Bioorganic Chemistry 90 (2019) 103090

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Synthesis and *in vitro* anti-tumor activity of carboranyl levodopa

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ARTICLE INFO

Keywords:
Neutron capture therapy (NCT)
Anti-tumor
Levodopa
Drug delivery
Bio-assessment

ABSTRACT

Reactions of *closo*-1-Me-2-Iodobutyl-1,2-*closo*-dicarborane, 1-Me-2-I$(CH_2)_4$-$C_2B_{10}H_{10}$, with L-dopa methyl ester can produce carboranyl L-dopa methyl esters in 54% yield in the presence of sodium hydroxide. The appended *closo*-carboranes can be decapitated with sodium hydroxide in a mixed solvent of ethanol and deionized water to produce highly water-soluble carboranyl levodopa in 64% yield. All the new compounds were characterized by ^{1}H, ^{13}C, ^{11}B NMR, FT-IR spectroscopy and elemental analysis. The highly water soluble carboranyl levodopa **4** shows promising efficacy of anti-tumors *in vitro* in the presence of slow neutron beams.

1. Introduction

Gliomas are a broad category of brain tumors that arise from the brain tissue itself. Based on histological characteristics, gliomas can be classified as World Health Organization (WHO) grades I-IV. Glioblastoma multiforme (GBM) is a WHO grade IV glioma, which is well recognized as the most malignant grade [1]. Although the causes of gliomas are not so far clear, it is believed that a prior radiation to the brain is one established environmental risk factor identified for the majority of malignant gliomas [2]. Thus, the GBM remained virtually untreatable and it is, therefore, inevitably lethal. These tumors tend to grow and infiltrate into the normal brain tissues. They infiltrate the brain so aggressively that surgeons are rarely able to remove all the cancerous tissues. These types of tumors are resistant to standard radiation treatment and chemotherapy and that complicates the treatment [3]. Therefore, a significant development of new effective drugs and therapy technologies are needed urgently for the treatment of malignant gliomas.

Boron Neutron Capture Therapy (BNCT) is a binary form of cancer treatment, in which a compound containing B-10 is selectively delivered to tumor tissues prior to irradiation by neutrons. Upon irradiation with thermal neutrons, interaction of a boron-10 atom with thermal neutron produces an α-particle, high energy Li-7 ion and low energy gamma γ rays (Eq. (1)). The linear energy transfer (LET) of these heavily charged particles has a range of one cell diameter which confines the radiant energy to the cell within which they arise, hence minimizing the damage to surrounding tissue. The capture cross section of boron for neutrons is more than three orders of magnitude higher than for other nuclei common to living tissue, so the target region can be dosed with neutrons at a sizeable flux and still have only minimal effect on boron-free regions in the beam path. Additional advantages of using B-10 compared to other nuclides (^{235}U, ^{6}Li) are that the fission products have high LET and the boron compounds can be synthesized having hydrolytically stable linkages between boron and other elements such as C, O and N. If boron can be transported to the target tissue with sufficient specificity using tumor selective agents and in sufficient quantity, tumor cell death may be accomplished [4,5]. The required boron concentration is generally estimated at ~ 10^{9} ^{10}B atoms (natural abundance 19.9%) per cell, which translates to approximately 35 µg ^{10}B per gram of tissue. To prevent damage to healthy tissue in the path of the neutron beam, the surrounding tissue should contain no more than 5 µg of boron-10/g of tissue.

$$^{10}B + {}^{1}n \rightarrow [^{11}B] \rightarrow {}^{4}He^{2+}(\alpha) + {}^{7}Li^{3+} + 2.31\ \text{Mev} \tag{1}$$

The BNCT is an innovative treatment that should provide opportunities and hope to patients and their doctors. Besides the treatment of difficult brain tumors, it has been found that BNCT treatment has an excellent response with patients' recurrent head and neck cancers, for whom there is no other treatment option available [6,7]. The BNCT is a potentially powerful form of radiotherapy involving the preferential incorporation of boron-10 containing compounds into tumor cells, followed by irradiation of the tumor by thermal neutrons. Several requirements must be met in order for this therapy to be effective: (i) a concentration of ~20–50 µg ^{10}B atoms/g of tumor must be achieved; (ii) a tumor/normal tissue (T/N) ratio of the boron delivery agent greater than 3 and tumor/blood (T/N) ratio of greater than 5 are

