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Boron accumulation in brain tumor cells through Boc-protected tryptophan as a carrier for boron neutron capture therapy

Chun-Ming Chio,^{a,†} Ying-Cheng Huang,^{b,†} You-Cheng Chou,^a Fu-Chun Hsu^a, Yen-Buo Lai^a and Chung-Shan Yu^{a,c*}

^aDepartment of Biomedical Engineering and Environmental Sciences, National Tsinghua University, Hsinchu 300, Taiwan

^bDepartment of Neurosurgery, Chang-Gung Memorial Hospital at Linkou, Chang Gung University, Taiwan.

^cInstitute of Nuclear Engineering and Science, National Tsing-Hua University, Hsinchu, 300, Taiwan.

KEYWORDS BNCT, tryptophan, boronodehalogenation, tumor to normal, U87-MG, protecting group

ABSTRACT: Boron neutron capture therapy (BNCT) is a binary therapeutic approach. Nonradioactive boron-10 atom accumulated in tumor cells combining with the neutron beams produce two high energetic particles that could eradicate the cell that takes it and the neighboring cells. Small molecules that carry boron atom e.g. 5- and 6-boronated and 2,7-diboronated tryptophans were assessed for their boron accumulation in U87-MG, LN229 and 3T3 for BNCT. TriBoc tryptophan, TB-6-BT, shows boron-10 at 300 ppm in both types of tumor cells with a tumor to normal ratio (T/N) of 5.19-5.25 (4 h). TB-5-BT and DBA-5-BT show boron-10 at 300 ppm (2 h) in U87-MG cells. TB-5-BT exerts a T/N of > 9.66 (1 h) in LN229 compared with the current clinical boronophenyl alanine with a highest T/N of 2.3 (1 h) and accumulation concentration of < 50 ppm. TB-5-BT and TB-6-BT warrant a further animal study.

Introduction

As it is the most common type of primary brain tumor in adults, glioblastoma multiforme (GBM) is classified as a World Health Organization grade IV astrocytoma. Its estimated incidence in North America is 3.0 per 100,000 people according to age-adjusted statistics. Due to its aggressive malignancy, GBM has an estimated 2-year survival rate of 8.7%. Even after maximum treatment, the median duration of survival is 12–18 months.¹ Whereas the tumor pathogenesis has been well deciphered, the median overall survival of patients has increased by only 3.3 months (from 11.3 months to 14.6 months) over the past 25 years. This disappointing prognosis is mostly associated with the prevalent recurrence of tumors after initial treatment with the maximum safe surgical resection, radiotherapy, and chemotherapy.¹ Moreover, the cause of death of glioma patients is not a distant metastasis but the failure of local control of the tumor. When the tumor repeatedly happens, additional systemic and local therapies as well as repeat surgery are commonly considered. While each of these therapies has potential benefits, each also carries associated risks, particularly surgery.²

The invasive nature of glioma often prevents total surgical excision, hence adjuvant therapy for the residual tumor is essential. However, conventional chemotherapy, immunotherapy, and radiotherapy have been proven to be of limited value.³ The highly proliferating malignant tumor cells may require significant substrates such as sugars and amino acids. If a specific upregulation of the amino acid transport system in malignant tumor cells does exist, it could be a molecular target for therapy.³

The leucine-type amino acid transporter (LAT1) is a Na-independent neutral amino acid transport agent and essential for

the transport of large neutral amino acids through the plasma membrane.^{4, 5} LAT1 exhibits high affinity for several nutritionally essential amino acids such as leucine, isoleucine, valine, phenylalanine, tryptophan and methionine.³ Thus, one such amino acid analog of phenylalanine bearing a boron-10 atom can be triggered to release energy upon irradiation with neutron beams.

