Treatment of Isografted 9L Rat Brain Tumors with β-5-o-Carboranyl-2'-Deoxyuridine Neutron Capture Therapy

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ABSTRACT

β-5-o-Carboranyl-2'-deoxyuridine (D-CDU) is a non-toxic pyrimidine nucleoside analogue designed for boron neutron capture therapy of brain tumors. In vitro studies indicated that D-CDU accumulates to levels 92- and 117-fold higher than the extracellular concentration in rat 9L and human U-251 glioma cells, respectively, and persists for several hours at levels 5-fold higher than the extracellular concentration. Furthermore, D-CDU was not toxic to rats injected i.p. with up to 150 mg/kg. On the basis of these studies, D-CDU was evaluated as a neutron capture therapy agent using rats bearing stereotactically implanted intracranial 9L tumors at single i.p. doses of 30 mg/kg and 150 mg/kg of D-CDU (20% 10 B enriched), given 2 h before irradiation with thermal neutrons. Boron concentrations in tumors 2 h after dosing were 2.3 ± 1.6 and 7.4 ± 1.3 μg boron/g tissue (mean ± SD), corresponding to tumor/burn ratios of 11.5 ± 3.6 and 6.8 ± 2.0 μg boron/g tissue for the low and high doses, respectively. All untreated animals died within 28 days, whereas half survived at days 32, 55, and 38 for groups receiving neutrons only, 30 mg/kg D-CDU, and 150 mg/kg D-CDU, respectively. Odds ratios of all treatment groups differed significantly from the untreated group (P < 0.002; logrank test). The median survival time for the 30 mg/kg-treated group but not for the 150 mg/kg-treated group was significantly longer than for rats treated with neutrons only (P = 0.036), which may correlate with the decreased tumor selectivity for D-CDU observed at the higher dose. Additional pharmacodynamic studies are warranted to determine optimal dosing strategies for D-CDU.

INTRODUCTION

The search for safer and more effective boron-containing compounds for BNCT3 is accelerating. However, none of the molecules developed to date are optimal for therapy. The two compounds being tested clinically in Japan and in the United States are p-boranophenylalanine, a modified amino acid containing one boron atom per molecule, and borocaptate sodium, a sulfhydryl-containing carborane (1–7). p-boranophenylalanine has not demonstrated toxicity at concentrations above those obtained in clinical use. Borocaptate sodium is also essentially nontoxic but lacks tumor specificity. D-CDU was developed based on the assumption that a nucleoside analogue containing 10 boron atoms per molecule would accumulate preferentially in cancer cells due to enhanced phosphorylation of the nucleotide moiety and would deliver 10 boron atoms per molecule accumulated in the tumor.

Desired properties for agents used for BNCT include preferential accumulation in tumors relative to nontumor tissue at boron concentrations sufficient for BNCT (estimated to be 5–30 ppm of 10 B, assuming uniform distribution in cells) and low toxicity to nontumor tissues (1, 6, 7). Previous studies have demonstrated that D-CDU (Fig. 1) has a low cellular toxicity, with IC50 of 71, >100, and 83.3 μM in exponentially growing human lymphoblastoid CEM cells, human U-251 glioma cells, and rat 9L glioma cells, respectively (8). Furthermore, D-CDU has been shown to be 5'-monophosphorylated in various cells (8) and, to a significant extent, by recombinant thymidine kinases (9). Although the precise mechanism of cellular accumulation has not been fully characterized, D-CDU accumulation in CEM cells was insensitive to the nucleoside uptake blockers 5'(4-nitrobenzyl)-6-thioinosine and dipyridamol and was 90% inhibited by the nucleoside base uptake inhibitor papaverine. Thus, accumulation may be largely mediated by a nucleoside base uptake process despite the highly lipophilic nature of D-CDU (10).

