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Boron Uptake

Remarkable Enhancement in Boron Uptake Within Glioblastoma Cells With Carboranyl–Indole Carboxamides

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Abstract: Novel boron-rich, carboranyl–indole carboxamide ligands were prepared and found to effectively target the 18 kDa translocator protein (TSPO), an upregulated mitochondrial membrane-bound protein which has been observed in variety of tumor cell lines and its expression appears to be proportional to the degree of tumorigenicity, emphasizing a key role in cancer cell proliferation. Both boronated compounds displayed remarkably high affinities for the TSPO. In addition, the in vitro uptake of these compounds into T98G human glioma cells was found to be 25- to 100-fold greater than that of clinical boron neutron capture therapy (BNCT) agents.

Introduction

An ideal anti-cancer therapy would show high selectivity and toxicity for cancer cells only, thus leaving surrounding healthy tissue unaffected by treatment. Given the raft of side-effects associated with modern chemotherapeutic and/or radiotherapeutic regimens, achieving high selectivity and toxicity toward tumor cells with minimal ill effects towards normal, healthy cells remains an elusive goal in cancer therapy. Boron neutron capture therapy (BNCT) is an experimental cancer treatment designed to take advantage of the physicochemical properties of molecular agents containing the non-radioactive ^{10}B isotope (19.8% natural abundance) in a binary therapy incorporating the delivery of boron compounds to the cancer site and subsequent targeted irradiation of the tumour mass with low energy (ca. 0.025 eV) thermal neutrons. Neutron capture by the ^{10}B nucleus results in a series of fission events that generate high linear energy transfer (LET) alpha particles ($^{4}He^{2+}$) and recoiling lithium-7 ($^{7}Li^{3+}$) nuclei. LET particles can travel approximately 5–9 μm in tissue, which is comparable to the size of a typical mammalian cell. Thus, high-energy LET particles produce destructive cellular effects that are limited to those boron-containing cells irradiated by thermal neutrons.[1] In principle, the targeted selectivity and toxicity of BNCT for malignant cells over neighboring normal cells provides a viable pathway in achieving high selectivity in tumor cell kill as long as suitable tumor-selective boronated compounds are used. Such agents require favorable physiological properties in order to act successfully as a useful anti-cancer therapy. Briefly, these features include: high uptake of boron into tumor cells (≈20 μg ^{10}B/g tumour, or ca. 10^{9} ^{10}B nuclei per cell), persistence of ^{10}B within the tumor during thermal neutron irradiation, and rapid clearance of the ^{10}B agent from the circulation and normal tissue. Clinically, BNCT has been focussed on the experimental treatment of high-grade gliomas, recurrent tumors of head and neck, melanoma, hepatic metastasis and mesothelioma. Currently, BNCT agents undergoing human trials include the boron-containing amino acid (*L*)-4-dihydroxyborylphenylalanine (BPA, **1**) and sodium mercaptoundecahydro-*closo*-dodecaborate (BSH, **2**), both of which have demonstrated only modest successes over the past few decades, particularly against the aggressive and intractable malignant brain tumor glioblastoma multiforme (GBM).[2]

The boron-rich cluster, dicarba-*closo*-dodecaborane (commonly known as *closo*-carborane) possesses pseudoaromatic character afforded by the cluster's intrinsic σ-electron delocalization. Carboranes exhibit low toxicity in vivo, are kinetically stable to metabolic degradation, and can improve drug bioavailability when utilized as a unique scaffold in medicinal chemistry.[3] Moreover, the incorporation of *closo*-carborane cage within a BNCT agent may allow greater boron loading capacity than single-boron agents such as BPA given the carbor-

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ane moiety contains 10 boron atoms per cluster. It has been shown that carborane-containing molecules can bind specifically to protein receptors overexpressed in tumor cells such as PSMA, EGFR, 5-lipoxygenase, and the folate receptor,[4] thus once an appropriate tumor target has been selected, carborane-containing molecules represent a practical method for enhanced boron delivery to tumor cells.[5,6]

