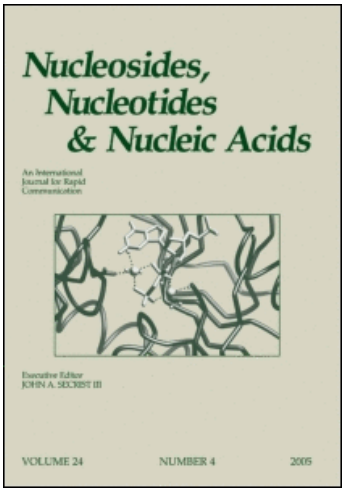

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CELLULAR PHARMACOLOGY OF THE D- AND L-ENANTIOMERS OF β-5-*o*-CARBORANYL-2'-DEOXYURIDINE

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Abstract: The cellular pharmacology of the D- and L-enantiomers of β-5-*o*-carboranyl-2'-deoxyuridine (CDU), compounds designed for boron neutron capture therapy (BNCT), were studied using human CEM lymphoblast and U-251 glioblastoma cells, at a physiologically achievable concentration (1 μM). Accumulation of the enantiomers was rapid and indistinguishable, reaching cellular concentrations > 40-fold higher than extracellular levels, with ~5% persisting in cells after incubation in fresh medium for more than 2 hr. Uptake was not affected by nucleoside uptake inhibitors, but was inhibited by the purine base uptake inhibitor papaverine.

Numerous boron containing compounds have been evaluated for their potential for neutron capture therapy (BNCT). Two compounds being tested in the clinic in Japan and preclinically in the USA are *p*-boranophenylalanine (BPA), a modified amino acid containing one boron atom per molecule and borocaptate sodium (BSH), a sulfhydryl-containing carborane.[1] BSH has an advantage of delivering ten boron atoms per molecule accumulated in cancer cells. There is a need to develop additional agents for BNCT since none of the molecules developed to date are optimal for therapy. Desired properties for agents used for BNCT include preferential accumulation in tumors relative to non-tumor tissue at boron concentrations sufficient for BNCT (estimated to be 5-30 ppm of ^{10}B), and low toxicity to non-tumor tissues.[1-3] Previous studies by our group have demonstrated that β-D-5-*o*-carboranyl-2'-deoxyuridine (D-CDU) has favorable pharmacological properties including phosphorylation in CEM cells and low cellular toxicity in African Green monkey kidney (Vero) cells, human lymphoblastoid (CEM)

cells, human glioma (U-251) cells, and human peripheral blood mononuclear cells, respectively.[4] The plasma pharmacokinetics of D-CDU is biexponential in rats with an initial distribution phase lasting 1 hr and terminal $t_{1/2}$ of 1.26 hr. The plasma concentrations at 1 hr following intravenous doses of 25 mg/kg and 5 mg/kg were 13 μM and 1.7 μM, respectively, indicating that the pharmacokinetics of D-CDU is linear in this dose range.[6]

Herein, we compared the accumulation of the D- and L-enantiomers of CDU in the adherent U-251 human glioma cell line and in the human CEM T-cell lymphoma line. CEM cells contain the *es* equilibrative nucleoside transporter, which is sensitive to 1 nM concentrations of *S*-(*p*-nitrobenzyl)-6-thio-inosine (NBTI), and does not contain the *ei* transporter that is blocked only at 20 μM concentrations of NBTI. This cell line also lacks sodium dependent concentrative nucleoside transporters. Concentrative nucleoside transporters have been identified primarily in specialized cells comprising the brush borders of the kidney, intestine, spleen, liver, and the choroid plexus tissue surrounding the brain and in only one leukemia cell line (L1210 murine leukemia cells).[7] There is a paucity of information on nucleoside transporters in glioblastoma cells, including U-251 cells. We report that cellular accumulation of CDU is not stereospecific and that it accumulates in sufficient amounts in cells to warrant further testing in BNCT animal models for cancers.