Pharmacokinetic studies in Fischer 344 rats injected i.v. with 25 mg/kg of D-CDU demonstrated a biexponential decline in plasma concentrations with a terminal half-life value of 1.26 h with 95% of compound bound to plasma proteins (11). The clearance values for total D-CDU and for the unbound fraction were 0.69 and 15.3 L/hr·kg−1, respectively. In this paper, we have demonstrated comparable cellular accumulation of D-CDU.

The abbreviations used are: BNCT, boron neutron capture therapy; D-CDU, β-5-o-Carboranyl-2'-deoxyuridine; HPLC, high-performance liquid chromatography; FBS, fetal bovine serum; DCP-AES, direct-current plasma atomic emission spectroscopy.
in rat 9L and human U-251 glioma cells. We also studied the in vivo distribution of D-CDU in rats bearing 9L intracranial brain tumor xenographs and the efficacy of BNCT with D-CDU using this animal system.

MATERIALS AND METHODS

Materials. D-CDU was synthesized by a previously published method (8). Radiolabeled D-CDU was custom synthesized by Moravek Biochemicals, Inc. (Brea, CA). 2-14C-D-CDU (45 mCi/mmol) was synthesized from 14C-labeled 5-ido-2'-deoxyuridine. 6-[3H]D-CDU (1.8 Ci/mmol, purity >99% by reverse phase HPLC) was radiolabeled with tritium in the 6-position and on the acidic proton of the carboranyl moiety by the hydrolysis of the lithium derivative of D-CDU-3',5'-di-O-benzoyl nucleoside with carrier-free tritiated water (8).

Scintillation fluid (Eco Lite), was purchased from ICN (Costa Mesa, CA). HPLC-grade acetonitrile was purchased from EM Science (Gibbstown, NJ), whereas HPLC-grade methanol was purchased from Fisher Chemical (Fair Lawn, NJ). 14C-labeled inosine (51 mCi/mmol, Moravek Biochemicals, Inc.) was used as an internal control. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. The U-251 human glioma cells were provided by Dr. J. Olson of Emory University School of Medicine (Atlanta, GA) and were grown in monolayer in high-glucose DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 15% heat-inactivated FBS (Summit Biotechnology, Fort Collins, CO), 2 mM l-glutamine, and antibiotics (Sigma Chemical Co.) at 37°C in 5% CO2-air. The 9L rat glioma cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, and antibiotics (12). The doubling times of the U-251 and 9L cells were 36 and 20 h, respectively. Median cell volumes were measured for suspensions of D-CDU in rats bearing 9L intracranial brain tumors consisted of Li2CO3 dispersed in polyethylene, 93% enriched in 6Li, 7.5% total Li by weight, 10.2 cm thick, with a conical aperture 12 cm in diameter at the reactor side tapering to 0.05 cm at the exit. The time of addition was taken as 0 min. For egress studies, cultures were preincubated in 1 μM D-CDU for 2.5 h 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 and 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 using disposable cell scrapers (Fisher Scientific, Pittsburgh, PA) and were washed into scintillation vials using 1 ml of 60% methanol in water. Ten milliliters of Ecolite scintillation fluid was added, and total radioactivity was measured using a Packard 2500 TR liquid scintillation counter (Packard Instrument Co., Meriden, CT). The number of cells per well was estimated by trypsinizing (1× trypsin/EDTA; Life Technologies; 10 min) the contents of triplicate untreated wells and counting using a hemocytometer. Egress rate constants (0.693/t1/2) were calculated as the slopes of ln(6-[3H]D-CDU) versus egress time (hr−1).

HPLC Analysis of Cell Extracts. Cell extracts of U-251 and 9L cells in 60% methanol were assayed using reverse-phase HPLC to measure the phosphorylation of D-CDU and total intracellular radioactivity. A Partisphere C18 5-μm column (Whatman, Clifton, NJ) was used, and detection was performed using a radiomatic Flo-one Beta detector (Packard Instrument Co.) capable of simultaneous detection of 14C and [3H] labels. D-CDU-5'-monophosphate was synthesized as previously described (8).