The 18 kDa translocator protein (TSPO) is a five transmembrane-domain protein consisting of 169 amino acid residues. Formerly known as the peripheral benzodiazepine receptor (PBR), it is chiefly localized at the contact sites between the outer and the inner mitochondrial membranes of steroid-producing cells and various other tissues.[7] TSPO is associated with specific mitochondrial proteins such as the voltage-dependent anion channel (VDAC) and adenine nucleotide transporter (ANT), and is implicated in the regulation of the opening of the mitochondrial permeability transition pore (MPTP). TSPO has been shown to play a role in various physiological functions, including cholesterol transport, steroidogenesis, calcium homeostasis, lipid metabolism, mitochondrial oxidation, cell growth and differentiation, apoptosis induction, MPTP opening, and regulation of immune functions.[7–10] Importantly, TSPO upregulation has been observed in variety of tumor cell lines and its expression appears proportional to the degree of tumorigenicity, emphasizing a key role in cancer cell proliferation which makes TSPO an attractive target for cancer therapy.[11–15]

PK11195 (**3**) is a widely-used ligand for TSPO studies (Figure 1). Despite having high affinity towards TSPO, PK11195 displays non-specific binding, variable kinetic behavior and low bioavailability. Several PK11195-derived carborane analogs were synthesized and probed for their potential to deliver boron in tumour cells and application towards BNCT.[16] However, the tumor cell line (breast cancer) utilized in those experiments are not amenable to current BNCT therapy, which focusses on cranial/extra-cranial tumors. Previously, we have reported that pyrazolopyrimidine-containing carborane compounds represent a new class of boronated agents that can effectively target the TSPO.[3] We observed that in vitro uptake of

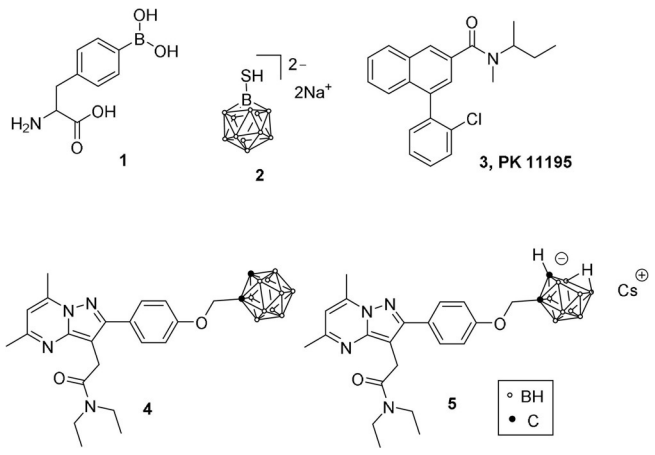


Figure 1. Chemical structures of BNCT agents 1 and 2, PK11195 (3), and previously-reported boronated TSPO ligands 4 and 5.

the carboranyl derivatives **4** and **5** into T98G human glioma cells was significantly higher than clinical agents currently used in BNCT. Compound **4** (19.75 ± 1.13 μg B mg^{-1} protein) was found to deliver one order of magnitude greater amount of boron into T98G cells than either BSH (1.49 ± 0.26 μg B mg^{-1} protein) or the BPA-fructose adduct (BPA-fr) (1.38 ± 0.24 μg B mg^{-1} protein). Moreover, the in vitro cytotoxicities of **4** and **5** were found to be low with $CC_{50} = 219.7 \pm 35.9$ μM and 220.9 ± 40.3 μM, respectively, against the T98G human glioma cell line. We also observed that conversion of *closo*-1,2-carborane **4** to the anionic *nido*-7,8-carborane **5** resulted in a slight loss of binding affinity, as well as a reduction in boron uptake of tumor cells.[3]

In our quest for highly selective, boron-rich, high-affinity ligands for TSPO with low cytotoxicity and an ability to deliver high concentrations of boron to cancer cells, we designed a series of novel carborane-containing indole-based ligands for the TSPO. Recently, a three-dimensional high-resolution structure of mammalian TSPO reconstituted in detergent micelles associated with its high-affinity ligand PK11195 has been reported.[17] The new ligands reported here are based on the ligand-based structure activity relationship (SAR) data of known TSPO ligands and a known pharmacophore model (Figure 2).[7,18] Herein we report the synthesis of two novel carboranyl-indole carboxamides, their affinity for the TSPO, and biological evaluation which specifically entails a study of their capacity to deliver high concentrations of boron in T98G human glioma cells in the absence of thermal neutrons.

