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Evaluation of the Potential of 2-Amino-3-(1,7-dicarba-*closo*-dodecaboranyl-1-thio)propanoic Acid as a Boron Neutron Capture Therapy Agent

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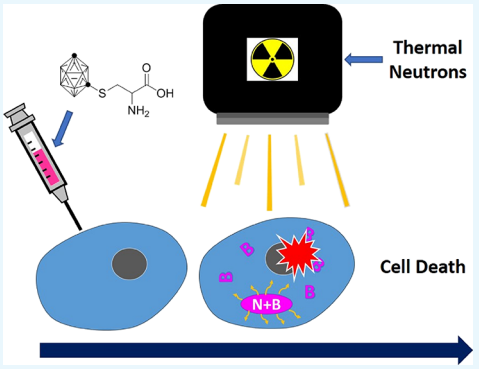
Supporting Information

ABSTRACT: A novel boron-rich α-amino acid (**3**) that serves as a boron delivery agent for boron neutron capture therapy (BNCT) has been designed and synthesized by substituting the side chain of cysteine with *m*-carborane. The uptake of this compound into neuronal U87 cells was determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) and showed intracellular concentrations of elemental boron at the picogram/cell level. To assess the cell-killing effect of **3**, U87 cells were incubated with varying concentrations of **3** and 1 mM of the known BNCT agent 4-borono-L-phenylalanine (BPA) for comparison. Cells were subsequently exposed to radiation with thermal neutrons at fluences varying from 1×10^8 to 2×10^9 neutrons/cm^2. Prior to neutron beam exposure, no cytotoxic effect was observed for BPA-treated cells, while a modest cytotoxic effect was observed for cells incubated with concentrations of **3** varying from 1 μM to 1 mM (resulting in cell viability reductions from 2.1% to 12.5%, respectively, relative to the control). An enhanced cell-killing effect (with a cell viability reduction of up to 47.8% relative to the control) was observed when **3**-treated cells were irradiated with thermal neutrons. This was attributed to the impact of α particle formation from **3** in response to neutron beam exposure. Lower concentrations of **3** exhibited a superior cytotoxic effect relative to BPA and at reduced levels of neutron fluences when compared to that used in conventional treatment. This work suggests the potential for a novel "chemo-radiotherapy" approach to the treatment of cancer by BNCT, whereby a 1000-fold lower neutron radiation fluence compared to typical BNCT can be used.



INTRODUCTION

Boron neutron capture therapy (BNCT) was first suggested in 1951 by Sweet from Massachusetts General Hospital as a treatment for malignant brain tumors, particularly glioblastoma multiforme (GBM).[1] It is a noninvasive cancer treatment approach that involves the selective tissue accumulation of a ^{10}B-containing agent, followed by irradiation of the tissue with thermal neutrons. Capture of the neutrons by ^{10}B results in boron degradation with the production of lithium nuclei and high linear energy transfer of α particles which results in the emission of a lethal radiation dose. Because the trajectory of the resulting particles is 9−10 μm which approximates the diameter of a single cell,[2] radiation damage is limited to the cells which contain the boron delivery agent. Thus, by exploiting this method, the side effects of conventional ionization radiation therapy can in principle be avoided or greatly reduced.

One essential aspect of this therapeutic modality is the delivery of boron into the tumor cells. With specific regard to the development of compounds that can be used for BNCT, several potential boron delivery agents have been prepared including borax ($Na_2B_4O_7{\cdot}10H_2O$), pentaborate ($NaB_5O_8{\cdot}4H_2O$) which was used in early clinical studies in the 1950's,[3−5] and aromatic boronic acids.[6] Shortly thereafter, Hawthorne et al. demonstrated the remarkable chemical stability of polyhedral borane anions ($B_{10}H_{10}^{2-}$ and $B_{12}H_{12}^{2-}$),[7,8] and attention was directed to incorporating these polyhedral clusters into a boron delivery agent because of their boron-rich nature. One proposed compound was sodium decahydrodecaborate ($Na_2B_{10}H_{10}$) which underwent evaluation in small animals.[9] More recently, "second generation" boron delivery agents have been evaluated including amino acids, nucleic acids, and liposomes.[10−14] Among these molecules, 4-borono-L-phenylalanine (BPA) and disodium mercaptoundecahydro-*closo*-decaborate ($Na_2B_{12}H_{11}SH$) are the only ones so far approved for human trials for the treatment of aggressive GBM.[15−20]