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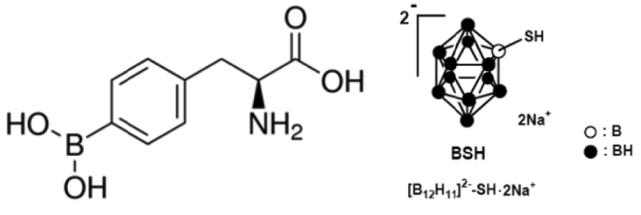
https://doi.org/10.1016/j.bioorg.2019.103090
Received 5 April 2019; Received in revised form 20 June 2019; Accepted 25 June 2019



L-BPA BSH

Fig. 1. Molecular structures of BPA (left) and BSH (right).

necessary; and (iii) the agent should be of low toxicity.

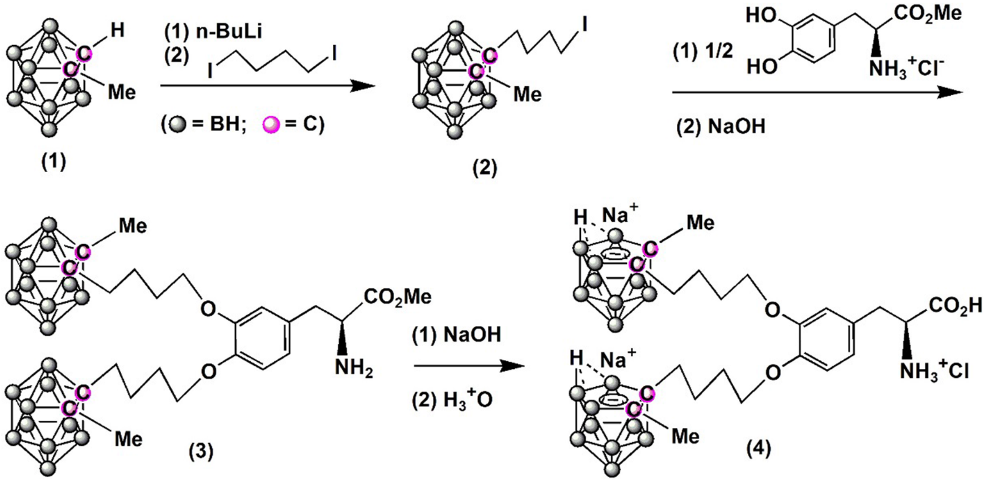
The primary focus of BNCT has been the treatment of malignant brain tumors because these tumors have little history for metastasizing to other organ sites and if tumor eradication could be achieved in the brain, then there should be a significant increase in life expectancy. Only two compounds $Na_2B_{12}H_{11}SH$ (BSH) and boronophenylalanine (BPA) (structures are shown in Fig. 1) are currently used in clinical trials. The two compounds use different mechanisms to enter the malignant cells. The BSH passively diffuses from the blood stream into malignant cells in the brain because these cells disrupt the blood–brain barrier (BBB). Meanwhile, BPA is a boron-labelled amino acid which can be transported through cell membranes to the tumor at much higher rates than those for healthy cells. The BPA concentration in malignant cells is about 2–4 times higher than in blood and other healthy cells. Unfortunately, neither BSH nor BPA show perfect selectivity and a uniform distribution in malignant cells. The overall results are not promising with these compounds, hence there is an urgent need for potential precursor molecules and more sophisticated technologies that can selectively destroy tumor cells and lead to the development of BNCT. A variety of carrier molecules have been used to deliver boron to tumor cells and these include carbohydrates, polyamines, nucleosides, antibodies, porphyrins, liposomes and amino acids [5,8–12]. Unfortunately, none of these has been used in clinical trials despite the voluminous literature development and, clinically, still only two FDA approved drugs, BSH and BPA, are being used worldwide. Therefore, more research efforts are necessary in the specific area seeking to develop novel boron agents with high therapeutic capacity.

