Notes

Synthesis and Evaluation of a Boronated Nitroimidazole for Boron Neutron Capture Therapy

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We postulated that nitroimidazoles, previously used for radiosensitizing solid tumors, may be interesting templates as carriers of ^{10}B for boron neutron capture therapy. To test this hypothesis, we synthesized a ^{10}B-enriched nitroimidazole, 1-[2-[(undecahydro-*closo*-dodecaborato)thio]ethyl]-2-methyl-5-nitroimidazole (imidocaptate), by coupling the Cs salt of BSH (Cs_2-$^{10}B_{12}H_{11}SH$) with 1-(2-bromoethyl)-2-methyl-5-nitroimidazole followed by purification of the adduct. Imidocaptate was taken up by V-79 cells in culture and showed no inherent toxicity under euoxic conditions up to 1.05 mM (126 μg of ^{10}B/mL of culture medium). Imidocaptate showed a dose-dependent decrease in D_0 when the treated cells were irradiated with a thermal neutron beam. At the highest dose tested (126 μg of ^{10}B/mL of culture medium), the ratio of control to sample D_0 values was 2.6 for both linear quadratic and single-hit multitarget models. At 33 μg of ^{10}B/mL, imidocaptate showed a control/treated D_0 ratio (1.5) equal to that observed with the disulfide form of BSH at 28 μg of ^{10}B/mL. Compared to BSH and its disulfide, the reduced toxicity and equipotency of imidocaptate suggest that this agent may be useful for boron neutron capture therapy of cancer.

Introduction

Binary systems for the improvement of cancer radiotherapy require the simultaneous presence of two agents, a radiation source and a target atom. The advantage of a binary approach is in the differential dose that can be established between the tumor and its surrounding normal tissue, provided that the compound, which carries the target atom, is avidly taken up in tumor, yielding a high tumor to normal tissue ratio.

Boron neutron capture therapy (BNCT) is one approach which exploits this advantage.[1,2] In BNCT, ^{10}B is the target atom which interacts with thermal neutrons. The α-particle and Li ion from the $^{10}B(n,\alpha)^7Li$ reaction are the high LET radiations which result in lethal damage to the tumor cells. These have very short ranges (approximately 10 and 5 μm, respectively). Therefore, cellular damage is restricted just to those cells in which the reaction occurs.

With most tumor-seeking agents, drug delivery is dependent upon sufficient vascularization within the tumor itself. Yet the morphology of most tumors is associated with a dense necrotic and hypoxic core, due to limited vascularity. Thus, most anticancer agents cannot be effectively delivered into the necrotic region. This can be a major impediment to any treatment because some tumor cells in this hypoxic region drop out of the proliferative compartment. These G_o cells are highly radioresistant and thus capable of regrowth if the target atoms required for the binary system are not delivered to these sites.

In order to address these limitations, our laboratory has focused on preparation of boron-containing nitroimidazoles as carriers of ^{10}B for BNCT. Nitroimidazoles have been widely used as antimicrobial chemotherapeutics[3,4] and as radiosensitizers for photon therapy of hypoxic tumors.[5−7] Nitroimidazoles also have properties that make them attractive potential candidates as boron carriers, especially for hypoxic solid tumors. First, nitroimidazoles readily penetrate tumors and can produce blood and intratumor concentrations approaching 1 mM.[8] Second, nitroimidazoles can undergo nitroreduction under hypoxic conditions to yield electrophilic substances which can damage protein and nucleic acids[9,10] and possibly enhance subsequent BNCT events. Finally, the metabolism and toxicology of nitroimidazoles, particularly metronidazole, has been characterized.[11,12] The addition of a carboranyl cage to the nitroimidazole structure will likely change the absorption, distribution, metabolism, and excretion of the complex from what would be observed in the parent nitroimidazole. However, knowledge of the behavior of the parent compound is instructive for estimating the behavior of the adduct. Similar rationales likely underly the design and synthesis of previously reported boron-containing hypoxia-affinic radiosensitizers as carriers of ^{10}B for neutron capture.[13−15]