Tissue Disposition of Radiolabeled D-CDU in Rats. Male Fischer 344 rats (200–250 g; Harlan Sprague-Dawley, Indianapolis, IN) bearing 9L intracranial tumors were injected i.p with 5 mg/kg 14C-D-CDU in 200 μl DMSO (12 μCi/animal) 14–17 days after implantation. Duplicate sets of animals were euthanized at 1, 3, and 6 h by pentobarbitol overdose (100 mg/kg, i.p.). Tissues were excised, rinsed with PBS, and freeze dried. Tissue samples were weighed and then solubilized in 1 M NaOH for 18–60 h with shaking, neutralized with an equal volume of 1 M HCl, extracted overnight in 12% methanol/water in a final volume of 1.6 ml with shaking, and centrifuged to clarify the supernatant. One milliliter of supernatant was combined with 15 ml Ecolite, and radioactivity was measured using a scintillation counter.

Toxicity of D-CDU in Rats. Pilot studies in rats demonstrated that single doses of D-CDU up to 150 mg/kg i.p. were well-tolerated with no abnormal behavior or loss in weight. Animals were monitored for 10 days posttreatment.

Treatment with D-CDU and Neutrons. 9L rat gliomas (10,000 cells) were stereotactically implanted in the right frontal lobe of Fischer 344 rats and grown for 14 days (13). Four groups with intracranial 9L tumors were treated as follows: Group 1 (n = 6) was untreated; Group 2 (n = 6) received neutron irradiation only; and Groups 3 (n = 10) and 4 (n = 9) received a single i.p. dose of 30 mg/kg and 150 mg/kg of D-CDU (20% 10B enriched), respectively, 2 h before neutron therapy in the Medical Reactor, Brookhaven National Laboratory (Upton, NY).

The collimator used for the neutron irradiation of brain tumors consisted of Li2O3 dispersed in polyethylene, 93% enriched in 6Li, 7.5% total Li by weight, 10.2 cm thick, with a conical aperture 12 cm in diameter at the reactor side tapering to
2 cm diameter at the rat irradiation point. The irradiation geometry and platform to hold the rat have been reported (14). At 1 MW reactor power, the entrance thermal neutron flux was \(7.17 \times 10^8\) cm\(^{-2}\) s\(^{-1}\). Assuming that the tumor extended from 3 mm to 7 mm beneath the skin surface, the geometric mean of the thermal neutron fluence rate in the tumor volume was \(5.1 \times 10^9\) cm\(^{-2}\) s\(^{-1}\), and the minimum fluence rate was \(4.4 \times 10^9\) cm\(^{-2}\) s\(^{-1}\).

At 3 MW reactor power, the mean dose rates to the tumor volume were: boron capture, 7.95 cGy/min/\(10^9\) B; fast neutrons, 27.0 cGy/min; total gamma, 13.8 cGy/min; and nitrogen capture, 18.3 cGy/min. All neutron irradiations were for 24 MW-min (3 MW reactor power was for 8 min).

**Measurement of Organ Boron Concentrations of D-CDU by DCP-AES.** A subset of rats treated i.p. with 30 and 150 mg/kg D-CDU (\(n = 3\)/group) were sacrificed at 2 h to measure levels in the tumor, normal brain, and blood using boron atomic emission spectroscopy as previously described (15). DCP-AES can measure natural-abundance boron at the level of \(\sim 0.1\) mg boron/ml of tissue digestate (15).

**Survival Analysis.** The number of animals surviving each day was determined over a period of 98 days. Kaplan-Meier survival curves were plotted and odds ratios were compared using a statistical package (The PHREG Procedure, Version 6, Cary, NC: SAS Institute, Inc., 1996).

**RESULTS**

**Cellular Accumulation and Egress of D-CDU.** Accumulation profiles were similar in both human U-251 and rat 9L glioma cells and reached pseudo-steady state within 10 min and 15 min for U-251 and 9L cells, respectively. Plateau levels after a 1-h incubation in U-251 and 9L cells were (mean \(\pm\) SD) 35 \(\pm\) 8.6 and 18.3 \(\pm\) 0.2 mg boron/10\(^9\) cells, respectively. These levels correspond to cellular concentrations equal to 9,200 and 11,700 mg/ml boron based upon measured volumes of 3,040 and 1,619 \(\mu\)m\(^3\) for U-251 and 9L cells, respectively.