Dopamine is an organic chemical of the catecholamine family and it also belongs to the phenethylamine family. As an important neurotransmitter, dopamine plays several important roles in the brain and body, such as memory, movement, and mood, and it is found in many locations throughout the body [13]. The L-DOPA, also known as levodopa, is an amino acid that is well recognized as one of the required biomolecules which are made and used as part of the normal biology of humans, as well as with some animals and plants. The L-DOPA is a precursor of dopamine. Unlike dopamine, L-DOPA shows high capacity to cross the protective blood–brain–barrier (BBB). It has been used as a delivery agent and/or precursor to increase drug concentrations in brain tumor and Parkinson's disease treatments [14–16]. The molecular structure of L-DOPA is very similar to that of BPA, and the conjugation of L-DOPA with carborane could be a feasible option for developing boron carrier agent [17]. Application of these new boron delivery agents could allow for high concentrations of boron to be taken up by the tumor cells; while decreasing side effects because the agents would exclude the possibility of the formation of disulfide bridges [17]. However, enzymes such as L-DOPA decarboxylase and monoamine oxidase can break down L-DOPA and thus limit the amount that is able to enter the central nervous system even though L-DOPA can cross the BBB and enter the brain very quickly [18]. Therefore, to develop a successful BNCT treatment, optimal doses of the carboranyl L-DOPA would be required. In this work, a carborane cluster has been attached to L-DOPA by forming a covalent C—O bond so as to enhance its penetration capability towards tumor cells. Thus, new type of conjugated boron agents are expected to demonstrate high potential for future application in BNCT. Herein, we report the synthesis, characterization and *in vitro* anti-tumor activity.

2. Results and discussion

The carboranyl L-DOPA compounds were synthesized according to the reaction routes shown in Scheme 1. Following literature procedures, commercially available *closo*-carborane, 1-H-2-Me-*closo*-$C_2B_{10}H_{10}$ (**1**) is deprotoned by *n*-butyllithium to form a carboranyl anion, and the *in situ* generated anion subsequently reacts with 1,4-diiodobutane to form the precursor, iodobutyl carboranes, 1-Me-2-I$(CH_2)_4$-*closo*-$C_2B_{10}H_{10}$ (**2**) [19]. The carboranyl iodides react with *L*-DOPA methyl acetate hydrochloride, in a mole ratio of 2:1, to produce *closo*-carborane-appended L-DOPA methyl acetate, *L*-3,4-Di(1′-Me-2′-$(CH_2)_4$-*closo*-$C_2B_{10}H_{10}$)-phenylalanine methyl ester (**3**) in 54% yield. The *closo*-carborane cage in (**3**) can be decapitated by NaOH in 90% ethanol under reflux condition. After neutralization, the *nido*-carborane-appended L-DOPA, *L*-3,4-Di(7′-Me-8′-$(CH_2)_4$-*nido*-$C_2B_9H_{10}$Na)-phenylalanine (**4**) is obtained as an off-white solid in 64% yield.

The compounds 3 and 4 were characterized by elemental analysis, NMR spectra and FT-IR spectra. All data are consistent with the formulations shown in Scheme 1. The ^1H and ^{13}C NMR spectra of compounds **3** and **4** show normal absorptions of methyl, butylene, ester, carboxylic functional groups and carborane cages. The ^{13}C NMR spectra of **3** show the absorptions of the carbons of carborane cage at $\delta = 75.66$ and 74.25 ppm, which are in the range of the reported C_{cage} resonances of the C_2B_{10} systems [19,20]. The ^{11}B NMR spectra are also consistent



Scheme 1. Syntheses of carboranyl L-DOPA agents.

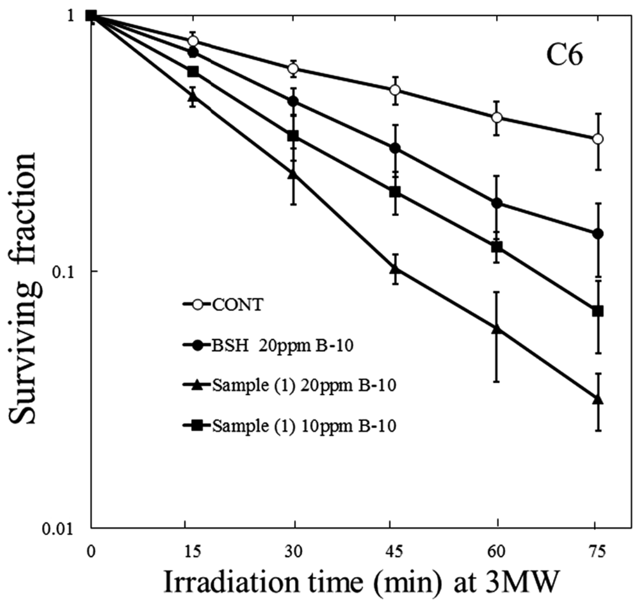


Fig. 2. A plot of surviving fraction vs. irradiation time.