Boron neutron capture therapy (BNCT) is a targeted radiation therapy that significantly increases the therapeutic ratio relative to conventional radiotherapeutic modalities (Fig. 1). BNCT is a binary approach: a boron-10 (¹⁰B)-labeled compound is administered that delivers high concentrations of ¹⁰B to the target tumor relative to surrounding normal tissues. This is followed by irradiation with thermal neutrons or epithermal neutrons which become thermalized at a depth in tissues. The short range (5-9 μm) of the α and ⁷Li particles released from the ¹⁰B(n, α)⁷Li neutron capture reaction make the microdistribution of ¹⁰B of critical importance in therapy.⁶

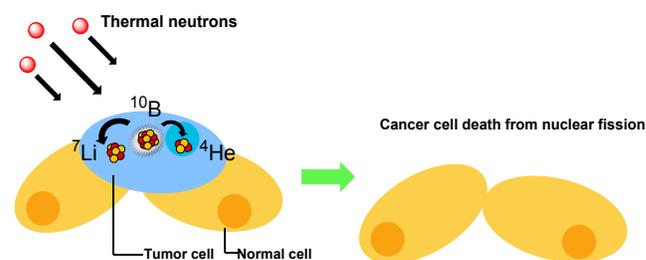


Figure 1. Illustration of the basic principles of boron neutron capture therapy (BNCT).

One of the advantages of a binary system is the potential that each component can be manipulated independently. Only when the timing is correct for achieving the maximal cytotoxic effect upon malignant cells, together with the maximum tolerated dose to contiguous normal cells, are the two components juxtaposed. If this approach is to succeed, it is essential that at least one of the components be confined rather specifically to tumor cells, while the second component is exposed to all cells in a particular area.⁷

The potential for developing NCT rests upon the fact that the various normal elemental constituents of tissue have very low capture cross-section values for thermal neutrons compared with boron-10 (3838 barns). The ideal boron agents should have the following features: (1) low intrinsic toxicity; (2) high tumor uptake ($\sim 20\text{--}50 \mu\text{g }^{10}\text{B}$) and low normal tissue uptake, ideally with tumor to normal tissue and tumor to blood boron concentration ratios of $> 3:1$; and (3) relatively rapid clearance from the blood and normal tissues along with persistence in the tumor for at least several hours during neutron irradiation.⁸ The present boron-bearing compounds used for BNCT are briefly divided to two types: small molecules e.g. boronophenylalanine (BPA) and cage-like boron clusters e.g. sodium borocaptate ($\text{Na}_2^{10}\text{B}_{12}\text{H}_{11}\text{SH}$, BSH).⁸ The clinically studied amino acid analog BPA exhibits acceptable *in vivo* tumor targeting and has proven to be sufficient in the treatment of brain tumors in humans.⁹ Although BPA and BSH remain unsatisfactory according to the above criteria, these two agents are the clinically used drugs. Other amino acid derivatives of tryptophan such as $\alpha\text{-}^{11}\text{C}$ -methyl-*L*-tryptophan (^{11}C -AMT), are amino acid PET tracers that can measure tryptophan metabolism via the immunomodulatory kynurenine pathway.¹⁰ It has been shown that ^{11}C -AMT accumulates because of both transport and metabolism in both untreated and recurrent World Health Organization (WHO) grade II–IV gliomas. A standardized uptake value (SUV) of 2.68 is distributed over the brain tumor along with a lesion to cortex ratio of 2.14 from a clinical study for 21 adults.

Although liposomal and nanoparticle compounds have been intensively developed as boron carriers, the accumulated boron in brain tumors remains low, *i.e.* $< 0.4\%$ injection dose/g.^{11,12} Satisfactorily high concentrations of 50 ppm ^{10}B have been achieved recently.¹³ However, the preparation is relatively uncommon to organic chemists, *i.e.* via grinding of a micron-sized ^{10}B -enriched rock-hard powder to give the particle size of 85 nm. Hence, we are encouraged to prepare single-boron carried small molecule via the common covalent-bond formation with the hope of improving tumoral accumulation. With respect to the similar features to those of BPA such as the transportation mechanism via LAT-1, essential nutrition source and insignificant cytotoxicity, we intend to prepare the amino acid-like boron derivatives of tryptophan (Fig. 2). The pseudotryptophan structure can be regarded as a prodrug that can diffuse through the BBB layer via LAT-1 mediated transportation.¹⁴ This is similar to the concept of a pseudonutrient that has been applied by DOPA, a clinically used prodrug of dopamine.¹⁵ Hence, the protecting groups are optionally attached to examine their effects on assisting the penetration across the cell membrane for accumulation. The present study is aimed to prepare boronopinacol tryptophan derivatives with protecting groups for assessing their potencies as BNCT agents. The relevant *in-vitro* assay includes the accumulation, cytotoxicity and the eradication effects through neutron irradiation.