It has been proposed that a suitable boron delivery agent is one that meets all of the following requirements: (1) high tumor selectivity; (2) low systemic toxicity; (3) high concentration of boron in tissues (~20 μg ^{10}B/g tissue); (4)

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rapid blood and tissue clearance; and (5) persistence of the boron compound in the tumor tissue.[2] In initiating the design of such a boron delivery agent, we proposed that the installation of a boron-rich moiety into an architectural framework that mimics those common in nature might enhance the biocompatibility and cell-entry characteristics of boron-rich compounds. Furthermore, it would yield a molecule that potentially has low systemic toxicity. We previously synthesized 2-amino-3-(1,7-dicarba-*closo*-dodecaboranyl-1-thio)propanoic acid (**3**)[21] by incorporating the *m*-carborane ($C_2B_{10}H_{12}$) moiety into the amino acid cysteine (Figure 1). It

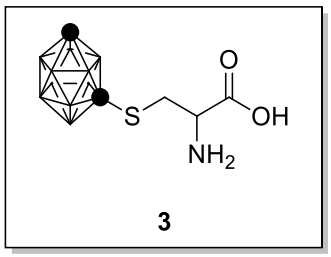

Figure 1. 2-Amino-3-(1,7-dicarba-*closo*-dodecaboranyl-1-thio)-propanoic acid (compound **3**).

was subsequently demonstrated that it crosses the membrane of neuronal U87 cells within 5 min of exposure and is retained within the cells 48 h post-treatment.[22] These features are important for a boron delivery agent because they enable the compound to respond to neutron radiation in a desired way (i.e., while contained within the cells), thus making it efficient in generating cell-killing α particles. We also demonstrated that aqueous solutions of this compound self-assemble into fibril constructs reminiscent of those observed in hydrogels.[21] This observation, which has heretofore not been reported for a BNCT agent, provides added advantages. First of all, the formation of these self-assembled architectures may be useful in facilitating the retention of the compound within the cell. In the event that these fibrils form within the tumor cells, the compound will be prevented from exiting the cell because of the size of the constructs. Second, in principle, it should be possible to install compound **3**-infused hydrogel patches in the vicinity of a tumor, followed by neutron beam exposure for a high-dose localized treatment. We have previously demonstrated the retention of boron in the glioblastoma cell line U87 by fluorescence microscopy.[22] At high doses, **3** exhibited cytostatic and cytotoxic effects, whereas at low concentrations, it resulted in little to no toxicity.[22] These results further suggested that a local high-dose treatment may yield a desirable cell-killing effect, while minimizing any negative impact on healthy tissues.

Of additional relevance was the revelation through transcriptomic analysis that compound **3** dramatically impacted U87 cells by promoting the upregulation of apoptosis-related genes, with simultaneous suppression of genes associated with cell proliferation.[22] This finding strongly implied the possibility of an enhanced cell-killing effect if neutron radiation was applied to cells after first having been exposed to compound **3**. While it has been estimated that a typical BNCT treatment requires neutron fluences to be $\sim 1 \times 10^{12}$ neutrons/cm^2,[14] the weakened state of cells after having been exposed to **3** might enable effective BNCT treatments to be accomplished with a lower neutron beam fluence, which would minimize the possible side effects on normal tissues.

In this work, we report on the evaluation of the cell-killing effects of **3** as a function of varying compound dosages and levels of neutron beam radiation. The effects of compound **3** were compared to those observed in analogous experiments using 4-borono-L-phenylalanine (BPA), a well-known compound developed as a BNCT agent.

RESULTS AND DISCUSSION

Quantification of Compound 3 Incorporation into U87 Cells. As previously shown, compound **3** seamlessly crosses the membrane of U87 cells within 5 min of exposure, and it remains within the cells after 48 h.[22] Because the impact of neutron beam exposure is directly correlated to the amount of ^{10}B within the cells, experiments were conducted to determine the amount of B that was taken up by the cells as a function of the concentrations of **3** to which they were exposed. It has been reported that the determination of B concentrations can be noninvasively estimated by positron emission tomography if an ^{18}F-labeled boron delivery agent is used.[23,24] Inductively coupled plasma−optical emission spectrometry (ICP−OES) has also been shown to enable accurate noninvasive quantification of elemental boron.[25,26] This proved to be a better option for estimation of B levels in this study, as compound **3** is not fluorinated. Thus, ICP−OES was used to determine the concentrations of **3** in U87 cells after they were treated with different concentrations of **3** for up to 1 h.