BNCT could be extended from a binary treatment protocol to a ternary protocol by application of hyperthermia between drug administration and irradiation. Hyperthermia can selectively collapse the neovascular-

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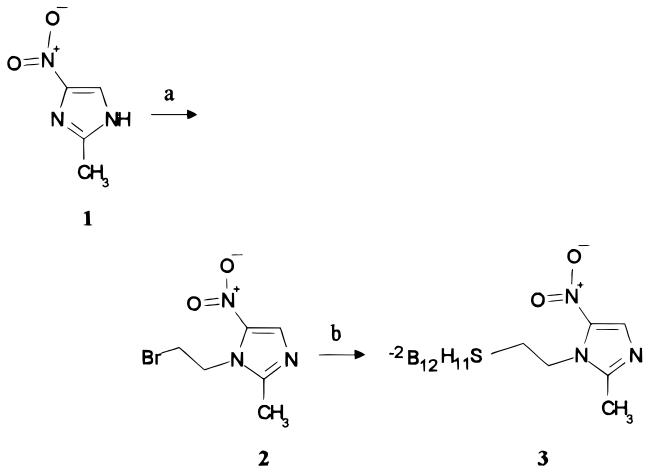
Scheme 1[a]



[a] (a) $BrCH_2CH_2Br$, dimethylformamide, reflux 4 h; (b) BSH, aqueous dimethylformamide, Na_2CO_3, 50 °C, 7 h.

ization of a tumor, trapping the nitroimidazole by bioreduction in the tumor[16] in a process that may be causative of the known synergy of nitroimidazoles on hyperthermia. Subsequent clearance of unbound drug from surrounding tissues could provide a relatively ^{10}B-free environment outside the target tumor.

The use of boronated nitroimidazoles for BNCT has been previously described in reports from other laboratories[13−15] and in a preliminary report from our group.[17] The compound described in this report is representative of agents which can be prepared by simple coupling reactions to yield a large series of agents for experimental BNCT.

Results and Discussion

Synthesis of Imidocaptate (3). The initial experimental design called for a metronidazole (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) derivative prepared by esterifying metronidazole with bromoacetyl bromide followed by coupling the bromoacyl function with Cs_2-$10B_{12}H_{11}SH$ (BSH, borocaptate).[1,2] The resulting boronated acylmetronidazole was found to have an unstable ester linkage at pH 6.0 ($t_{1/2}$ *ca.* 15 h at 37 °C; data not shown). Consequently, we decided to prepare **3**, which had a more stable thioether linkage by a procedure outlined in Scheme 1 (production of 4-isomer not shown). Direct reaction of 1,2-dibromoethane with 2-methyl-5-nitroimidazole (**1**) in refluxing dimethylformamide/1,2-dibromoethane led to the production of **2** in relatively poor yield. The crude reaction contained starting material and 1-(2-bromoethyl)-2-methyl-5-nitroimidazole as the major product; the latter was about 2.5 times the concentration of **2** in the reaction and was characterized by EIMS and by its λ_{max} (methanol) of 298 nm, *cf.* 309 nm for **2** (data not shown). Extended refluxing consumed more of the 2-methyl-5-nitroimidazole starting material, but yields of the two isomers leveled off and significant darkening of the reaction mixture occurred.

Coupling of **2** with BSH in 50% aqueous dimethylformamide (Scheme 1) containing Na_2CO_3 proceeded smoothly with consumption of both reactants as shown by analytical HPLC. Na_2CO_3 (pK_a = 10.3) serves to partially deprotonate the sulfhydryl (pK_a, 13.4),[18] thereby enhancing the reaction rate. Na_2CO_3 also inhibits