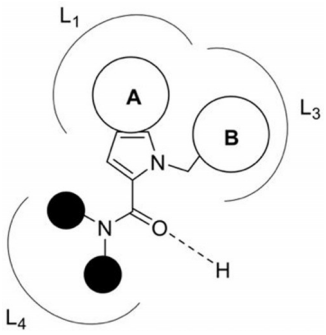
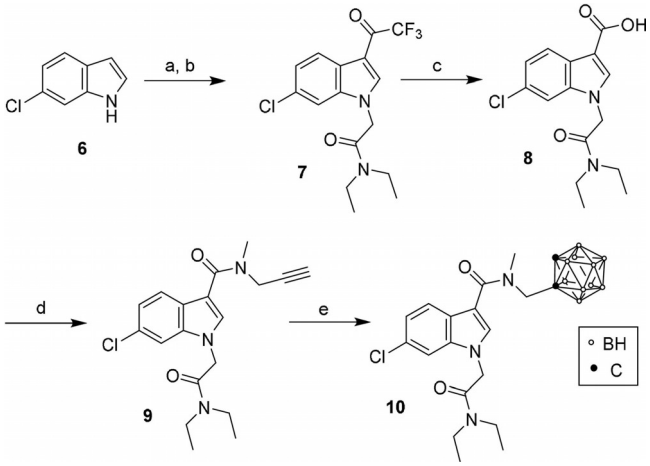


Figure 2. Design of TSPO ligands. As per the TSPO pharmacophore model:[7,18] pocket L_1 is occupied by ring A, pocket L_3 is occupied by ring B and pocket L_4 is occupied by alkyl substituents on the amide nitrogen. H-bond donor group of the amide carbonyl is crucial for the activity and any functional modification results in the loss of activity.

Results and Discussion

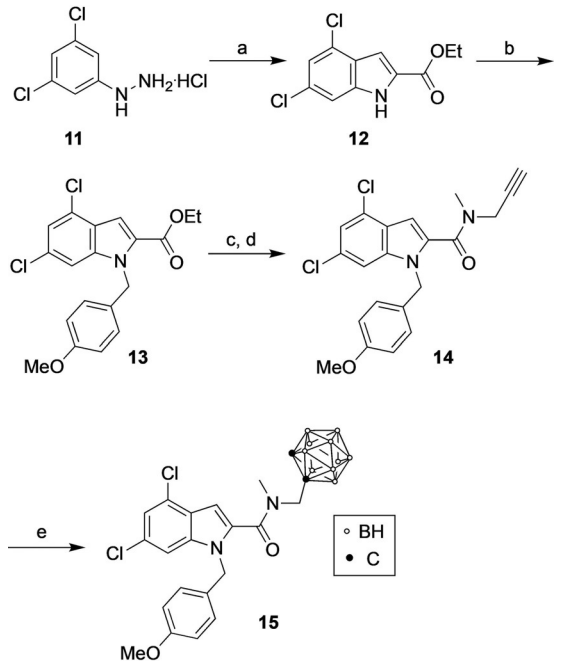
The synthesis of carboranyl indole 3-carboxamide was commenced from the commercially available 6-chloroindole, as shown in Scheme 1. *N*-alkylation of indole **6** was achieved using sodium hydride (NaH) and *N*,*N*-diethylchloroacetamide in DMF followed by addition of trifluoroacetic anhydride to afford 3-trifluoroacetyl indole **7**. Basic hydrolysis of trifluoromethyl ketone furnished acid **8** which upon treatment with oxalyl chloride generated the acid chloride derivative, which was followed by amidation with *N*-methylpropargylamine to yield



Scheme 1. Synthetic route to carboranyl indole 3-carboxamides. Reagents and conditions: (a) 1. NaH, *N*,*N*-diethyl chloroacetamide, 0 °C to RT, 1 h 2. Trifluoroacetic anhydride, 0 °C, 2 h, quantitative; (b) KOH, EtOH:H_2O, reflux, 4 h, 70%; (c) 1. Oxalyl chloride, CH_2Cl_2, RT, 2 h (d) *N*-Methylpropargylamine, Et_3N, CH_2Cl_2, 0 °C to RT, 2 h, 75%; (e) *nido*-decaborane, CH_3CN, toluene, reflux, 24 h, 35%.

indole 3-carboxamide **9**. *Closo*-1,2-carboranyl indole **10** was prepared by the reaction of **9** with the decaborane-acetonitrile adduct (prepared in situ from *nido*-decaborane and CH_3CN) under reflux conditions. Purification by means of silica column chromatography gave the desired **10** as a pale-yellow solid.