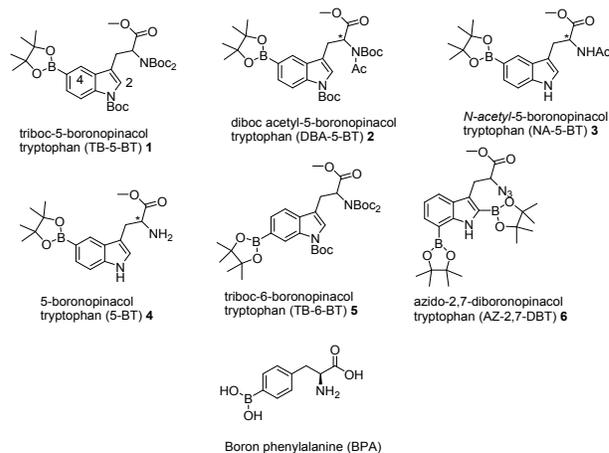


Figure 2. Boronopinacoltryptophan derivatives described in this work.

Electrophilic substitution on the indole-based tryptophan is apt to take place at the pyrrole ring or the 5- or 6- position of the benzene ring. However, borylation on 5- and 6- positions via regioselective control over a common intermediate remains difficult.¹⁶ In general, the reactivity of the two fused rings has been speculated (Fig. 3). The intermediates formed from 4- or 7-substitution lead to the entire loss of aromaticity of both rings. Experimental data¹⁷ and theoretical calculation¹⁸ predicts that the reactivity under the catalysis of Pd(0) is in an order of $\text{C-4} > \text{C-6} > \text{C-5} > \text{C-7}$. Furthermore, Pd(0)-catalyzed oxidative addition for the boronopinacol group at the six-membered ring moiety suffers from other disadvantages. For example, Pd(0) is deactivated by electron donors, such as -NH_2 through its coordination to Pd, thereby rendering the metal insertion into R-X inefficient. The bulky electron-withdrawing Boc as the protecting group for -NH_2 not only blocks its capacity for coordination but also weakens the electron delocalization to the halogenated carbon, thereby retaining the polarizability of the C-Br bond (Scheme 1 (A)).

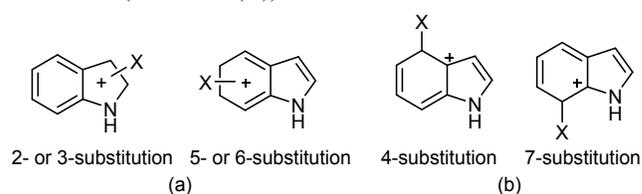
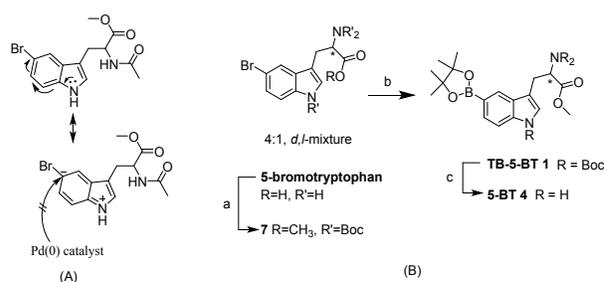


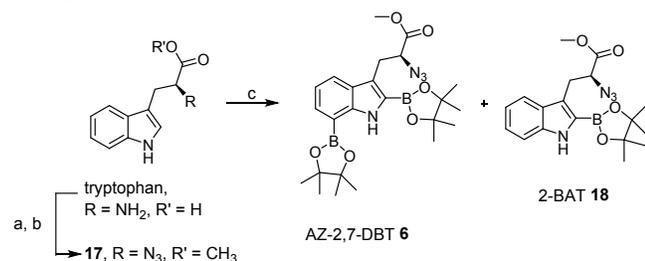
Figure 3. Electrophilic substitution prefers the route via a cation intermediate (a) over that of a cation (b).