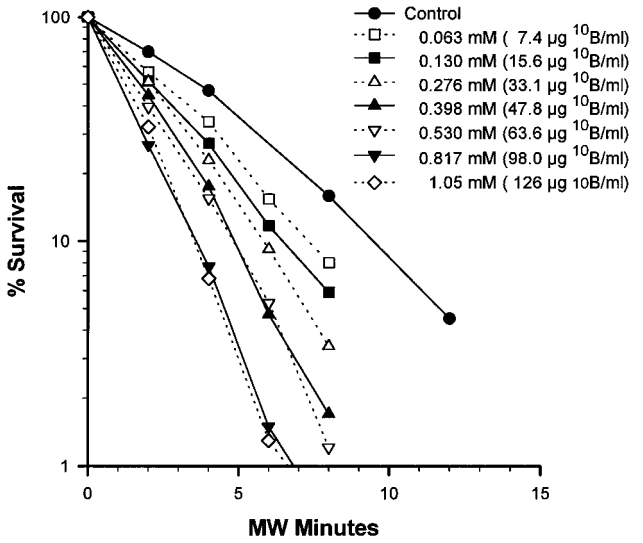


Figure 1. Dose−response for imidocaptate as a carrier of ^{10}B for *in vitro* BNCT assay. Cultured V-79 cells were incubated with imidocaptate (sodium salt, 57% ^{10}B by weight) as described in the Experimental Section. The medium was replaced, and the cells were irradiated with a neutron beam. Survivals were determined by replating assay as described in the text.

formation of a sulfur-centered radical in the disulfide product of BSH,[19] the consequences of which radical are not known for this reaction. The coupling of **2** with BSH proceeded in a fashion consistent with sulfhydryl character of the latter, in contrast to the amine-like reactivity suggested by Gabel *et al.*[18] It should be noted however, that when Gabel and his co-workers carried out alkylations of BSH under relatively mild conditions, reasonable yields of monoalkylated BSH were obtained.[18]

While purification can be accomplished by recrystallization (not shown), a more effective method is preparative HPLC on a C18 matrix using a triethylammonium acetate buffer, followed by lyophilization to yield the bistriethylammonium salt of **3**. NMR, electrospray MS, and elemental analysis were carried out on the bis(triethylammonium) salt of **3**. Elemental analysis was satisfactory, and the electrospray MS showed molecular ions consisting of **3** complexed with two triethylammonium ions. The NMR spectrum was dominated by the triethylammonium protons which compromised analysis of the B−H signals. Accordingly, the triethylammonium salt **3** was converted to the sodium salt by passing through a Dowex 50 (Na^+) column. None of the B−H protons was exchanged in D_2O, and the ^{10}B-decoupled spectrum, the broad 11-proton multiplet at 0.4−1.4 ppm collapsed to two 5-proton singlets and a 1-proton singlet as expected for the monosubstituted cage. Compound **3** was stable when stored under atmospheric conditions on the laboratory shelf for 4 months as evidenced by electrospray mass spectrometry.

BNCT Studies. Figure 1 is a cell survival curve showing the biological efficacy of **3** after incubation with different concentrations of the sodium salt of the drug, followed by neutron irradiation. Greater lethality was observed at the higher concentrations, where more ^{10}B was made available to the cells. Even at the higher levels of boron uptake, no toxicity from the drug was observed in the absence of neutron irradiation. This is indicated by the plating efficiencies shown in Table 1.

Table 1. Cell Survival Statistics from LQ and SHMT Models Relative to [^{10}B] for Imidocaptate, H_3BO_3, and BSSB

imidocaptate (disodium salt) mM (μg of ^{10}B/mL)	MW min at 10% survival	ratio at 10% survival (control/sample)	D_0 (MW min)	ratio of D_0 (control/sample)	% plating efficiency
vehicle control	9.2 ± 0.11		3.39		65
0.063 (7.4)	7.3 ± 0.19	1.3 ± 0.04	2.9 ± 0.19	1.1 ± 0.08	66
0.130 (15.6)	6.6 ± 0.16	1.4 ± 0.04	2.7 ± 0.12	1.2 ± 0.07	71
0.276 (33.1)	5.8 ± 0.03	1.6 ± 0.02	2.1 ± 0.07	1.5 ± 0.07	69
0.398 (47.8)	5.0 ± 0.19	1.9 ± 0.07	1.8 ± 0.09	1.9 ± 0.11	78
0.530 (63.6)	4.8 ± 0.20	1.9 ± 0.09	1.7 ± 0.17	1.9 ± 0.20	72
0.817 (98.0)	3.5 ± 0.10	2.6 ± 0.07	1.4 ± 0.07	2.4 ± 0.15	96
1.05 (126)	3.6 ± 0.25	2.6 ± 0.18	1.3 ± 0.04	2.6 ± 0.12	73
H_3BO_3 (27 μg of ^{10}B/mL)	1.7 ± 0.05	5.5 ± 0.18	0.6 ± 0.06	5.2 ± 0.51	27
BSSB (28 μg of ^{10}B/mL)	6.1 ± 0.40	1.5 ± 0.10	2.1 ± 0.16	1.5 ± 0.12	28