The synthetic route for carboranyl indole 2-carboxamide (Scheme 2) began with commercially-available 3,5-dichlorophenyl hydrazine hydrochloride **11** which was converted to hydrazone using ethyl pyruvate under reflux conditions, followed by the Fischer indole cyclization using polyphosphoric acid at 120 °C to give indole ester **12**. *N*-benzylation of indole ester **12** occurred upon treatment with NaH and PMB-chloride to provide ester **13**. Basic hydrolysis of **13** under reflux conditions gave the corresponding acid, which was converted to the acid chloride using oxalyl chloride. This was followed by amidation with commercially available *N*-methylpropargylamine to provide the indole carboxamide **14**. *Closo*-1,2-carboranyl indole **15** was obtained by reaction of propargyl amide **14** with decaborane-acetonitrile adduct under reflux conditions to yield carboranyl indole 2-carboxamide **15** as an off-white solid.



Scheme 2. Synthetic route to carboranyl indole 2-carboxamides. Reagents and conditions: (a) 1. AcONa, EtOH, reflux, 2 h 2. PPA, 120 °C, 30 min, 70%; (b) NaH, 4-methoxybenzyl chloride, DMF, 0 °C to RT, overnight, 81%; (c) KOH, EtOH:H2O, reflux, 2–8 h, 91%; (d) 1. Oxalyl chloride, CH_2Cl_2, RT, 2 h 2. *N*-methylpropargyl amine, Et_3N, CH_2Cl_2, 0 °C to RT, 75%; (e) *nido*-decaborane, CH_3CN, toluene, reflux, 24 h, 27%.

The structures of both **10** and **15** were confirmed by multinuclear (^1H, ^{13}C and ^{11}B) NMR spectroscopy and high-resolution ESI-MS, and their purity was confirmed by HPLC prior to biological evaluation. In the $^{13}C\{^1H\}$ NMR spectrum of **10**, the resonances at 75.2 and 61.1 ppm are attributed to the two carborane cluster carbon atoms. Similarly, $^{13}C\{^1H\}$ NMR spectrum of **15** displayed resonances at 73.7 and 60.3 ppm. Compounds **10** and **15** exhibit five unique boron atom environments between −1 to −13 ppm in their $^{11}B\{^1H\}$ NMR spectra, indicating the presence of an intact *closo*-1,2-carborane moiety.

Having confirmed their molecular structures, boron-rich indole carboxamides **10** and **15** were then assessed for their ability to bind TSPO in a competitive membrane binding assay using [^{3}H]-PK11195 as a radioligand using mitochondrial fractions isolated from the rat kidney as the receptor source. Both compounds competitively inhibited the binding of [^{3}H]-PK11195 and displayed high affinity for TSPO. Compounds **10** and **15** competitively inhibited the binding of [^{3}H]-PK11195 with an affinity of 0.49 ± 0.05 nM and 294.5 ± 30.0 nM, respectively. Unlike compound **4**, which revealed a unique two-site binding configuration (a high-affinity and a low-affinity binding site), the displacement of [^{3}H]-PK11195 by compounds **10** and **15** was best fit to a one-site model. The binding affinity of compound **15** is in accordance with the previously-reported carboranyl TSPO ligands.[3,16] However, compound **10** with a $K_i = 0.49 \pm 0.05$ nM displays the highest TSPO affinity of a boron-containing ligand reported to date.