Egress rates after a 2.5-h incubation with 1 \(\mu\)M compound were biphasic in both cell lines with about 40% of the compound egressing from the cells within 10 min (Fig. 2). However, 9L and U-251 cells retained 6% and 5% of compound for more than 24 h, respectively. HPLC analysis of cell extracts indicated the presence of a less lipophilic metabolite with a similar retention time to that of D-CDU-5'-monophosphate in 9L cells (0.2% of total intracellular compound) and in U-251 cells incubated with 1 \(\mu\)M D-CDU for 24 h. This metabolite was susceptible to digestion with alkaline phosphatase to produce D-CDU, as characterized by its HPLC retention time and UV absorbance.

**Concentrations of \(^{14}\)C-D-CDU in Organs and Fluids.** Duplicate sets of rats were implanted with 9L rat glioma cells and were treated with 5 mg/kg \(^{14}\)C-labeled D-CDU. Animals were sacrificed using 100 mg/kg i.p. doses of pentobarbitol. The serum concentration was 0.43 nmol/ml (equivalent to 0.043 mg/g boron) at 3 h. The concentration of D-CDU in 9L tumors 3 h after administration was 13-fold higher than in nontumor brain tissue and 2-fold higher based on tissue wet weight than in serum, indicating a favorable therapeutic ratio for D-CDU for treating brain tumors (Fig. 3). This corresponds to an average tumor concentration of 0.1 mg of boron/g tumor (wet weight) 3 h after administration. The average tumor wet weight was 25 \(\pm\) 19 mg (range). The maximum lengths of tumors were 0.5 \(\pm\) 0.2 cm at the time of removal. High levels of D-CDU were found initially in the liver, kidney, stomach, spleen, lung, heart, and adrenals. As expected, D-CDU levels declined with time in all fluids and organs examined. However, 51% of the 1-h concentration remained in the tumor 6 h after dosing compared with 26% in nontumor brain and 6.4% in the serum. Corresponding half-lives of decline were 5.1, 2.6, and 1.3 h, respectively. Based
upon this initial distribution study with 5 mg/kg, doses of 30 and 150 mg/kg were selected for remaining experiments to target approximate tumor boron concentrations of 0.6 and 3.0 mg/g.

**Organ Concentrations of D-CDU by DCP-AES in Rats Receiving Treatment Doses of D-CDU.** Concentrations of boron in tumor tissues 2 h after dosing were 2.3 ± 1.6 and 7.4 ± 1.3 µg boron/g (mean ± SD), and tumor/brain ratios were 11.5 ± 3.6, and 6.8 ± 2.0 µg boron/g for Groups 3 (30 mg/kg) and 4 (150 mg/kg), respectively (Table 1). These concentrations agree with levels predicted from the 5 mg/kg 14C-D-CDU experiment and the previously published terminal t₁/₂ of 1.6 h in rats (11).

**Survival of Rats Treated with D-CDU BNCT.** The median survival time for animals receiving no treatment was 20 days with all animals dead within 28 days (Group 1). The median survival times were 32, 55, and 38 days for animals receiving neutrons only (Group 2), 30 mg/kg D-CDU plus neutrons (Group 3), and 150 mg/kg plus neutrons (Group 4), respectively (Fig. 4). One-third of the rats treated with D-CDU (30 mg/kg) survived significantly longer than those treated with neutrons only (P = 0.036). The delay for the higher D-CDU dose was not significantly different from that of the radiation-treated group.