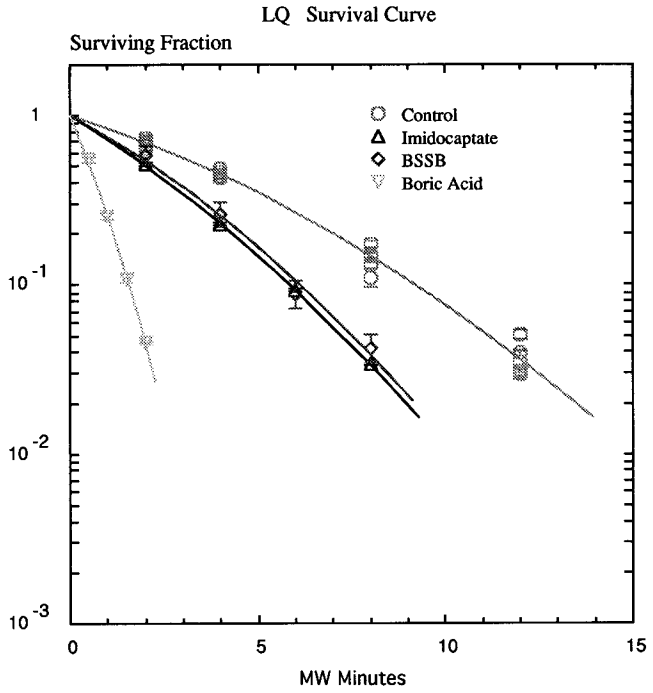


Figure 2. Comparison of the uptake of imidocaptate with BSSB and borate in V-79 cells. Imidocaptate at 33 μg of ^{10}B/mL, BSSB at 28 μg of ^{10}B/mL, and borate at 27 μg of ^{10}B/mL were incubated with V-79 cells in culture as described in the text. The medium for containing imidocaptate and BSSB was replaced, and the cells were washed, while the medium containing ^{10}B borate was not replaced to provide a reference for even distribution of ^{10}B. Cells were irradiated and survivals were determined by replating assays.

Also shown in Table 1 is a comparison of the irradiations times (MW min) required to reduce cell survival to 10%. For reactor irradiations, MW min, dose, and thermal neutron fluence are proportional. At the highest concentrations of drug, the irradiation time required to achieve 10% survival was reduced by a factor >2.5. The similarity in the response between the 98 and 126 μg of ^{10}B/mL concentrations suggests that saturation might have been reached at these levels, although one cannot from these data rule out a statistical anomaly. The slope of the linear portion of the curve is defined by the value D_o, which is the irradiation time required to reduce survival by a factor of l/e. Steeper slopes are also observed for the higher concentrations of drug (Table 1).

Cell survival curves showing **3** in comparison with the disulfide, BSSB, can be seen in Figure 2. BSSB is the dimer of BSH, a boron compound currently being used in clinical BNCT trials in Japan, which is also under consideration for clinical trials in Europe.[2] Intratumor uptake of BSSB was shown to be greater than that for BSH.[20,21] Cells with **3** responded similarly to those with BSSB at approximately the same ^{10}B concentrations. However, BSSB toxicity increases at higher drug concentrations (personal communication, D. D. Joel), whereas the same is not true for **3**. Thus, further studies with **3** are warranted to find toxic limits.

