral targets (35). One of the best physicochemical parameters to define these essential properties is the octanol/water partition coefficient log P (36). The log P values of compounds 1-6 and 7-12 were determined by a reversed-phase HPLC method developed by Teijeiro et al. (19). They increased as a function of the length of the methylene spacer except for compounds 1 and 7, which had slightly higher log P values than compounds 2 and 8 (Table 2). The optimal log P value for compounds designed to penetrate into the central nervous system has been determined empirically to be ~2 (35). Interestingly, the log P value of compound 10 is 2.09, indicating that it might be a good candidate for the treatment of brain tumors by neuron capture therapy.

Cellular Cytotoxicity. The inhibitory activities of the boronated dThds on the proliferation of the murine fibroblast L929 cells and the human T-lymphoblast cell line CCRF-CEM and their corresponding variants L929 TK− and CCRF-CEM TK−, respectively, which lack

Table 2. Physicochemical properties of boronated dThds and their in vitro cytotoxicity data in L929 and CCRF-CEM wild-type cell lines and their TK1 deficient sub lines.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log P (HPLC)*</th>
<th>L929 §</th>
<th>CCRF-CEM §</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type TK</td>
<td></td>
<td>Wild-type TK</td>
</tr>
<tr>
<td>1</td>
<td>1.872</td>
<td>142 ± 6</td>
<td>&gt;160</td>
</tr>
<tr>
<td>2</td>
<td>1.847</td>
<td>&gt;160</td>
<td>&gt;160</td>
</tr>
<tr>
<td>3</td>
<td>2.147</td>
<td>119 ± 7</td>
<td>134 ± 6</td>
</tr>
<tr>
<td>4</td>
<td>2.445</td>
<td>56 ± 9</td>
<td>127 ± 5</td>
</tr>
<tr>
<td>5</td>
<td>2.954</td>
<td>39 ± 8</td>
<td>37 ± 4</td>
</tr>
<tr>
<td>6</td>
<td>3.226</td>
<td>20 ± 5</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>7</td>
<td>1.468</td>
<td>&gt;160</td>
<td>&gt;160</td>
</tr>
<tr>
<td>8</td>
<td>1.27</td>
<td>&gt;160</td>
<td>&gt;160</td>
</tr>
<tr>
<td>9</td>
<td>1.522</td>
<td>&gt;160</td>
<td>&gt;160</td>
</tr>
<tr>
<td>10</td>
<td>2.087</td>
<td>113 ± 11</td>
<td>&gt;160</td>
</tr>
<tr>
<td>11</td>
<td>2.411</td>
<td>67 ± 6</td>
<td>63 ± 6</td>
</tr>
<tr>
<td>12</td>
<td>2.606</td>
<td>38 ± 5</td>
<td>56 ± 3</td>
</tr>
</tbody>
</table>

* Log P values were determined by HPLC as described in Materials and Methods.
† Cell growth inhibition data are expressed as mean ± SDs of three experiments, each performed in triplicate. Cells were incubated with compounds 1-12 for 72 hours and cell survival was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in Materials and Methods.
‡ The IC50 value (μmol/L) is defined as the concentration of the compound that inhibited cell growth by 50% following 72 hours of incubation.
§ The TK-specific activities in L929 and CCRF-CEM wild-type strains were 227 ± 16 and 202 ± 15 pmol/min/mg total proteins, respectively, whereas in strains lacking TK1, the activities were 9 ± 3 and 5 ± 1 pmol/min/mg total proteins, respectively. Data represent the mean ± SDs of three independent experiments.

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was further supported by the fact that the attachment of additional hydrophilic groups (compounds 7-12) reduced the cytotoxic effect. Interestingly, the IC_{50} values for compounds 4 and 10 observed in L929 wild-type cells were significantly lower compared with L929 TK^{-} cells. This finding is in agreement with the kinetic properties of both compounds and indicates higher metabolic activity than for all other compounds. Growth inhibition could have been related to significant differences in the intracellular accumulation of phosphorylated products of 4 and 10, which may have interfered with cellular processes, and this currently is under investigation.

In general, the compounds tested were only moderately cytotoxic. Furthermore, the cytotoxicity assay that was used in this study detected cell viability after 72 hours of exposure to the test compounds, a time course that is at least three times longer than that necessary to obtain sufficient intracellular boron accumulation. During the time period used for boron-uptake studies, no cytotoxic effect was detected (data not shown), indicating that the boronated dThds may be cytostatic rather than cytotoxic agents.

**In vitro Uptake and Retention Studies.** Because the cellular uptake of sufficient amounts of {sup}10{sub}B is a prerequisite for a boron delivery agent for neutron capture therapy, *in vitro* uptake and retention studies were performed as reported in a companion article in this issue (37). Compounds 4, 6, 10, and 12 were evaluated in L929 wild-type and L929 TK^{-} cell cultures (Table 3). A significant 5- to 30-fold difference in uptake between L929 wild-type and TK^{-} cells was observed. In addition, 29 to 46% of these compounds were retained for at least 12 hours in L929 wild-type but not in TK^{-} cells. The uptake of compounds 10 and 12 occurred at 1.3- and 1.7-fold higher rates, respectively, in comparison to their counterparts lacking wild-type and L929 TK^{-}/H11002

In vitro uptake and retention of boronated dThd analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>L929 Wild-type</th>
<th>L929 TK^{-}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uptake (μmol/L)</td>
<td>Retention (%)</td>
</tr>
<tr>
<td>4</td>
<td>68.4 ± 11.0</td>
<td>45</td>
</tr>
<tr>
<td>6</td>
<td>92.0 ± 20.0</td>
<td>29</td>
</tr>
<tr>
<td>10</td>
<td>91.1 ± 12.0</td>
<td>46</td>
</tr>
<tr>
<td>12</td>
<td>160.1 ± 17.0</td>
<td>29</td>
</tr>
</tbody>
</table>

Table 3 **In vitro uptake and retention of boronated dThd analogues**

Additionally, 29 to 46% of these compounds were retained for at least 12 hours in L929 wild-type but not in TK^{-} cells. The uptake of compounds 10 and 12 occurred at 1.3- and 1.7-fold higher rates, respectively, in comparison to their counterparts lacking wild-type and L929 TK^{-}/H11002. The uptake of compounds 4 and 10 were taken up by the wild-type L929 cells and were trapped in the cells, presumably because of intracellular phosphorylation by TK1. *In vivo* experiments, described in a companion paper in this issue (37), in which compound 10 was administered to F98 glioma-bearing rats by convection-enhanced delivery, resulted in the selective retention of boron concentrations within the tumor that are sufficient for BNCT. Studies are planned to evaluate compound 10 in therapy studies using the F98 glioma model.

**ACKNOWLEDGMENTS**

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