Because of the ubiquity of boron (primarily from borosilicate glass), great care was taken to try to avoid exposure to glass utensils. Therefore, the experiments were conducted using polystyrene Petri dishes and polyethylene Falcon tubes. Known numbers of cells were treated with compound **3** at concentrations of 1 μM, 100 μM, and 1 mM for 1 h. After removing the media containing the various concentrations of **3**, cell lysis was conducted to release boron from within the cells by acidification with 1% (v/v) nitric acid. The suspension was centrifuged to precipitate cellular debris, and the concentration of boron in the resulting supernatant was then measured by ICP−OES. The amount of **3** within

Table 1. Concentrations of Elemental Boron Incorporated into U87 Cells After a 1 h Exposure to Varying Concentrations of 3, as Determined by ICP−OES[a]

concentration of **3** to which cells were exposed	elemental boron concentration	calculated compound **3** concentration	elemental boron concentration per cell	calculated compound **3** concentration per cell
0 (no treatment)	6.01 ± 0.42 μM			
1 μM treatment	4.48 ± 0.13 μM	448 ± 13 nM	48.38 ± 1.4 pg	117.82 ± 3.42 pg
100 μM treatment	8.05 ± 0.08 μM	805 ± 8 nM	86.94 ± 0.86 pg	211.72 ± 2.10 pg
1 mM treatment	42.92 ± 0.43 μM	4.29 ± 0.04 μM	463.54 ± 4.64 pg	1.13 ± 0.01 ng

[a]From the amounts of elemental boron observed and the number of cells present, the intracellular concentration of **3** could be estimated.

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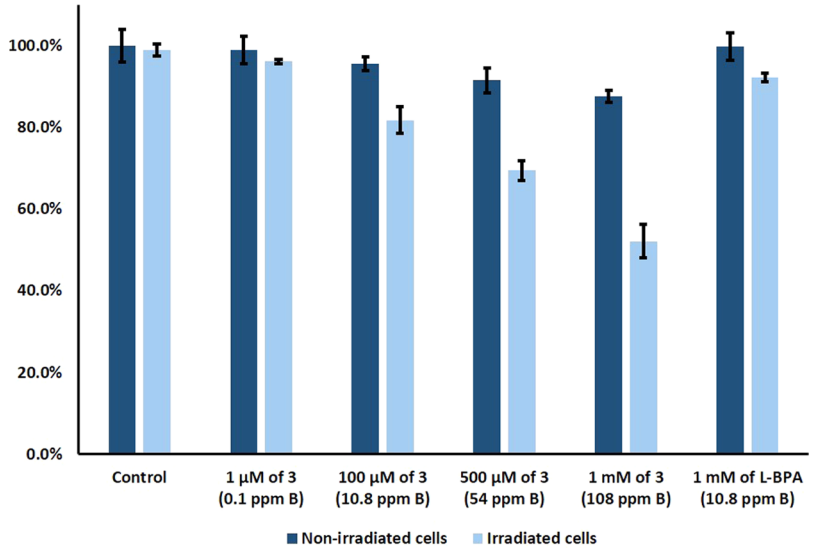

Figure 2. Viability profile of U87 cells incubated for 1 h with compound **3** at concentrations ranging from 1 μM to 1 mM, or with 1 mM BPA, followed by irradiation with thermal neutrons. For irradiated cells, viability was assessed 48 h post neutron beam exposure, and for the non-irradiated controls, 48 h after incubation with compound (i.e., **3** or BPA). Elemental boron levels in ppm are indicated in parentheses. Cells that were not exposed to neutron radiation were used as a control. The neutron fluence was 1×10^9 neutrons/cm^2.

each cell was estimated based on the number of cells, and the ratio of elemental B to the number of boron atoms in each molecule of 3 (i.e., 1:10). The results are presented in Table 1.