The response of the cells to boric acid is also included in Figure 2. Boric acid is assumed to distribute the boron atoms uniformly throughout the cell; that is, nucleus, cytoplasm, and external environment.[22] Cells treated with BSSB or **3** were washed thoroughly to remove free extracellular drug and were irradiated in boron-free medium, but the boric acid samples were continuously exposed to boron and irradiated in boron-containing medium. The data in Table 1 show neutron capture-mediated toxicity for boric acid that has not been washed from the cells. The BSSB- and **3**-treated cells demonstrated significant toxicity in response to neutron irradiation; thus, uptake of both BSSB and **3** was predominantly intracellular. Statistical values for the curves in Figure 2 are shown in Table 1.

To verify that the effect from the **3** was not due to radiosensitization, and was strictly a function of the boron reaction, cells with and without **3** were irradiated with ^{137}Cs-γ rays.[22] Cells with **3** responded identically to the controls, as expected for these euoxic cells.

We have shown that **3** is capable of delivering boron atoms intracellularly under euoxic conditions and is not toxic to cells at concentrations up to 1.05 mM (126 μg of ^{10}B/mL). It is clear that similar experiments will have to be carried out under hypoxic conditions in order to assess the value of **3** for delivering boron to hypoxic cells.

Clearly, future work with this compound is necessary, given the high response in the *in vitro* BNCT assay relative to the clinical candidate, BSSB. *In vivo* studies will include optimization of the drug delivery system, route, and the appropriate dose to be administered. *In vitro* studies will be continued to evaluate the uptake of boron under hypoxic conditions.

Imidocaptate represents a class of compound with higher water solubility than the carborane series[13,14] and can be prepared by simple sulfhydryl coupling strategies[18] that will permit synthesis and evaluation of a large number of structural analogs with a requirement for only minimal synthetic effort.

Experimental Section

Melting points were determined on a Fisher-Johns apparatus and were uncorrected. EI mass spectra were obtained on a Finnigan 4000 mass spectrometer, and electrospray ionization mass spectra were obtained on a Finnigan MAT 900

instrument. ^{1}H NMR spectra were obtained on a Brüker AM500 instrument with multinuclear capability. UV spectra were obtained with Pye-Unicam SP1750 or Hitachi U2000 spectrophotometers. HPLC analysis was carried out with a Shimadzu HPLC system equipped with an SPD 10AV dual-wavelength photometer set for 210 nm (to detect the BSH component) and at 309 nm (to detect nitroimidazole component). Analytical HPLC was carried out with a Millipore 8 × 10 RCM C18 cartridge eluted at 4 mL/min with 30% methanol in water containing 50 mM triethylammonium acetate, pH 6. Semipreparative HPLC was carried out with a 25 × 10 RCM C18 cartridge eluted with the same solvent at 4 mL/min. Elemental analysis was performed by M-H-W Laboratories, Phoenix, AZ, and were ±0.4% of the calculated composition.

Caution: *1,2-Dibromoethane is a potential carcinogen and mutagen and should be handled under appropriate containment. The toxicities of the other compounds to humans is unknown.* 1-(2-Bromoethyl)-2-methyl-5-nitroimidazole (2). 2-Methyl-5-nitroimidazole (5 g, 0.04 mol; Aldrich Chemical Co.) was dissolved in 100 mL of *N*,*N*-dimethylformamide and refluxed with 1,2-dibromoethane (100 mL, 1.3 mol; Aldrich) for 4 h. After removal of solvent by rotary evaporation, the crude mixture was fractionated on a 4- × 50-cm column of Si60 eluted with 5% methanol in dichloromethane. The band eluting first from the column was primarily **2** with some contamination by the major product, the 4-nitro isomer. The solvent was removed by evaporation, and the crude **2** was dissolved in 50% methanol (20 mL). A 5-mL aliquot was purified by rechromatography on C-18 with 40% methanol. Evaporation of solvent and refrigeration of the residual oil gave pure **2** as yellow crystals (1.4 mmol, 14%): analytical HPLC showed 97.5% purity; UV (methanol) $\lambda_{max} = 309$ nm, $\epsilon = 8900$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.48 (s, 3 H), δ 3.83 (t, $J = 5$ Hz, 2 H), δ 4.70 (t, $J = 5$ Hz, 2 H), δ 8.05 (s, 1 H); EIMS *m*/*e* (relative intensity) 233, 235 (M^+, 88, 91), 187, 189 (100), 154 (38), 107, 109 (96, 94).