Experimental

Materials: D-CDU was synthesized by a previously published method,[4] whereas the synthesis of L-CDU will be reported elsewhere.[5] The compounds were radiolabeled with tritium in the 6-position and on the acidic proton of the carboranyl moiety by Moravek Biochemicals, Inc. (Brea, CA), by hydrolysis of the lithium derivative of the respective CDU-3',5'-di-*O*-benzoyl enantiomers with carrier-free tritiated water. The specific activities were 1.8 Ci/mmol and 23.1 Ci/mmol, for the D- and L-enantiomers, respectively. Their purity was > 99% by reverse phase HPLC (see below).

Silicone oil (specific gravity, 1.035 to 1.045) was purchased from William F. Nye Co. (New Bedford, MA). Scintillation fluid (Eco Lite), was purchased from ICN Pharmaceuticals (Costa Mesa, CA). HPLC grade acetonitrile was purchased from EM Science (Gibbstown, NJ), while HPLC grade methanol was purchased from Fisher Chemical (Fair Lawn, NJ). ^{14}C-labeled inosine (51 mCi/mmol), used as an internal control, was obtained from Moravek Biochemicals, (Brea, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture: CEM T-lymphoblast cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in suspension in (25 ml) Falcon 75 cm^2 tissue culture flasks (Becton Dickenson, NJ) in RPMI 1640 medium (Cellgro, Herndon, VA) at 37°C in 5% CO_2 in air. Ten percent heat inactivated fetal bovine serum (Grand Island Biological Company, Gaithersburg, MD), 2 mM L-glutamine (Grand Island Biological Company, Gaithersburg, MD) and antibiotics (penicillin 100 I.U./ml and streptomycin 100 μg/ml, Cellgro/Mediatech, Hernden, VA) were added to the medium. The doubling time of the CEM cells was about 18 hr.

The U-251 human glioblastoma cells were kindly provided by Dr. J. Olson of Emory University School of Medicine. U-251 cells were grown in monolayer in Falcon 75 cm^2 tissue culture flasks (Becton Dickenson, NJ) in high glucose Dulbecco's Modified Eagle Medium (Grand Island Biological Company, Gaithersburg, MD) at 37°C in 5% CO_2-air. Fifteen percent heat inactivated fetal bovine serum, 2 mM L-glutamine and antibiotics were added to the media. The doubling time of the U-251 cells was about 36 hr. Median cell volumes (± SD) were measured for suspensions of exponentially growing CEM cells and trypsinized U-251 cells using a Coulter Multisizer (Coulter Electronics, Miami, FL.).

The Spin Through Oil Technique: This methodology involved centrifuging cells that had been incubated in medium containing radioactive compounds for a specific time through a layer of silicone oil, and measuring the amount of compound accumulated in the cells using a liquid scintillation counter (Tri Carb 2500 SR, Packard Instrument Company, Downers Grove, IL).[8,9,10] Samples were harvested in quadruplicate, and counts per min were converted to amount of drug using a regression analysis of scintillation counts against a known amount of the extracellular ^3H-compound containing medium. Regression curves were determined for each individual experiment.

Accumulation and Efflux in CEM Cells: To determine the accumulation of L-CDU and D-CDU in CEM cells, the cells were washed and resuspended in RPMI 1640 medium at a density of 10^6 cells/ml. Accumulation experiments were conducted in serum free medium, or medium containing 2% serum at 37°C. Samples of the cell suspension (200 μl, in quadruplicate) were removed at noted times with the addition of soluble compounds (1 μM) and the accumulation of CDU per 10^6 cells measured using the "spin through oil" technique (see above). Pilot HPLC analysis of the extracellular medium after centrifuging cells indicated that extracellular concentrations did not vary noticeably during the experiments.

The efflux of D- and L-enantiomers of CDU were measured in CEM cells. Cells were washed and resuspended at a density of 10^6 cells/ml in prewarmed serum free RPMI 1640 medium. Radiolabeled compound (1 μM) was added and the cells were incubated for 2.5 hr. Cells were then centrifuged at 800 x *g* for 5 min and resuspended in prewarmed (37°C) RPMI 1640 medium containing 10% FBS (t = 0 min). Cell suspensions were kept at 37°C for the remainder of the experiment. Quadruplicate samples of 200 μl cell suspension were removed at noted times and the accumulation of CDU per 10^6 cells measured using the "spin through oil" technique. Samples of the supernatant medium was used to create a calibration curve of cpm *versus* pmoles of compound.