with literature data [19,20] with the chemical shifts of δ = –33.73 and − 40.27 ppm as observed for *nido*-carborane clusters of **4**. In FT-IR spectra, the B—H bond stretching absorptions ($\nu_{\text{B-H}}$) are found at 2591 and 2529 cm^{-1} for **3** and **4**, respectively. In addition to NMR and FT-IR spectroscopy, chemical analysis was carried out to further characterize the new products, and the results were consistent to support their molecular structures.

It has also been observed that compound **4** can easily dissolve in highly polar solvents such as DMSO and water to form clear and transparent solutions. The water solubility of the carboranyl L-DOPA has been dramatically improved when compared with the pristine carborane clusters, *closo*-1-Me-1,2-*closo*-dicarborane and *closo*-1-Me-2-Iodobutyl-1,2-*closo*-dicarborane.

The preliminary *in vitro* killing effects and IC_{50} of compound **4** were examined by using C6 gliosarcoma cells. An *in vitro* survival study was completed with a thermal neutron fluence ranging from 0 to 4.2×10^{12}n/cm^2. The γ-ray dose presented in the experiment was monitored by thermoluminescent dosimeters (TLDs) attached to the tubes and ranged from 0 to 5 Sv. As shown in Fig. 2, compound **4** demonstrated significantly improved killing effects for tumor cells. A lowest surviving fraction of 0.03 was reached by compound **4** after irradiation for 75 min. Compared with the current clinically used BNCT drug, BSH, the killing effects has been approximately doubled under the same operating conditions.

Testing of boron uptake of **4** was conducted by using rat glioma cells (C6). Clinically used BSH and 4-borono-L-phenylalanine (L-BPA) were employed as comparative examples. The fractional amounts delivered to cells were found to be 1.53, 2.14 and 0.67 μg/10^7 cell, for **4**, BSH and BPA respectively. According to the results, **4** could permeate C6 tumor cells and showed good bio-availability *in vitro*. Despite there are very limited studies in the mechanism of the carboranyl compounds crossing the BBB, anionic *nido*-carboranyl compounds such as cesium *N*-(*nido*-7,8-carboranylmethyl)-2-chloro-5-methoxybenzamide, have been reported to provide high *in vivo* bio-availability for central nervous system [21,22], our results are consistent with the literature, and thus compound 4 may be potentially applicable to BNCT.

3. Conclusions

The work has demonstrated that it is a feasible option to conjugate *nido*-carborane clusters to L-DOPA. The resulting water-soluble carborane-appended complex has been found to be an effective C6 tumor cells-killing agent. These results indicate that such compounds should be further investigated as effective boron carriers for BNCT treatment. Since boron-10 isotope is active for BNCT only, the ^{10}B-enriched compound **4** will be investigated in the future for clinic tests. Additional bioassessments such as biodistribution and cytotoxicity studies, are currently being investigated in our laboratories.

4. Experimental section

4.1. General

All reactions were carried out under an argon atmosphere using standard Schlenk techniques. Tetrahydrofuran and diethyl ether were heated over sodium/benzophenone until a blue color was sustained, and distilled under nitrogen just before use. The 1,4-Diiodobutane, *N*-butyllithium (1.6 M in hexanes), L-DOPA methyl ester hydrochloride, and other reagents and organic solvents, were used as received. The 1-Methyl-*closo*-1,2-$C_2B_{10}H_{11}$ was purchased from KatChem Ltd. and used as received. The FT-IR spectra were measured using a BIO-RAD spectrophotometer with KBr pellets or organic solvent films. Elemental analyses were measured using a EURO EA equipment. The ^{1}H, ^{13}C, and ^{11}B NMR spectra were recorded using a Bruker 400 analyzer at 400.13, 100.62, and 128.38 MHz, respectively. Inductively coupled plasma-optical emission spectroscopy (ICP-OES) measurements were made using a VISTA-MPX machine.