As indicated in Table 1, in the nontreated cells, the concentration of elemental boron was determined to be 6.01 ± 0.42 μM, which was of the same order of magnitude as that observed for the 1 μM treatment. This result likely reflects the high level of background B because of its ubiquity in the laboratory setting. However, the 100 μM and 1 mM treatments showed intracellular levels of B that were above background and which corresponded to intracellular concentrations of **3** at the picogram level per cell. These findings confirmed the results observed by fluorescence microscopy that **3** enters cells and that exposure to higher concentrations of **3** results in increased uptake.[22]

Cell Viability Determination after Thermal Neutron Radiation. With confirmation that **3** entered cells, it was anticipated that α particles produced in response to exposure of the cells to thermal neutrons would result in cell death, at a level commensurate with the amount of ^{10}B present within the cells and the neutron beam fluence. Thus, using the crystal violet assay, the viability of cells after exposure to thermal neutrons and as a function of varying concentrations of **3** was determined. Cells were first treated for 1 h with **3**, and following replacement of the media with media that did not contain **3**, were exposed to neutron radiation with a fluence of 1×10^9 neutrons/cm^2. BPA, an early generation BNCT agent to which cells were exposed at a concentration of 1 mM, was used for comparison. Nondrug-treated cells were used as a control. The results of these experiments, which were performed in triplicate, are illustrated in Figure 2.

In Figure 2, the results for the control indicated that neutron radiation in and of itself did not appear to have a cytotoxic effect because the cell viability was similarly high for both irradiated- and nonirradiated untreated cells. In the case of the 1 mM BPA-treated cells (which were exposed to BPA for 1 h), and in the absence of radiation, there was no effect at 48 h post BPA incubation, and there was only a 7.7% reduction in cell viability 48 h post neutron beam exposure. As the dose of **3** to which the cells were exposed was increased, there was a downward trend in cell viability (98.9% for 1 μM, 95.6% for 100 μM, 91.5% for 500 μM, and 87.5% for 1 mM) which was independent of neutron beam exposure. Within the experimental error, the results for the nonirradiated 1 mM BPA experiment were identical to those of the non-BPA non-irradiated control. For the compound **3**-treated nonirradiated cells, the downward trend in cell viability is consistent with the results reported previously,[22] showing a cytostatic effect after 24 h, followed by cell death at 48 h. It is also consistent with the microarray and transcriptome results shown in previous studies,[22] which indicated downregulation of cell cycle-related genes and upregulation of those associated with apoptosis.

A more dramatic negative effect on cell viability was observed with the application of the neutron beam, as indicated by the increasing reductions in cell counts as a function of increasing concentrations of **3** at a constant neutron beam fluence (96.1% for 1 μM, 81.7% for 100 μM, 69.4% for 500 μM, and 52.1% for 1 mM). These observations indicate that: (1) α particle exposure resulted in cell death and (2) cell death is, as expected, correlated with the levels of **3** within the cells. Comparing the results for **3** and BPA at the 1 mM concentration and while under the influence of neutron radiation showed **3** to be about twice as effective in promoting cell death. This is likely related in part to the fact that at comparable molar concentrations, **3** contains 10 times more boron than BPA. Thus, the results for the two compounds at 100 μM **3** and 1 mM BPA would be a more apt comparison because the amounts of B would be equivalent. Nevertheless, it should be noted that even though the elemental boron levels are the same for 1 mM of BPA and 100 μM of **3**, there was an enhanced cell-killing effect for compound **3** both with and without neutron radiation. We posit that this is a consequence of the impact of **3** (relative to BPA) on the transcriptome of U87 cells (i.e., upregulation of genes associated with apoptosis and downregulation of cell cycle genes). To compare the effects of the two compounds at the same boron level for 500 μM and 1 mM of **3** would require BPA concentration to be over 5 mM, which is not achievable in aqueous solution, and

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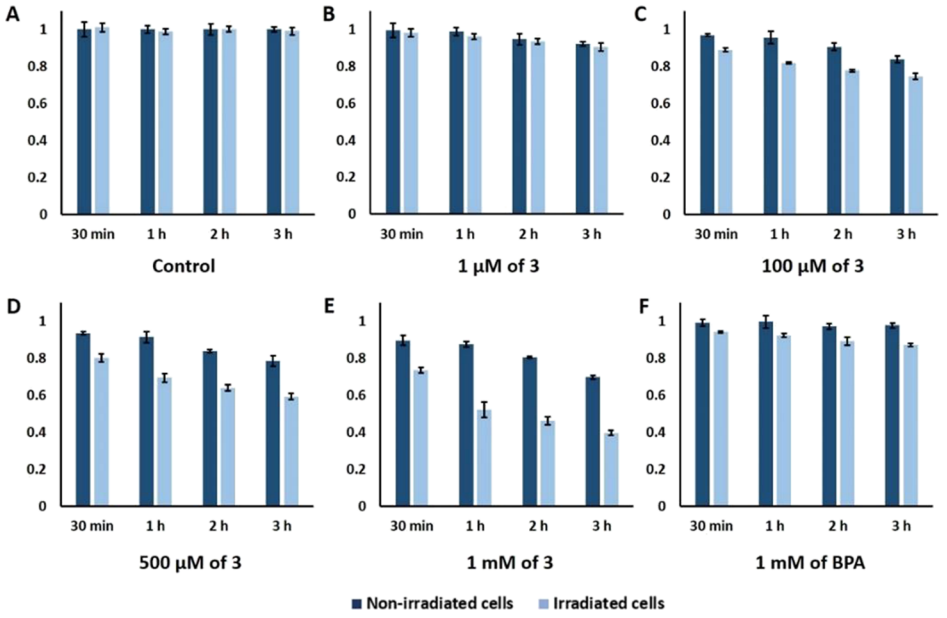