Accumulation was measured in CEM cells in the presence and absence of uptake inhibitors to determine whether accumulation of CDU enantiomers was dependent upon known equilibrative nucleoside, or nucleoside-base carriers.[11-13] Inhibitors of nucleoside uptake tested included NBTI (20 μM) and dipyridamole (DPM, 20 μM) and the nucleobase uptake inhibitor, papaverine hydrochloride (19 mM).[14,15] For NBTI and DPM, cells were washed and suspended in RPMI 1640 medium at a density of 10^6 cells/ml and incubated at 37°C for 30 min in medium containing either of the nucleoside uptake inhibitors, NBTI, or DPM. D- or L-CDU was then added (1 μM), and the accumulation was measured 60 sec later using the "spin through oil" technique. Cellular accumulation was compared to that obtained in medium in the absence of inhibitors. Papaverine base is almost insoluble in water, while the hydrochloride salt has a solubility of 66.5 mM.[16] Since papaverine is not soluble in medium, the accumulation of D-CDU was determined in normal saline with and without papaverine (19 mM).

Determination of Accumulation and Efflux in U-251 Cells: The adherent U251 human brain tumor cells were measured in duplicate monolayer cultures. Cells were seeded at 2 x 10^5 cells/well into 6-well plates (Nunc, Intermed, Naperville, IL). After 24 hr, the medium was replaced with fresh, prewarmed, serum-free medium containing 1 μM of D-CDU or L-CDU. For accumulation studies, the time of addition was taken as 0 min. For efflux studies, cultures were preincubated in 1 μM L-CDU or D-CDU for 2.5 hr in the presence of 2% FBS. The medium was replaced with fresh prewarmed medium containing 2% FBS (t = 0 min). At designated times, cells were placed on ice, washed twice with ice-cold serum-free medium before the addition of 1 ml of cold 60% methanol in water. The contents of the wells were then scraped into scintillation vials using disposable cell scrapers (Fisher Scientific, Pittsburgh, PA), and the surface of the wells were washed into scintillation vials using 1 ml of 60 % methanol in water. Ten ml of

scintillation fluid was added and the radioactivity measured using a liquid scintillation counter, as described for CEM cells. The number of cells per well was determined by trypsinizing (1X trypsin/EDTA, 10 min) the contents of triplicate untreated wells and counting using a hemacytometer.

HPLC Analysis: HPLC was used to determine whether the D- and L-enantiomers of CDU were phosphorylated in CEM and U-251 cells. CEM cells were suspended at 1 x 10^6 cells/ml in RPMI 1640 medium. Medium containing 10% FBS and 1 μM radiolabeled D- or L-CDU was added and the cells incubated for up to 24 hr. Cells were then washed twice in ice-cold PBS, centrifuging at 800 x *g* for 5 min. The CEM cell pellet was then lysed with 400 μl of ice-cold 60% methanol in water and stored at -70°C. U-251 cells were plated as above in 6-well plates 24 hr before the addition of 1 μM radiolabeled compound, and further incubated for up to 24 hr. For U-251 cells, the plates were kept on ice and the cells were washed twice with ice-cold serum free medium. The cell monolayer was lysed with 400 μl of 60% ice-cold methanol in water and stored at -70°C until assayed. Samples were thawed at 4°C and centrifuged for 10 min on a desk top centrifuge at 16,000 x *g*. ^{14}C-labeled inosine (0.05 nmoles, 51 mCi/mmol) was added as an internal standard before drying the samples overnight at room temperature using a Speed Vac centrifuge dryer (Model SC110, Savant Instruments Inc., Farmingdale, NY). The dry extract was then dissolved in 40 μl of 100 mM tris buffer containing 20 μM $MgCl_2$ and subdivided into two aliquots. To dephosphorylate possible nucleotides of D- or L-CDU, 22 units of alkaline phosphatase from calf intestinal mucosa (EC 3.1.3.1, type XXX-A, Sigma, Saint Louis, MO) was added to an aliquot, prior to incubation at 37°C for 2 hr. The solution was then diluted to 100 μl with buffer and injected into the HPLC. A gradient consisting of mobile phase A (0.05 M triethylamine adjusted to pH 7 with acetic acid in water) and mobile phase B (50% acetonitrile diluted with 2-fold buffer A) was used. The starting solvent ratio was 5% solvent B which increased to 30% solvent B over 40 min. A Whatman Partisphere C_{18}, 5 μm column (Whatman, Clifton, NJ) was used and detection of radiolabeled compound was achieved by means of a radiomatic Flo-one *Beta* detector (Packard, Downers Grove, IL), capable of simultaneous detection of ^{14}C and ^{3}H labels.