Scheme 1. (A) Deactivating Pd(0) through electron delocalization. (B) TriBoc protected 5-boronopinacol tryptophan analog TB-5-BT **1** and its deprotection leading to 5-BT **4**. Reaction conditions a: SOCl_2 , CH_3OH ; Boc_2O , DMAP, CH_3CN , CH_3CN , 50% over two steps; b: diboronopinacol, $\text{Ferrocene}_2\text{PdCl}_2$, DMSO, KOAc, 100°C , 2 h, 90%. c. As listed in Table 1.

deiodinated byproduct (~ 10%) even after HPLC purification. After borylation, the 6-isomer TB-6-BT **5** was obtained as the sole product from column chromatography.

The third target compound A,Z-2,7-DBT **6** with two boron atoms is thought to be more useful than the above single boron tryptophan derivatives (Scheme 4). Ir-assisted direct borylation on tryptophan has been thoroughly documented in several reports.⁴⁰⁻⁴² However, intrinsic instability is associated with the diboronopinacol moieties, especially when oxidizing using H₂O₂.⁴¹ Whereas Ir-involved borylation did not produce a satisfactory yield of the diboron product AZ-2,7-DBT **6**, the quantity was enough for the *in-vitro* biological analysis.



Scheme 4. 2,7-Diboronopinacol tryptophan analog AZ-2,7-DBT **6** prepared from tryptophan. Reaction conditions: a: SOCl₂, CH₃OH; b: TfN₃, CuSO₄, K₂CO₃, CH₃OH, rt, over 2 steps, 69%; c. h: [Ir(COD)OMe]₂, (CH₃)₄phen, B₂pin₂, THF, 80 °C, 11% for AZ-2,7-DBT **6** and **18**, respectively.

Cytotoxicity

The cytotoxicities of these tryptophan derivatives were assessed in three cell lines including two tumor cell lines: U87-MG and LN-229 from human and one control cell line: 3T3 fibroblast cell from mice. The reduction potential of mitochondrial was evaluated by the formation of a dark yellow precipitate when treating WST-1 reagents with aliquots of compounds in a series of dilutions from 1 nM to 100 μM. The cell viability was thus obtained as shown in Supp. Fig. 22 and Table 2. To prepare the samples as a solution phase, compounds were first dissolved in 100% DMSO to give 10 mM as a stock for the following dilutions. The most concentrated aliquots (100 μM) contains 1% DMSO. Except for the control 3T3 cells, both tumor cells tolerate the presence of 1% DMSO well. Furthermore, the Boc-protected derivatives, e.g., TB-6-BT **5**, TB-5-BT **1** and DBA-5-BT **2**, are slightly toxic against 3T3 cells but insignificant when compared with the control compound BPA. BPA was generally regarded as nontoxic.⁴³⁻⁴⁸

Table 2. Compound toxicity (100 μM) against tumor (U87-MG and LN-229) and normal (3T3) cells