Figure 3. Viability profile of U87 cells treated with various concentrations of **3** and 1 mM BPA before and after irradiation with thermal neutrons, as a function of the amount of time during which cells were exposed to the indicated concentration of the compounds. Panel (A): control; Panel (B): cells treated with 1 μM of **3**; Panel (C) cells treated with 100 μM of **3**; Panel (D): cells treated with 500 μM of **3**; Panel (E): cells treated with 1 mM of **3**; and Panel (F) cells treated with 1 mM BPA.

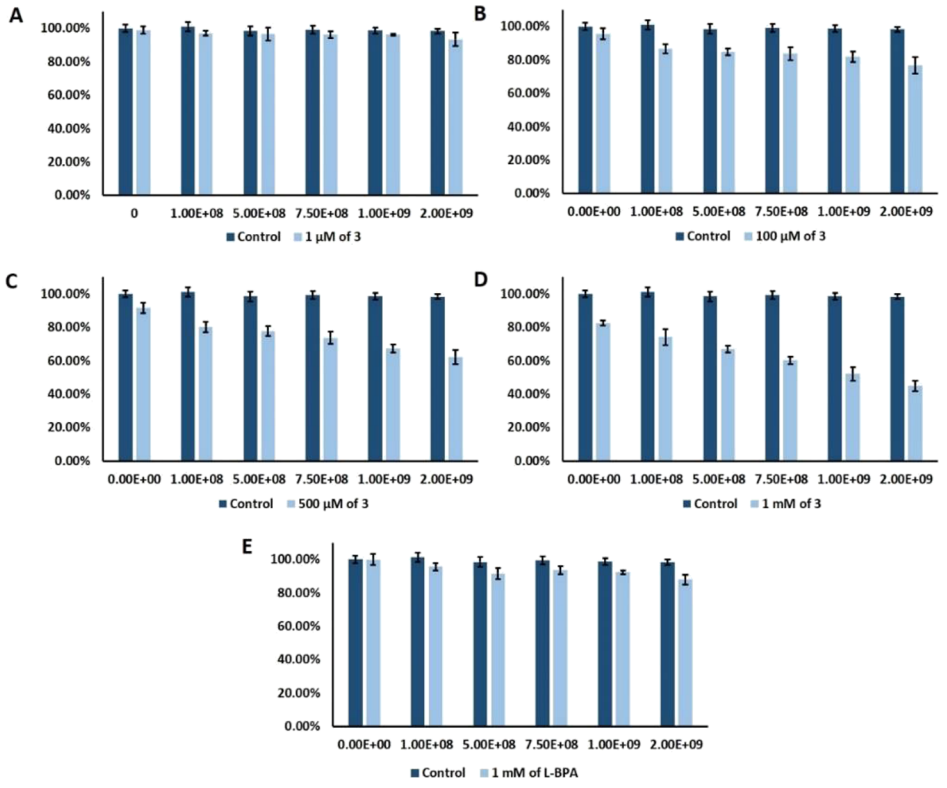

Figure 4. Viability of U87 cells irradiated with thermal neutrons, with non-irradiated cells serving as controls. The *x*-axis represents the fluence level in neutron/cm^2 and the *y*-axis represents cell viability. Panel (A): treatment with 1 μM of **3**; Panel (B): treatment with 100 μM of **3**; Panel (C): treatment with 500 μM of **3**; Panel (D): treatment with 1 mM of **3**; and Panel (E): treatment with 1 mM of BPA.

exceeds the typical concentrations ($\leq$2 mM) used in BNCT studies.