Neutral lipid content in CEM and U-251 cells: The relative amounts of intracytoplasmic neutral lipids between CEM and U-251 cells were measured by staining with Oil red O (National Aniline Division, Allied Chemical and Dye Corp., New York, NY).[17] The working solution was prepared by dissolving 0.2 g of Oil red O in 57 ml of isopropyl alcohol using a published procedure.[18] U-251 cells were trypsinized (1X

trypsin/ EDTA) and quadruplicate aliquots of 5 x 10^6 cells were suspended and fixed in suspension with 10% formaldehyde in neutral isotonic phosphate buffered saline (PBS) for 1 hr at 37°C. Quadruplicate suspensions of 5 x 10^6 CEM cells that had been prewashed in PBS was fixed in a similar manner. The cell suspensions were washed three times in distilled water and stained for 2 hr in a working solution of Oil red O at 60°C. Cells were then washed three times in 10 ml of fresh distilled water (centrifuge cycles were 800 x *g*, 5 min). Dyed cells were resuspended in 1 ml water, transferred to microcentrifuge tubes and dried overnight on a Speed Vac lyophilizer. The relative amounts of neutral lipids in the cytoplasm were estimated by extracting the cell pellets in 1 ml of isopropyl alcohol at 60°C, centrifuging (16,000 x *g*, 5 min) and measuring the absorbance of the supernatant at 510 nm using a Beckman DU 640 spectrophotometer (Beckman Instruments, Schaumburg, IL). Similarly treated extracts from fixed non-dyed cells were used as a blank. Absorbance values were in the linear range for the spectrophotometer.

Results and Discussion

Cellular accumulation of D- and L-CDU remained linear in the concentration range tested (0.1 to 10 μM) (data not shown). At a 1 μM extracellular concentration, accumulation profiles for the enantiomers of CDU were similar in both cell lines and reached pseudo-steady state within 3 and 10 min, for CEM and U-251 cells, respectively. Accumulation was not affected by the addition of 2% FBS (data not shown). Plateau levels at 1 hr of incubation were approximately 45 pmol and 220 pmol per 10^6 CEM and U251 cells, respectively (FIG. 1).

Efflux rates following a 2.5 hr incubation with 1 μM compound were biphasic for the enantiomers in both cell lines. About 40% of the compound effluxed from the cells within 10 min. However, CEM cells and U-251 cells retained 6% and 5% of compound for at least 2 hr and 24 hr, respectively (FIG. 2 and 3). The similar accumulation and efflux profiles of D- and L-enantiomers of CDU indicates that uptake and efflux was not stereospecific.

The 60-sec accumulation of CDU enantiomers were not significantly inhibited by the nucleoside uptake inhibitors NBTI (20 μM) and DPM (20 μM) (data not shown). However, papaverine (19 mM) decreased the 1 min accumulation of D- and L-CDU by 89.9 ± 0.97 and 88.6 ± 3.7%, respectively. Papaverine has previously been used in an "inhibitor stop" assay to block the accumulation of purine bases and to demonstrate that