Low cytotoxicity of boronated agents is a pivotal parameter for BNCT that allows for high boron levels to be accumulated within the tumor. Compounds **10** and **15** displayed no discernible in vitro cytotoxicity at concentrations of 100 μM against T98G human glioma cells, a clinically-amenable tumour cell line for BNCT, or SVGp12 human glial cells. When compared to the lipophilic phosphonium-carborane ligands, *closo*-1,2-carboran-1-ylmethyl)triphenylphosphonium bromide (CC_{50} = of 21.1 ± 1.4 μM) and 7,8-dicarba-*nido*-undecaborane-7-ylmethyl)-triphenylphosphonium ($CC_{50} = 60.6 \pm 19.0$ μM) which contain a *closo*- and *nido*-carborane cage, respectively, compounds **10** and **15** display far less cytotoxicity against the same cancer cell line.[19] PK11195-derived *closo*-carboranyl ligands have also been reported to display higher cytotoxicity ($CC_{50} = 116.0 \pm 4.1$ μM) against breast tumor cell lines.[16]

Cellular accumulation studies of **10** and **15** within cultured T98G cells were conducted in order to determine the boron

delivery characteristics of these novel compounds within human glioma cells (Figure 3). T98G cells were treated with **10** and **15** for 72 h at concentrations of 100 μM, followed by determination of boron levels by means of inductively-coupled

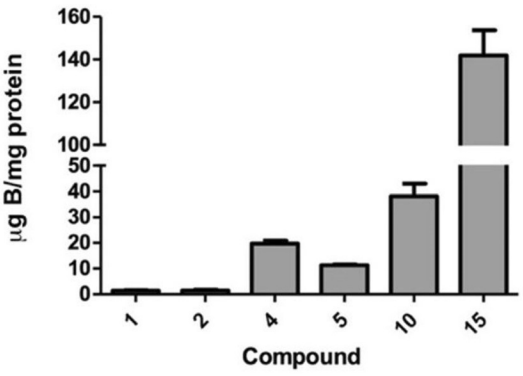


Figure 3. ICP-OES determination of boron levels in T98G human glioma cells treated with **1**, **2**, **4**, **5**, **10** and **15**.

plasma optical emission spectroscopy (ICP-OES). Compounds **10** and **15** exhibited remarkably greater boron uptake in T98G cells when compared to clinical BNCT agents **1** and **2**. Compound **10** (38.18 μg B mg^{-1} protein) was found to deliver a 25-fold greater amount of boron into T98G cells than either BSH **2** (1.49 ± 0.26 μg B mg^{-1} protein) or BPA **1** (1.38 ± 0.24 μg B mg^{-1} protein). Similarly, when compared to **4** (19.75 ± 1.13 μg B mg^{-1} protein) and **5** (11.28 ± 0.28 μg B mg^{-1} protein), compound **10** delivered higher amounts (two to four-fold, respectively) of boron to T98G cells. Furthermore, compound **15** (141.86 μg B mg^{-1} protein) delivered remarkably high amounts of boron into T98G cells, with boron levels determined to be two orders of magnitude greater than that of the clinical BNCT agents **1** and **2**. For comparison, lower levels of boron were found for **10** (3.12 ± 0.19 μg B mg^{-1} protein) and **15** (33.52 μg ± 1.11 B mg^{-1} protein) in normal glial cells (SVGp12), indicating that high accumulation of these compounds in T98G cells is inconsistent with a passive diffusion mechanism alone. Indeed, the clinical BNCT agents **1** and **2** are transported into tumor cells by an active L-amino-acid transport mechanism and passive diffusion, respectively, neither of which are likely to be dominant in the case of **10** or **15**.[1] In comparison with the high-affinity TSPO ligands **4** and **5**, compound **15** was found to deliver even greater amount of boron (7- to 14-fold) into T98G cells. Intracellular boron accumulation of species **10** and **15** was also found to exceed that of mitochondrially targeted *closo*-carborane-phosphonium salts by 5- to 17-fold within the same tumor cell line.[19]