**DISCUSSION**

The accumulation and efflux of D-CDU were similar in human U-251 and rat 9L glioma cells. This suggests that rats bearing intracranial 9L gliomas may be suitable animal models for BNCT with D-CDU for malignant gliomas. 9L cells incubated in the presence of 1 µM D-CDU accumulated ~0.2 µmol/10⁷ cells (equivalent to 20 µg boron/10⁷ cells) within 10

**Table 1** Tissue levels of boron in rats bearing 9L tumors intracranially at 2 h after i.p. dosing of D-CDU and median survival time after neutron irradiation

<table>
<thead>
<tr>
<th>D-CDU</th>
<th>µg boron/g of tumor (mean ± SD)²</th>
<th>µg boron/g of brain (mean ± SD)²</th>
<th>µg boron/g of blood (mean ± SD)²</th>
<th>Median survival time (days)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Neutrons only</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>30 mg/kg + neutrons</td>
<td>2.3 ± 1.6</td>
<td>0.17 ± 0.12</td>
<td>1.70 ± 0.70</td>
<td>55²</td>
</tr>
<tr>
<td>150 mg/kg + neutrons</td>
<td>7.43 ± 1.31</td>
<td>1.13 ± 0.23</td>
<td>6.67 ± 0.65</td>
<td>35</td>
</tr>
</tbody>
</table>

² Boron levels were determined by DCP-AES in rats (three per group) that did not receive neutrons.

³ Rats were exposed to neutrons 2 h after D-CDU administration (six rats per group).

² Significantly different (P = 0.036) than for rats receiving neutrons only.
The plasma concentration of [3H]D-CDU for rats treated 2 h after D-CDU. The 10B in D-CDU was natural (20%). Neutrons were administered; min, corresponding to a cellular concentration ~100 times higher than the extracellular concentration. Approximately 5% of the accumulated compound did not egress easily from the cells. This fraction is important because it corresponds to a cellular concentration that is 5 times greater than the original extracellular concentration, and it may contribute to a therapeutic advantage. Furthermore, some compound persisted in cells for more than 24 h. We have previously shown that D-CDU accumulates in cells via a process that is sensitive to the nucleoside-base uptake inhibitor papaverine but not to the nucleoside uptake inhibitors S-(4-nitrobenzyl)-6-thioinosine or dipiridamol. The precise mechanism for persistence of compound in cells is not fully understood because <0.3% of the compound is phosphorylated in U-251 cells, but it could involve complexation of the lipophilic carbonyl group with intracellular membranes (10).

The nontoxic properties of D-CDU in the absence of neutrons together with a relatively short terminal half-life may be advantageous because BNCT is a binary process in which cell toxicity is only desired in target tissues when both boron and neutrons are present. Therefore, neutrons may be administered when the ratio between tumor and surrounding brain tissue D-CDU concentrations are maximal. Previous studies in rats had also indicated that elimination from plasma was biexponential, becoming monoexponential between 2 and 3 h. The onset of the terminal monoexponential decline phase is indicative of the time taken for the compound to reach pseudo-equilibrium between plasma and the majority of peripheral tissues, and therefore, 2 h after D-CDU dosing was selected as the time of irradiation with neutrons. The terminal t1/2 value in plasma was 1.26 ± 0.28 h (11). The plasma concentration of [3H]D-CDU for rats treated with 5 mg/kg i.p. was 0.43 pmol/ml at 3 h, which agrees with a 0.81 pmol/ml prediction from the previous pharmacokinetic study with i.v. dosing, assuming linear pharmacokinetics (11).

This result suggests that differences in tissue concentrations are not substantial when D-CDU is dosed i.p. rather than i.v. once tissue equilibration has occurred. On the basis of an average tumor concentration of 0.1 μg boron/g tumor (wt weight) observed 3 h after an i.p. injection of 5 mg/kg 14C-D-CDU and linear extrapolation, 30 mg/kg and 150 mg/kg doses of D-CDU should result in tumor concentrations of about 0.6 and 3.0 μg total boron/g tumor, respectively. The concentrations of D-CDU achieved in the 9L tumors 2 h after i.p. injection were 2.3 ± 1.6 and 7.4 ± 1.3 μg total boron/g for 30 and 150 mg/kg of D-CDU, respectively, based on atomic emission of boron. Considering the expected heterogeneity in blood-brain-barrier disruption and tumor perfusion expected among brain tumors, the concentrations determined using scintillation counting of 14C-labeled compound and DCP-AES were similar (16, 17). Agreement in D-CDU predictions between experiments using the 14C-labeled compound and those using boron measurements are not surprising because the 14C is placed on the base of D-CDU heterocycle using a chemically stable covalent linkage to the carbonyl moiety.