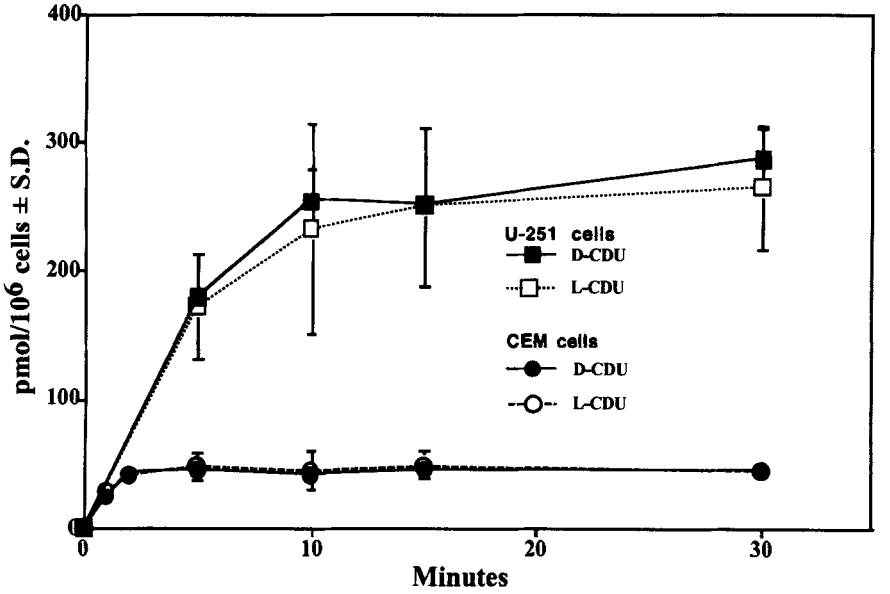

FIG. 1. Uptake of D- and L-enantiomers of CDU (1 μM extracellular) in CEM human T-lymphoblast cells (O) and in U-251 human glioblastoma cells (□). Closed symbols represent D-CDU and open symbols represent L-CDU. Shown are averages (± SD) from 3 experiments, each performed in quadruplicate.

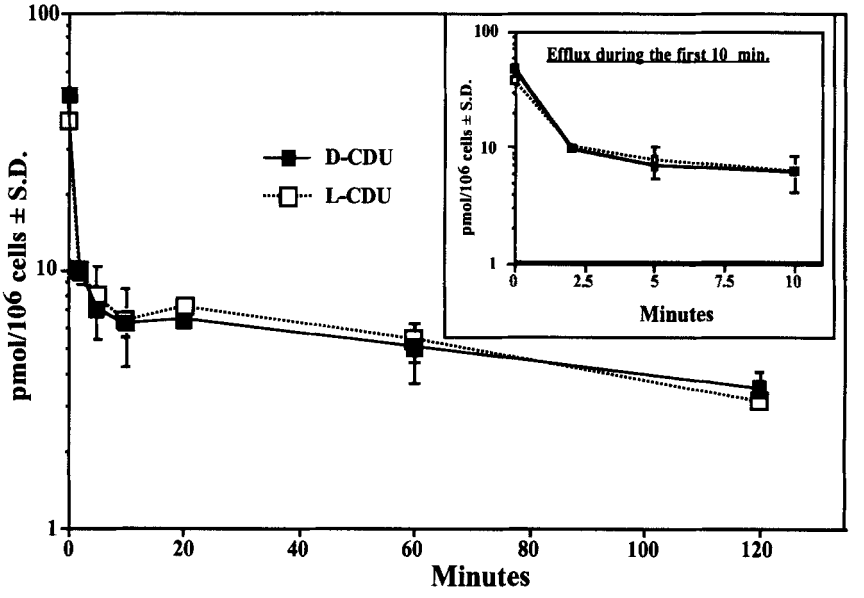

FIG. 2. Efflux of D- and L-enantiomers of CDU from CEM human T-lymphoblast cells following 2.5 hr incubation in compound (1 μM) (0 to 120 min, inset is 0 to 10 min). Time of resuspension in fresh, prewarmed medium was taken as 0 min. Shown are averages (± SD) from 3 experiments, each performed in quadruplicate.