The impact of neutron beam irradiation on cell viability for **3**- and BPA-exposed U87 cells, as a function of the length of time the cells were exposed to the drugs, was then assessed. U87 cells were incubated with **3** or BPA for a duration ranging from 30 min to 3 h, and this was followed by exposure to a neutron beam with a fluence of 1 $\times$ 10^9 neutrons/cm^2. The results, which are presented in Figure 3, indicated that: (1) cell viability for nondrug-treated cells was unaffected by neutron beam irradiation (Panel A); and (2) the longer the cells were exposed to compound **3** and BPA, the lower were the cell

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viabilities for both irradiated and nonirradiated cells. For example, compared to a reduction of 26.4% in cell viability post-irradiation when cells were incubated with 1 mM of **3** for 30 min, viability reductions for 1, 2, and 3 h incubations with **3** at the same concentration were 47.9%, 53.7% and 60.3%, respectively (Panel E). For BPA, no obvious effect was observed in the absence of radiation, and the increase in the duration of the exposure of cells to BPA only resulted in very modest reductions in cell viability (i.e., 5.7%, 7.7%, 10.8%, and 12.7% for 30 min, 1, 2 and 3 h incubations with BPA, respectively—Panel F). The results align with the earlier finding that longer drug exposure times result in increased uptake. Greater amounts of intracellular drug would yield correspondingly higher levels of α particles on neutron beam irradiation, which in turn would result in increased cell death.

Cell Viability as a Function of Neutron Fluence. Thermal neutron radiation studies were also conducted to evaluate the effect of different levels of neutron fluence in order to optimize exposure. The results of the cell-killing effects of compound **3** and BPA at neutron beam fluence levels ranging from 1×10^8 to 2×10^9 neutrons/cm^2 are presented in Figure 4. As anticipated, increasing neutron fluences resulted in an increase in the cell-killing effect at all the concentrations of compound **3** that were tested. For example, the reduction in viability of cells treated with 1 mM of **3** (Panel D) increased from 26% at 1×10^8 neutrons/cm^2 to 55.5% at 2×10^9 neutrons/cm^2, relative to the control. A similar trend was observed for cells treated with 500, 100, and 1 μM of compound **3** (Panels C, B, and A, respectively), although in the last case, the reduction at the highest fluence was only 6.7%. On the other hand, cells treated with 1 mM of BPA (Panel E) only showed modest reductions in cell viability ranging from 4.4% to 12.5% with increasing neutron fluences from 1×10^8 to 2×10^9 neutrons/cm^2. Interestingly, the results demonstrated that cells treated with 1 μM of **3** exhibited an effect similar to that of 1 mM BPA-treated cells, indicating that compound **3** is more effective than BPA and can achieve desirable results at lower concentrations.

From the presented results, it was concluded that compound **3** exhibited a cell-killing effect on U87 cells even in the absence of thermal neutron radiation at concentrations as low as 1 μM, while BPA did not exhibit any toxic effect on U87 cells without the neutron radiation. This observation is likely because of the shutdown of cell cycle in response to compound **3** exposure.[22] This cytotoxicity was amplified with irradiation because of the generation of α particles from intracellular **3**. Furthermore, compared to BPA-exposed cells that were treated under identical conditions, **3** exhibited a greater than two-fold negative impact on cell viability when the neutron beam was applied. It would be of interest to determine whether similar effects would be observed with cell lines other than U87, and this is the subject of continuing studies in our laboratory.

The results from the ICP−OES experiments suggest that the concentrations of compound **3** incorporated into cells can be as low as the femtomolar level for cell death to be observed. The neutron fluences used in this study (with the highest fluence being 2×10^9 neutrons/cm^2) are significantly lower than previously reported neutron fluence levels for a typical BNCT treatment ($\sim 1 \times 10^{12}$ neutrons/cm^2).[14] Cell viability reductions were observed with a neutron fluence as low as 1×10^8 neutrons/cm^2. The reason for the cell-killing activity at such relatively low neutron fluences is proposed to be a consequence of the impact that **3** has on cell cycle gene regulation in U87 cells, as previously described.[22] Thus, the cell-killing effect is a combined response of the cell cycle suppression and α particle generation.