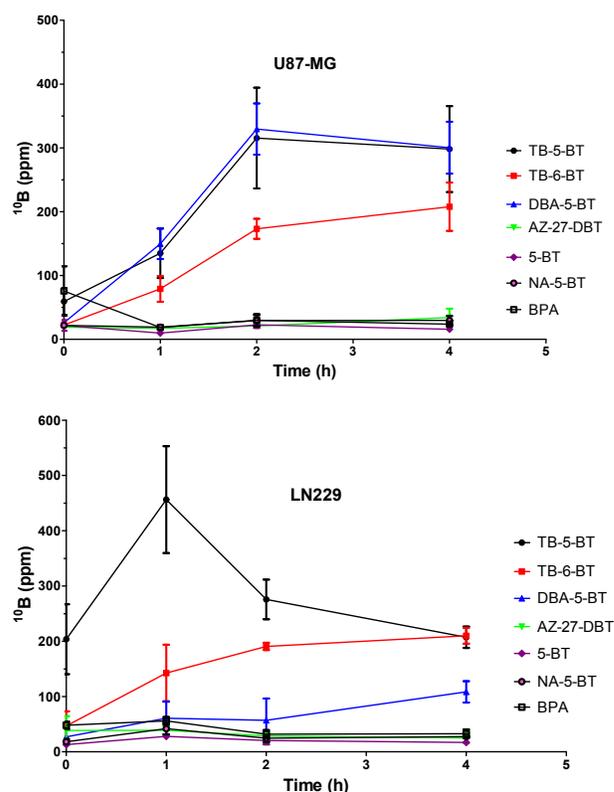
Cell lines	Cell viability (%)							
	TB-6-BT	TB-5-BT	DBA-5-BT	NA-5-BT	5-BT	AZ-2,7-DBT	BPA	DMSO (1%)
U87	60.1 ± 1.9	67.4 ± 1.6	70.2 ± 0.1	66.2 ± 3.2	65.8 ± 3.3	61.3 ± 5.0	67.0 ± 2.0	97
LN229	53.2 ± 2.0	60.7 ± 5.2	70.8 ± 1.4	69.9 ± 2.9	67.3 ± 1.9	57.2 ± 6.2	79.6 ± 3.6	91
3T3	63.6 ± 0.9	60.2 ± 2.3	66.6 ± 1.8	42.9 ± 0.7	47.6 ± 1.5	109.9 ± 1.1	53.0 ± 1.6	57

Results were tabulated from the most concentrated (100 μM) aliquots in 1% DMSO after 24-h treatment. Data were obtained in quadruplicate and were expressed as mean ± SD. GraphPad Prism 5 software was used.

In-vitro accumulation

The cellular accumulation of boron tryptophan derivatives was evaluated by determining the boron concentration using induced coupled mass spectrometry (ICP-MS). The three tryptophan

derivatives with Boc groups including TB-5-BT **1**, DBA-5-BT **2** and TB-6-BT **5** showed better accumulation than the partial protected derivatives 5-BT **4** and NA-5-BT **3** (Fig. 4). Both the triBoc-protected TB-5-BT **1** and TB-6-BT **5** accumulated more than 100 ppm of ¹⁰B after 1 h incubation in LN229 cell. Moreover, TB-5-BT **1** and DBA-5-BT **2** reach a high level of 300 ppm after 2 h incubation in U87-MG cells, and TB-5-BT **1** exhibited an even higher accumulation level of 450 ppm post 1 h of incubation in LN229 cell. By comparison with the 6-boronopinacol derivative, 5-boronopinacol derivatives accumulated preferentially in U87-MG cells. Setting the 3T3 cells as a control, accumulation selectivity indices defined by the tumor vs. normal (T/N) ratio of TB-6-BT **6** are >5 (T/N) 4 h post addition in both types of tumor cells (Fig. 5). TB-5-BT **1** showed an average T/N >2 and an impressive T/N > 9 in LN229 cells before 1 h. Thus, the most highly accumulated and selective TB-5-BT **1** warrants further study. Furthermore, to serve as a clinically useful BNCT agent, plenty of dosages (>1g/kg) are commonly given to patients over two hours prior to neutron irradiation. Based on this rationale, the 6-boronopinacol derivative TB-6-BT **5** is potent because of its increasing accumulation trend. Due to the slight toxicity of 100 μM of some tryptophan derivatives (Table 2), half concentration (50 μM) was employed for neutron irradiation experiments. The results of the BNCT were assessed using WST-1 assay. After neutron irradiation, more cells treated with the most accumulated TB-5-BT **1**, DBA-5-BT **2** and TB-6-BT **5** were rendered nonviable than those treated with BPA (Fig. 6). Unlike TB-6-BT **1** and DBA-5-BT **2** both showed enhanced cancerous eradication, TB-5-BT **5** in U87-MG cell is somewhat inferior probably due to the bias as indicated by the internal standard of DMSO with increasing survival rate of greater than 100%.