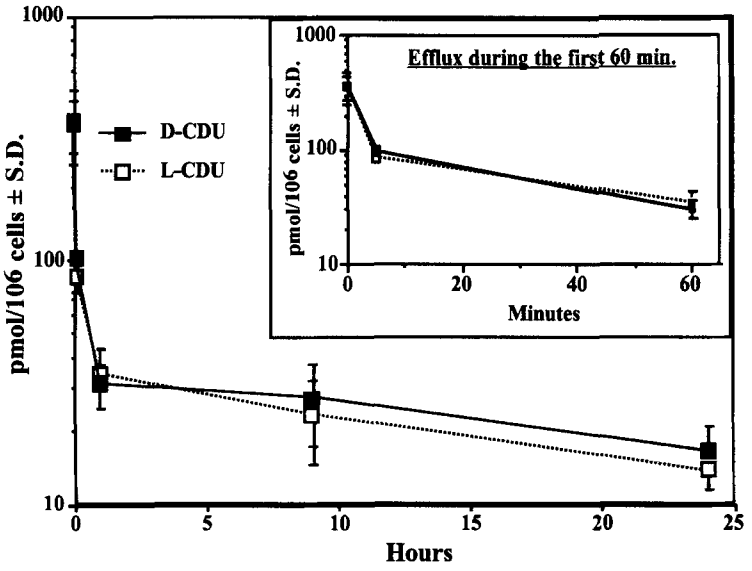

FIG. 3. Efflux of D- and L-enantiomers of CDU from U-251 human glioblastoma cells following 2.5 hr incubation in compound (1 μM) (0 to 24 hr, inset is 0 to 60 min). Time of replacement of medium with fresh, prewarmed medium was taken as 0 min. Shown are averages (± SD) from 3 experiments, each performed in quadruplicate.

the accumulation of the acyclic nucleoside derivative, acyclovir, is mediated by a nucleoside-base uptake process.[14,15] That CDU accumulation is not affected by the nucleoside inhibitors NBTI, but is inhibited by papaverine, a compound that blocks nucleoside-base accumulation, suggests that the accumulation of CDU enantiomers is mediated to a large extent by a nucleoside-base transport process. However it remains possible that accumulation may be partially mediated by passive diffusion or complexation with the cell membrane, as predicted by the highly lipophilic nature of D-CDU (octanol/water partition coefficient = 3.05 x 10^3) or by other carrier-mediated processes.

HPLC analysis of cell extracts indicated the presence of a less lipophilic metabolite in cell extracts of CEM cells incubated with 1 μM D-CDU for 24 hr (FIG. 4). The levels of the metabolite were lower in U-251 cells than in CEM cells. The retention times for *nido*-CDU, CDU-monophosphate and *closo*-CDU, previously synthesized in our laboratory,[4] were 22, 35 and 42 min, respectively. The peak corresponding to D-CDU-monophosphate accounted for about 5% of the total radioactivity in CEM cells and was

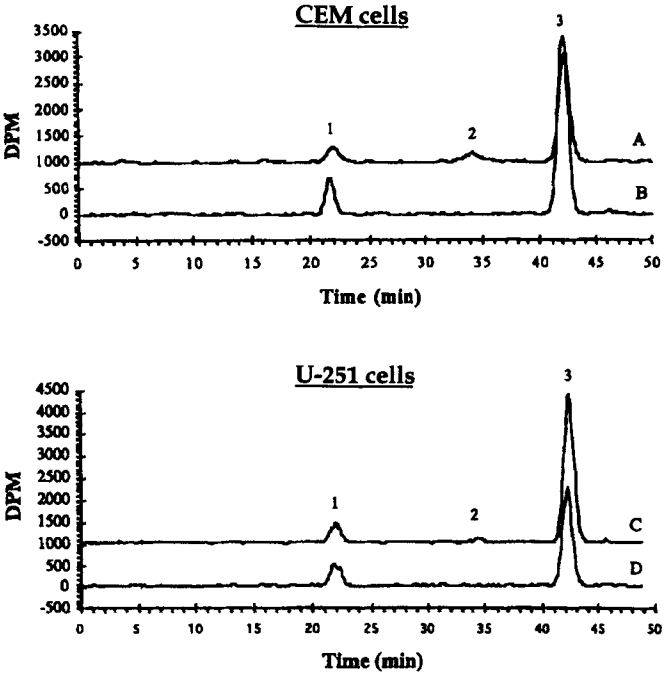

FIG. 4. HPLC analysis of cell extracts of cells incubated with D-CDU (1 μM) for 24 hr. The retention times for *nido*-CDU (peak 1), phosphorylated CDU (peak 2) and *closo*-CDU (peak 3), were 22, 35, and 42 min, respectively. Chromatogram A: HPLC analysis of CEM cell extracts. Chromatogram B: HPLC analysis of CEM cell extracts after treatment with alkaline phosphatase. Chromatogram C: HPLC analysis of U-251 cell extracts. Chromatogram D: HPLC analysis of U-251 cell extracts after treatment with alkaline phosphatase.