Previously, Hattori et al. reported the biological evaluation of dodecaborate-containing amino acids for BNCT (also using BPA for comparison).[14] They observed by visual assessment using the crystal violet assay that the amino acids showed greater toxicity in human oral squamous cell carcinoma, B16 (mouse melanoma), and C6 (rat glioma) cell lines than did BPA (in response to neutron beam irradiation). It should be noted that in contrast to the present study, amino acid concentrations of 2 mM were used, and neutron fluence levels ranging from 1×10^{12} to 4×10^{12} neutrons/cm^2 (over 1000 times greater than the fluences reported here) were applied. Furthermore, the amino acids were ^{10}B-enriched, which would have resulted in a factor of 5 increase in the number of α particles produced compared to that in this study.

The fact that **3** promoted a desirable cell-killing effect at significantly lower concentrations and reduced fluences of applied radiation than those that have been previously reported may be attributed to the gene regulatory effects it exerts. The results invite consideration of a novel "chemo-radiotherapy" strategy to the application of BNCT, whereby the boron delivery agent itself exhibits a cytostatic and/or cytotoxic effect, and its effectiveness is further amplified when a neutron beam is applied. This allows much lower beam fluences to be used and hence may reduce side effects. Furthermore, the self-assembly of **3** to form hydrogels[21] provides opportunities for a treatment comprised of local application of **3** into the tumor (via injection), followed by neutron radiation to achieve an enhanced cell-killing effect. Full characterization of the hydrogel formed from **3** and assessment of the activity of **3** in vivo (i.e., animal studies) are subjects of ongoing investigations. Another line of inquiry is the mechanism by which **3** enters cells. The results of transcriptomic analysis implicate the involvement of amino acid transport proteins, and this is currently being investigated.

METHODS

Materials and Tissue Culture. 2-Amino-3-(1,7-dicarba-*closo*-dodecaboranyl-1-thio)propanoic acid (**3**) was prepared and characterized as previously described.[21] BPA was purchased from Sigma-Aldrich (St. Louis, MO, USA). The human primary glioblastoma U87 cell line was purchased from ATCC (Manassas, VA, USA). Cells were grown in Eagle's minimum essential medium (EMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate. Cells were maintained at 37 °C in a humidified environment of 5% CO_2.

The solid form of compound **3** was added directly to EMEM media which was sonicated until the compound dissolved. The final concentration was 1 mM. The mixture was then sterile filtered and used directly. For different dose treatments, the 1 mM stock solution was diluted to concentrations of 500, 100, and 1 μM. EMEM media containing BPA was prepared in the same fashion.

ICP−OES-Facilitated Determination of Intracellular 3 Concentrations. Sample analysis was carried out using a PerkinElmer Optima 5300DV inductively coupled plasma optical emission spectrometer (PerkinElmer, Shelton, CT). The detailed experimental method is described in the Supporting Information.

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Neutron Capture Therapy. A flux of thermal neutrons was produced using the 30° beam line at the University at Albany-SUNY Dynamitron accelerator. A description of the configuration of the accelerator and mounting of the samples is provided in the Supporting Information.

Viability Studies of Irradiated 3- and BPA-treated U87 Cells. U87 cells were inoculated at 20 000 cells/well in 24-well plates. The cells were then treated with various concentrations of compound **3** and 1 mM BPA for a duration ranging from 30 min to 3 h, 48 h after plating. Nontreated cells served as controls. After incubation with the compounds, the drug-containing media was removed, fresh media was applied, and the cells were then irradiated with thermal neutrons with total fluences ranging from 1×10^8 to 2×10^9 neutrons/cm^2. The cells that were treated with compounds but were not irradiated were used as controls.

To assess cell death upon irradiation, U87 cells were fixed with 2% glutaraldehyde (Fisher Scientific, Hampton, NH) in PBS for 20 min at room temperature (48 h after the thermal neutron radiation) and then stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO) in deionized water for 30 min. After rinsing with water, the plates were dried overnight. The crystal violet stain was solubilized in 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in distilled water for 30 min with gentle shaking (250 rpm). The absorbance was recorded with a Victor V 1420 Multilabel counter (PerkinElmer Inc., Waltham, MA) at 590 nm. For the control experiments, the same procedure was followed.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b03407.

A description of the ICP−OES-facilitated determination of intracellular **3** concentrations, and descriptions of the configuration of the thermal neutron beam and that of the analyzed samples relative to the beam, is provided (PDF)

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Author Contributions

R.A.M. conceived of the project, performed experiments, and wrote the manuscript with T.H. T.H. performed experiments.

Notes

The authors declare no competing financial interest.

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