The ratios of D-CDU in tumor relative to nontumor brain tissue were 11.5 ± 3.6 (mean ± SD) and 6.8 ± 2.0 for the low and high dose, respectively. This indicates that, although accumulation into tumor increases at higher doses, accumulation in nontumor brain tissue may exceed the threshold for the onset of the nuclear reaction, thereby increasing the amounts of normal tissue destroyed in the process.

Survival odds ratios differed between the untreated group and all treatment groups, including the group treated with neutrons only (P < 0.002; logrank test). Therefore, certain animals received some benefit from treatment with neutrons in the absence of boron administration, probably as a result of the gamma or fast neutron components of the neutron beam. Although the possible effects of the immune system on the 9L tumors cannot be discounted (12), both groups receiving D-CDU in combination with neutrons survived longer than the group that received neutrons only, with the group that received 30 mg/kg D-CDU surviving significantly longer than the neutron-only group (P = 0.036).

Existing dosimetry studies assume a uniform distribution of boron across the cell and predict that the total amount of 10B achieved in tumors in this pilot study (0.41 and 1.48 μg/g tumor for the 30 and 150 mg/kg/day doses, respectively) would be too low to have a therapeutic effect (<5 to 30 μg/g) (18). However, D-CDU is a complex delivery agent. D-CDU is a derivative of the nucleoside β-D-2'-deoxyuridine and is monophosphorylated, producing an anionic metabolite in cells. Furthermore, the carbonyl moiety may be reduced to the anionic nido form. Therefore, it is possible that the subcellular distribution of these charged D-CDU metabolites is not uniform. Subcellular localization or complexation with cellular organelles and/or macromolecules could produce localized subcellular microenvironments in which boron concentrations are higher than average.

The delay in mortality from the highest D-CDU dose group (150 mg/kg) was not significantly greater than that of the neutron-treated group. This may be a result of increased accumulation in nontumor brain tissue at the higher dose because if sufficient boron accumulates in the surrounding brain tissue, neutron irradiation would compromise both tumor and brain.
tissue. An optimal dose should produce a maximal boron concentration in tumor and maintain selectivity of D-CDU for tumor relative to brain. For 20% $^{10}$B-enriched D-CDU, this dose lies below 150 mg/kg under the current experimental conditions. However, it is anticipated that a 100% enriched product would deliver a 5-fold higher tumor level of $^{10}$B for each molecule of D-CDU accumulated. Provided that the decreased survival of rats receiving the higher dose results from a decreased specificity for brain tumor cells, a 100% enriched product would be beneficial. D-CDU then could be given at a dose enabling sufficient $^{10}$B to accumulate in brain tumors and maintain a high brain tumor/normal brain $^{10}$B ratio, resulting in an increased therapeutic ratio for D-CDU. The initial studies reported here are encouraging enough to warrant the synthesis of $^{10}$B-enriched D-CDU.

In summary, these studies indicate that a nontoxic, effective, $\sigma$-carboranyl-containing nucleoside analogue can be designed for the treatment of gliomas. These pilot studies justify the additional effort and expense of synthesizing 100% $^{10}$B-enriched compound, which will enable further dosing strategies to be investigated for D-CDU using the rat glioma model. Furthermore, the use of D-CDU and related compounds should be evaluated for other malignancies including liver and prostate cancers and should be compared with standard compounds such as borocaptate and p-boronophenylalanine.

**REFERENCES**

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