Bis(triethylammonium) 1-[2-[(undecahydro-*closo*-dodecaborato)thio]ethyl]-2-methyl-5-nitroimidazole (3). Compound **2** (117 mg, 0.5 mmol) was dissolved in 5 mL of argon-purged 50% aqueous dimethylformamide. $Cs_2{}^{10}B_{12}H_{11}SH$ (BSH, 237 mg, 0.55 mmol; Boron Biologicals, Inc., Raleigh, NC, >95% ^{10}B enriched) was dissolved in 5 mL of the same solvent, and the two solutions were mixed. Na_2CO_3 (70 mg) was added, and the reaction was stirred at 50 °C. The course of the reaction was followed by analytical HPLC with product **3** well separated from BSH and **2**, showing retention times of 3.36, 4.74, and 7.02 min, respectively. After 7 h, the reaction mixture was evaporated to dryness and resuspended in water (10 mL), and 6 mL was fractionated on the semipreparative column by sequential runs using 2-mL injections. Product **3** ($t_R = 28.6$ min) was well separated from BSH ($t_R = 38$ min) and **2** ($t_R = 50.5$ min). After lyophilization, pure **3** as the bis-(triethylammonium) salt was isolated as an amorphous yellow powder (69.9 mg, 43%): mp 128−130 °C dec; UV (H_2O) $\lambda_{max} = 320$ nm, $\epsilon = 5950$, (methanol) $\lambda_{max} = 311$ nm, $\epsilon = 6200$; ^{1} H NMR (as Na^+ salt from ion-exchange resin, 500 MHz, DMSO-d_6) δ 0.4−1.4 (m, 11 H), 2.54 (s, 3 H), 2.57 (t, $J = 6.6$ Hz, 2 H), 4.3 (t, $J = 6.6$ Hz, 2 H), 7.97 (s, 1 H), ^{10}B-decoupling collapsed the broad multiplet (0.4−1.4 ppm) to three singlets at 0.73 (1 H), 0.87 (5 H), and 1.08 (5 H), the NMR of the triethylammonium salt was dominated by the ethyl protons, which obscured the B−H protons, but was otherwise the same as that of the Na^+ salt; electrospray mass spectrometry *m*/*e* 522 (MH^+), 544 (MNa^+) showed retention of the triethylammonium counterions. Anal. ($^{10}B_{12}C_{18}H_{51}N_5O_2S$) C, H, N.

***In Vitro* BNCT Studies.** Details of the cell culture technique are described previously.[22] Briefly, V-79 Chinese hamster cells in exponential growth were incubated in DME complete medium with varying concentrations of **3** for ~16 h (concentrations shown in legend of Figure 1). After vigorously washing the cells three times with phosphate buffer saline to remove free extracellular drug, cells were trypsinized, harvested, and diluted to cell density of 3×10^5 cells/mL. One-milliliter cell suspensions were placed in 1.5 mL Eppendorf microfuge tubes for irradiation over a range of doses at the Brookhaven Medical Research Reactor. The irradiation procedure and apparatus are described elsewhere.[23] Microfuge tubes were positioned in a lucite rotator and irradiated for different times with thermal neutrons from the BMRR at a reactor power of 1 Megawatt (MW). Since the length of time of neutron exposure and the thermal neutron fluence are both proportional to dose, cell survival curves are presented in megawatt minutes (reactor power of 1 MW × 1 min neutron exposure = 1 MW min) to simplify the complexities associated with determining the "effective dose" from a mixed radiation field.[24] Experiments were carried out under euoxic conditions. Cells were plated for clonogenic determination after irradiation and permitted undisturbed growth for 5 days prior to counting colonies.[22] Measurements of boron uptake in cells were carried out using the prompt γ facility of the BMRR as described elsewhere.[25] Curves fit the linear-quadratic (LQ) and single-hit multitarget (SHMT) models equally well. Software for analysis has previously been described.[26]

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