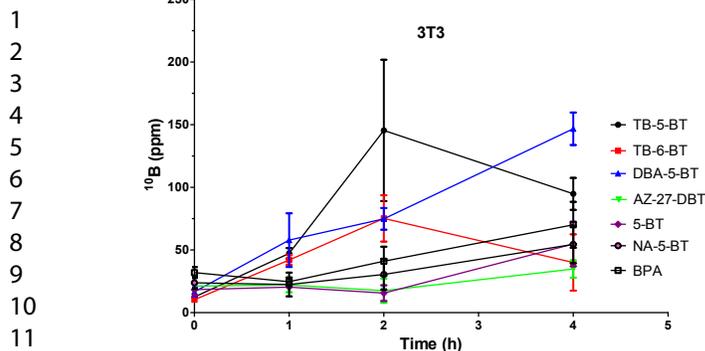


Figure 4. Schematic comparison of boron accumulation by boronotryptophan derivatives in tumor (U87-MG and LN229) and normal brain cells (3T3).

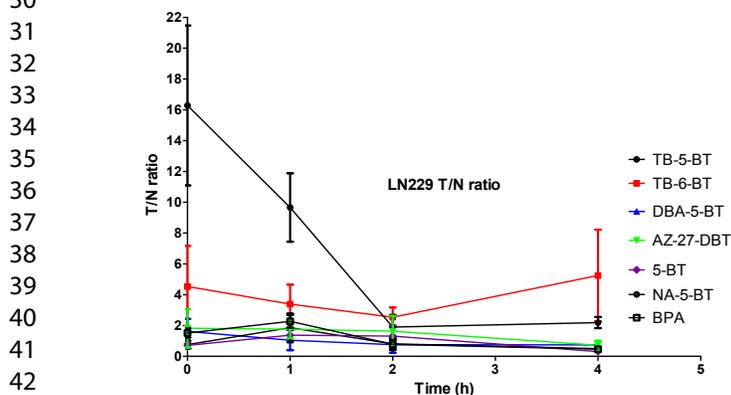
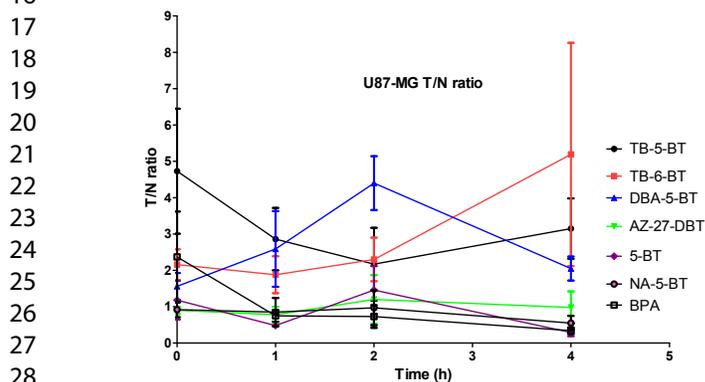
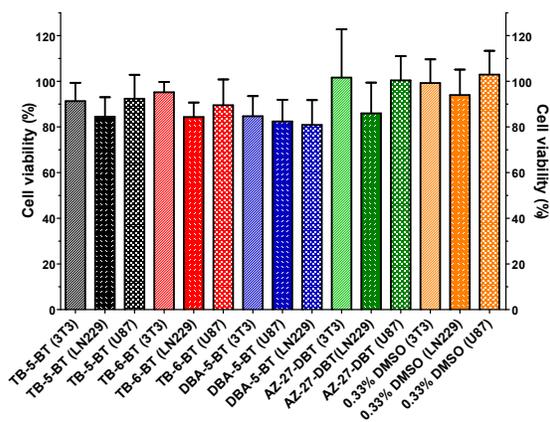
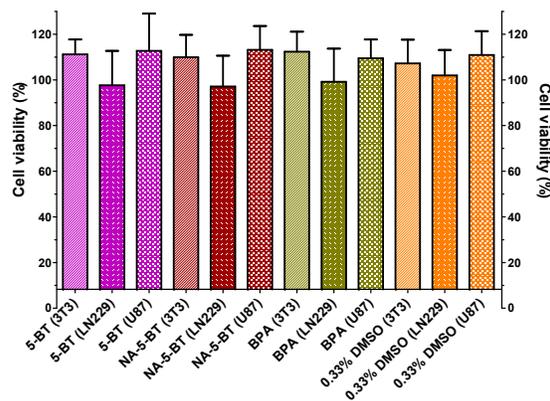