4.2. Synthesis of 2

A literature procedure [19] was adopted to synthesize compound **2**. Accordingly, 1.05 g (6.64 mmol) of 1-Me-*closo*- $C_2B_{10}H_{11}$ was added to a 250-mL three-necked round-bottom flask equipped with a magnetic stirring bar, and the compound was dissolved in 60 mL of THF. The solution was cooled to − 78 °C, and then 4.35 mL (6.96 mmol) of *n*-BuLi (1.60 M in hexanes) solution was added dropwise. The resulting reaction mixture was continuously stirred for 30 min at − 78 °C before allowing to warm to room temperature. After 4-h, the mixture was cooled to 0 °C, and then 0.98 mL (~7.0 mmol) of 1,4-diiodobutane was carefully added using a syringe. After this addition, the reaction mixture was stirred for 30 min at 0 °C, slowly allowed to warm to room temperature, stirred for an additional 1 h, and then refluxed for 3 h. The mixture was then cooled to 0 °C and quenched with sufficient quantity of deionized water. The reaction mixture was dried in vacuum and the residue was purified by thin-layer chromatography (using silica gel), developed with an *n*-pentane/ethyl acetate (v/v = 6/1) solvent mixture to isolate product (**2**) as a colorless oil. The product was identified by comparing its ^{1}H NMR spectrum with that published in the literature [19].

4.3. Synthesis of 3

Carboranyl butyl groups were appended to L-DOPA molecule using a classical Williamson ether synthesis between iodide (**2**) and L-DOPA salts. To prepare compound **3**, L-DOPA methyl ester hydrochloride (123.8 mg, 0.50 mmol) and sodium hydroxide powder (62.0 mg, 1.55 mmol) were added to a 100-mL three-necked round-bottom flask containing 60 mL dimethylacetamide (DMA) and the resulting mixture was stirred for 0.5 h before adding 1-methyl-2-iodobutyl-*o*-carborane (423.3 mg, 1.00 mmol) to the flask. The reaction mixture was heated to 90 °C and allowed to react for 40 hrs with continuous stirring. The solvent was then removed under reduced pressure and the residue was purified by flash chromatography (SiO_2, DCM/THF/Hexane) to isolate the off-white powder product (**3**), *L*-3,4-Di(1′-Me-2′-$(CH_2)_4$-*closo*-$C_2B_{10}H_{10}$)-phenylalanine methyl ester (172.4 mg) in 54% yield. Elemental Anal: Calcd for $C_{24}H_{53}B_{20}NO_4$: C, 45.33; H, 8.40; N2.20. Found: C, 45.10; H, 8.52; N 2.15. ^{1}H NMR ($CDCl_3$, relative to $SiMe_4$, ppm): *δ* 7.31–7.22 (m, 3H, $-C_6\underline{H}_3-$), 3.95 (t, 4H, 2 $C\underline{H}_2O$), 3.82 (s, 3H,

$O C\underline{H_3}$), 3.64 (m, 1H, $C\underline{H}N$), 3.41–1.18 (m, 42H, $C\underline{H_2}{-}C_6H_3$, $6C\underline{H_2}$, $2C\underline{H_3}\text{-}C_{cage}$, $N\underline{H_2}$, 2 $C_{cage}B_{10}\underline{H_{10}}$). ^{13}C NMR ($CDCl_3$, relative to $SiMe_4$, ppm): δ 172.22 (1C, $\underline{C}O_2$), 146.09, 145.64, 127.78, 125.83, 120.71, 117.53 (6C, $\underline{C_6}H_3$), 75.66, 74.25 (2C, $\underline{C}_{cage}$), 67.78 (2C, $O\underline{C}H_2$), 56.14, 55.89 (2C, $O\underline{C}H_3$, $C_6H_3{-}\underline{C}H_2{-}$), 39.70 (1C, $\underline{C}H{-}N$), 33.04 (2C, $C_{cage}\text{-}\underline{C}H_2{-}$), 31.27, 29.36, (4C, 4 $\underline{C}H_2$), 22.25 (2C, 2 $C_{cage}\text{-}\underline{C}H_3$). ^{11}B NMR ($CDCl_3$, relative to $BF_3{\cdot}OEt_2$, ppm): δ −4.64 (1B, $^1J_{BH} = 158$ Hz); −4.76 (1B, $^1J_{BH} = 166$ Hz); −9.17 (2B, $^1J_{BH} = 97$ Hz); −10.26 (2B, $^1J_{BH} = 112$ Hz); −11.72 (4B, $^1J_{BH} = 146$ Hz). IR (film on KBr, cm^{-1}) 3469 (s, s), 3225 (s, s), 2897 (s, m), 2976 (s, s), 2864 (m, s), 2591 (vs, s, ν_{BH}), 1962 (w, s), 1845 (w, s), 1753 (s, vs), 1722 (s, vs), 1708 (s, s), 1552 (s, s), 1410 (s, s), 1375 (s, s), 1355 (m, s), 1291 (m, s), 1258 (s, s), 1219 (s, m), 1199 (s, m), 1170 (s, w), 1010 (br, m), 834 (s, s), 784 (m, br), 733 (s, s), 673 (m, s), 646 (s, w), 591 (s, s).