not present following treatment with alkaline phosphatase. Phosphorylation of the L-enantiomer of CDU in CEM or U-251 was not detected by HPLC. These studies indicate that phosphorylation of CDU is slow, stereospecific and dependent upon cell type. Although phosphorylation was observed only in CEM cells treated with D-CDU and not with the L-enantiomer, the total amount of non-diffusible metabolite was similar for both compounds. This suggests that other processes may be involved in the formation of the nondiffusible fraction, such as the conversion of the neutral *closo* form of the boron cage into its *nido* form bearing a negative charge or non-covalent binding to cellular components, as a result of the highly lipophilic nature of the *o*-carborane unit. We have noted that cells incubated with the less lipophilic *nido*-form of CDU accumulate less than 5% of compound relative to cells incubated with *closo*-CDU (data not shown).

Since cell lipid content could in part explain differences in the amount of compound associated with each cell, these parameters were assessed. CEM cells accumulated 60 ±

6% (mean ± SD) of the lipophilic dye Oil red O compared to the U-251 (100 ± 16%), indicating a higher lipid content in U-251 cells. However, it is also possible that CDU may also partition into other lipophilic cellular compartments that are not stained by Oil red O, such as cell membranes. Although CDU may become sequestered in the cell membrane, there is support for some CDU entering the cytoplasm. These include the marked decrease in accumulation in the presence of the uptake inhibitor papaverine, together with the detection of phosphorylated CDU.

U-251 cells had larger volumes ($3.04 \pm 1.25 \times 10^3$ μm^3) than CEM cells ($1.10 \pm 3.67 \times 10^3$ μm^3). When cell volumes are taken into account, the cellular boron concentrations were 4.09 and 7.24 ppm for CEM and U-251 cells, respectively. Based on estimates of cell volumes, average plateau cellular concentrations reached by the D- and L-CDU were approximately 41- and 72-times higher for the CEM and U-251 cells, respectively, than the concentration in the medium (1 μM). Since approximately 5% of the CDU persisted for more than 2 hr, the average cell concentrations of non-diffusible metabolite is between 2.1- to 3.6-times higher than the initial extracellular concentration of CDU. This suggests the possibility that malignant cells may retain significant levels of CDU after plasma concentrations begin to decline.

Previous studies have shown that borocaptate sodium (BSH), a sulfhydryl-containing carborane, penetrates malignant brain tumors to a larger extent than tissue containing an intact blood-brain-barrier.[19,20] This indicates that despite the high octanol/water partition coefficient of the carboranyl moiety, small molecules containing a carborane moiety may not cross the blood-brain-barrier easily. Protection of non-tumor brain tissue by an intact blood-brain-barrier combined with a large accumulation of CDU in cancer cells could be therapeutically advantageous. Furthermore, the relatively slow terminal egress phase of CDU may permit scheduling of the neutron irradiation of tumor bearing tissue to take place when tumor concentrations are at a maximum, and not necessarily when most of the CDU has been cleared from the plasma. BNCT is a binary process in which cell killing requires both accumulation of boron into target tissue and irradiation of the accumulated boron with epithermal neutrons.[1] Since only tissue neighboring the brain tumor will be irradiated, the boron contained in the bulk of the blood and other non-brain tissue should not cause dose-limiting toxicity.

The approximate cellular boron content following incubation in 1 μM CDU was 4.1 and 7.2 ppm for CEM and U-251 cells, respectively, based upon the measurements of cellular uptake as a function of cell number and volume. It is generally assumed that

between 5 to 30 ppm of boron is required for BNCT.[1-3] Since accumulation for CDU remained proportional to extracellular concentrations in the range tested (0.1 to 10 μM), sustained extracellular concentrations in the tumor that equal or exceed 1.5 to 4.0 μM for these compounds may be adequate for effective therapy. Thus, further preclinical development is warranted for D- and L-CDU as potential agents for BNCT.

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