Figure 5. Schematic comparison of boron accumulation ratio (T/N) in tumor (U87-MG and LN229) vs normal brain cells (3T3).



(A) Less polar tryptophan derivatives



(B) More polar tryptophan derivatives

Figure 6. Comparison of the cell survival ratio from the treatment of boron agents (50 μ M) with neutron irradiation (A and continued part B). Data were collected from quadruplicate of three sets and two sets of experiments for cancer cells (LN229 and U87-MG) and normal cell 3T3, respectively.

LAT-1 expression assay

It is necessary to examine whether these tryptophan derivatives can affect the expression of LAT-1. Although no direct binding assay was performed, the protein expression by Western blot can generate a qualitative comparison (Fig. 7). In general, no significant inhibition of LAT-1 was observed over the 2-h time course except 3T3 cell. Especially, the two more T/N selective TB-5-BT **1** and TB-6-BT **5** can be due to the differential expression of LAT-1. In addition, the preferential accumulation of these tryptophan derivatives in tumor cells can be accounted for by two reasons. First, the hydrophobic characteristics of these compounds can facilitate diffusion across the cell membrane. Second, tumor cells tend to ingest more tryptophan derivatives as nutrition sources than normal cells do.

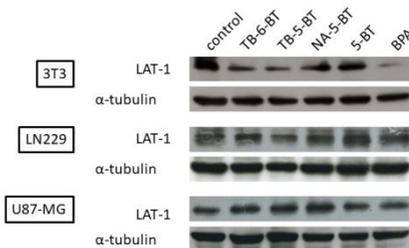


Figure 7. Western blot of the LAT-1 expression level of the three cell lines after 2 h incubation of the selected tryptophan derivatives.

Primary antibody LAT-1 (1:2500, Cell Signaling Tech) and α -tubulin (1:5000, GeneTex).

Conclusion

The three Boc-protected tryptophan derivatives TB-5-BT **1**, DBA-5-BT **2** and TB-6-BT **5** are not soluble in aqueous solution, and hence, the question now rests on the formulation optimization for injection in an animal study. In brief, TB-5-BT **1** and DBA-5-BT **2** as well as TB-6-BT **5**, showed better tumor accumulation and selectivity, and their *in vivo* application should occur in due course.



Figure 8. Aqueous solubility test for the tryptophan derivatives (1 mM, 20% EtOH in saline).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Experimental procedures for the preparation of both described tryptophan derivatives and biological data including the cell survival assay, cellular accumulation assay, neutron irradiation and LAT-1 blotting are provided. Figures 1-21, $^1\text{H-NMR}$, $^{13}\text{C-DEPT-135 NMR}$, LC-ESI-HRMS and chromatogram; Figure 22, Cytotoxicity of boronopincaol tryptophan derivatives in various concentrations ranging from 1 nM to 100 μM (PDF).

AUTHOR INFORMATION

Corresponding Author

* To whom correspondence should be addressed.
csyu@mx.nthu.edu.tw; Tel: 886-3-5715131ext35582.

Author Contributions

†These authors contributed equally.

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ABBREVIATIONS

BNCT, boron neutron capture therapy; LAT1, leucine-type amino acid transporter 1; T/N, tumor to normal accumulation ratio; BPA, boronophenyl alanine.

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