4.4. *Synthesis of 4.*

A 1.00 g sample of sodium hydroxide was dissolved in 50 mL of 90% ethanol in water, and the resulting solution was added to 0.20 g (0.31 mol) of **3** with constant stirring for 40 min. The resulting mixture was heated to reflux for 12 hrs and allowed to cool to 0 °C and then neutralized with aqueous HCl to a pH of about 5.0 and stirred further for 5 hrs and removed unreacted NaOEt. Removal of volatiles under reduced pressure produced a residue that was recrystallized from ethanol and then dried *in vacuo* to give compound **4** as pale yellow waxy solid (135.4 mg) in 64% yield. Elemental Anal: Calcd for $C_{23}H_{52}B_{18}ClNO_4Na_2$: C, 40.46; H, 7.68; N2.05. Found: C, 40.18; H, 7.33; N 2.10. ^1H NMR (D_2O, relative to $SiMe_4$, ppm): δ 7.20–7.08 (m, 3H, $-C_6\underline{H_3}-$), 4.39 (m, 1H, $C\underline{H}N$), 3.87 (t, 4H, $2C\underline{H_2}O$), 3.35–0.09 (m, 38H, $C\underline{H_2}{-}C_6H_3$, 6 $C\underline{H_2}$, $2C\underline{H_3}\text{-}C_{cage}$, $2C_{cage}B_9\underline{H_9}$), −2.30- −3.50 (br, 2H, $2BH_{bridge}$). ^{13}C NMR (D_2O, relative to $SiMe_4$, ppm): δ 171.30 (1C, $\underline{C}O_2$), 145.07, 144.87, 126.18, 123.66, 120.51, 115.47 (6C, $\underline{C_6}H_3$), 64.74 (4C, $O\underline{C}H_2$), 61.78 (br), 56.14 (br) (4C, $4C_{cage}$); 55.39, 53.76 (4C, $2\underline{C}H{-}N$, $2C_6H_3{-}\underline{C}H_2{-}$), 38.62 32.17 (2C, $C_{cage}\text{-}\underline{C}H_2{-}$), 30.21, 28.37, (4C, $4\underline{C}H_2$), 23.15 (2C, $2C_{cage}\text{-}\underline{C}H_3$). ^{11}B NMR (D_2O, relative to $BF_3{\cdot}OEt_2$, ppm): δ −12.44, −18.35, −33.73, −40.27 (br). IR (film on KBr, cm^{-1}) 3584 (s, br), 3356 (s, s), 3215 (s, s), 2921 (s, s), 2887 (s, s), 2529 (vs, s, ν_{BH}), 1955 (m, s), 1839 (m, s), 1742 (s, s), 1713 (s, s), 1621 (s, s), 1544 (m, s), 1437 (s, br), 1360 (m, s), 1347 (s, s), 1287 (s, s), 1265 (m, s), 1191 (m, s), 1159 (s, s), 1024 (m, br), 827 (m, s), 796 (w, br), 750 (w, s), 653 (m, s).

4.5. *Evaluation of the bioactivity*

4.5.1. *In-vitro survival study*

The C6 gliosarcoma cells were incubated in subconfluent condition in standard minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), at 36 °C, 5% CO_2 atmosphere for overnight. Aliquots of the boron sample solutions were added over a period of 6 hrs into the respective Petri dishes for uptaking of tumor cells in dose-dependent manners. The boron concentrations in the media were planned to be 20 ppm of ^{10}B and were confirmed by prompt γ spectroscopy (PGA). The tumor cells were washed twice after boron loading. Aliquots containing 5×10^3 cells/mL were pipetted into cylindrical Teflon tubes of 1 cm in diameter and 3 cm high which did not generate any secondary radiation when subjected to thermal neutrons. The inability of cells to adhere to Teflon allows precise quantitative manipulation of cells without trypsinization. The thermal neutron fluence was determined by averaging the activity of two gold films symmetrically attached to the Teflon tube surface along the thermal neutron axis. The thermal neutron fluence ranged from 0 to 4.2×10^{12} n/cm^2. For estimation of the neutron energy spectra, 8 kinds of activation films and 14 kinds of nuclear reactions were used: $^{197}Au(n, \gamma)^{198}Au$ for the thermal neutron regions; $^{115}In(n, \gamma)^{116m}In$, $^{197}Au(n, \gamma)^{198}Au$, $^{58}Fe(n, \gamma)^{59}Fe$ and $^{63}Cu(n, \gamma)^{64}Cu$ for the epithermal neutron region; and $^{115}In(n, n^*)^{116}In$, $^{54}Fe(n, p)^{54}Mn$, $^{27}Al(n, p)^{27}Mg$, $^{27}Al(n, \alpha)^{24}Na$, $^{47}Ti(n, p)^{47}Sc$, $^{48}Ti(n, p)^{48}Sc$, $^{24}Mg(n, p)^{24}Na$, $^{63}Cu(n, \alpha)^{60}Co$, $^{58}Ni(n, p)^{58}Co$ and $^{197}Au(n, 2n)^{196}Au$ for the fast-neutron region. The neutron absorbed dose (Gy) was calculated using the flux-to-dose conversion factor [23,24]. The elemental composition of the tumors was assumed to be 10.7% hydrogen, 12.1% carbon, 2% nitrogen, 71.4% oxygen and 3.8% others [23,24]. The γ-ray dose was monitored by thermoluminescent dosimeters (TLDs) attached to the tubes and it ranged from 0 to 5 Sv. Immediately after irradiation, 500 cells were seeded in 6-cm Petri dishes (Corning, NY, USA) and incubated for 10 days in a humidified 37 °C atmosphere of 95% air/5% carbon dioxide to allow colony formation. The colonies were fixed and stained with a 10% formaldehyde/1% toluidine blue solution and then counted microscopically.

4.5.2. *Cytotoxicity*

The IC_{50} (moles/liter, *M*), that is the concentration that inhibited the growth of C6 gliosarcoma cells by 50% after 3 days of continuous exposure, was determined. Suspensions of 10^5 C6 cells/200 µl MEM containing 20% FCS were incubated for 3 days using 96-well culture plates with various concentrations of the samples. The damaged cells were detected at 490 nm optical absorbance by AQueous One Solution Cell Proliferation Assay. The IC_{50} for compound **4** is 8.93×10^{-4} M.

4.5.3. *Uptake of boron by tumor cells*

The 1.7×10^7 rat glioma cells (C6) were seeded and cultured at room temperature (37 °C., 5% CO) for 24 hrs. This culture fluid was removed therefrom by suction, culture fluids containing 1.5 mM of each boron agent (**4**, BSH and L-BPA) were added and continued culturing for 24 hrs under same conditions. These culture fluids were removed by suction and the cells were washed three times with PBS and treated with trypsin to recover the cells. The number of the cells recovered was counted, and HNO_3 (2 N, 1.5 mL) was added and the resulting mixture was heated at 80 °C for 12 hrs. After filtering with a membrane filter, the boron concentration was determined by ICP-AES.

4.5.4. *Statistics*

The *in vitro* BNCT was carried out in triplicate. Values are the mean ± SEM from three independent experiments. The significance of differences in survival was assessed by Student's *t* test. The statistical analyses were performed using Prism 3.0 (GraphPad Software Inc., CA, USA).

Declaration of Competing Interest

The authors declare that they have no conflict of interest to disclose.

Acknowledgement

This work was supported by an FDCT grant from the Science and Technology Development Fund of Macau (Project code 0030/2018/A1).

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