Conclusions

Two novel boron-rich, carboranyl–indole carboxamide ligands were synthesized and found to effectively target TSPO. Both compounds (**10** and **15**) displayed very high affinities for the TSPO with K_i values of 0.49 ± 0.05 nM and 294.5 ± 30.0 nM, respectively. The in vitro uptake of **10** and **15** into T98G human glioma cells was found to be 25- to 100-fold greater than clinical BNCT agents **1** and **2**, and also displayed 4- to 12-fold greater boron uptake than that determined in a normal glial cell line. Both compounds **10** and **15** were also found to be significantly less toxic toward cultured T98G human glioma cells and exhibited no discernible cytotoxicity up to 100 μM. Carboranyl indole **15** had a lower binding affinity for TSPO when compared to **10**, however the cellular accumulation of **15** was found to be approximately four-fold higher. This result is most likely attributed to the differences associated with measuring TSPO binding affinities using rat kidney mitochondrial fractions versus cellular uptake using intact human tumor cells. Furthermore, drug parameters such as lipophilicity are unlikely to play a major role in the significant cellular uptake of **10** and **15** into T98G cells by means of passive diffusion as much lower levels of boron uptake were found in the normal glial cell line. In conclusion, this particular study demonstrates a significant advance in the development of tumor-cell targeted BNCT agents through the targeting of a key mitochondrial receptor protein. In vivo studies involving thermal neutrons are currently being planned and will be reported in due course.

Experimental Section

Syntheses

General

All glass apparatus were oven-dried prior to use. All chemicals used were purchased from Aldrich Chemical Co. Ltd. (St Louis, MO, USA). All solvents were distilled by standard techniques prior to use. Where stated, reactions were performed under an inert atmosphere of nitrogen using syringe-septum cap techniques or a Schlenk manifold. ^{1}H NMR spectra were recorded at 300 MHz and 400 MHz, ^{13}C{^{1}H} NMR spectra were recorded at 75 MHz and 125 MHz, and ^{11}B{^{1}H} NMR spectra were recorded at 128 MHz using Bruker 300 and Bruker 500 spectrometers. Chemical shifts (δ) are quoted in parts per million (ppm). Tetramethylsilane (TMS) was used as an internal reference for ^{1}H and ^{13}C{^{1}H} NMR spectra and $BF_3{\cdot}OEt_2$ was used for ^{11}B{^{1}H} NMR spectra (0 ppm). All coupling constants (J) are given in Hertz and the splitting patterns are designed as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), dt (doublet of triplets), q (quartet), and m (multiplet). Low- and high-resolution electrospray ionization (ESI) MS was carried out using a Bruker (USA) Daltronics BioApexII with a 7T superconducting magnet and an analytical ESI source. Thin-layer chromatography was performed using Merck aluminium backed plates, precoated with silica (0.2 mm, 60F_{254}), which were developed using one of the following techniques: UV fluorescence (254 nm) and iodine vapors. Flash chromatography was performed on silica gel (Merck silica gel 60 H, particle size 5–40 μm).

2-(6-chloro-3-(2,2,2-trifluoroacetyl)-1H-indol-1-yl)-*N*,*N*-diethylacetamide (7)

To a stirred solution of 6-chloroindole **6** (1.0 g, 6.60 mmol) in DMF (10 mL) was added sodium hydride (528 mg, 13.19 mmol) in portions at 0 °C and the mixture was stirred at RT for 15 min. 2-Chloro-*N*,*N*-diethylamide (0.952 mL, 6.93 mmol) was then added to the mixture at 0 °C and it was stirred for 30 min. Trifluoroacetic anhydride (1.11 mL, 7.92 mmol) was then added dropwise whilst main-

taining the temperature at 0 °C, and the mixture was stirred for 3 h. Upon completion (TLC), the reaction was then quenched with a saturated solution of sodium carbonate in H_2O (10 mL). The precipitate formed was filtered off and air-dried to afford **7** as a creamy-brown solid in quantitative yield which was used in the next step without any further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.30 (d, J = 8.7 Hz, 1H), 7.95 (d, J = 1.5 Hz, 1H), 7.32 (dd, J = 8.6 Hz, J = 1.7 Hz, 1H), 7.23 (d, J = 1.5 Hz, 1H), 4.92 (s, 2H), 3.48–3.39 (m, 4H), 1.33 (t, J = 7.2 Hz, 3H), 1.17 ppm (t, J = 7.1, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 164.2, 139.7, 138.1, 131.1, 125.6, 124.9, 124.0, 110.5, 48.5, 41.9, 41.4, 14.8, 13.2 ppm.

6-chloro-1-(2-(diethylamino)-2-oxoethyl)-N-methyl-N-(prop-2-ynyl)-1H-indole-3-carboxamide (9)

A suspension of 2-(6-chloro-3-(2,2,2-trifluoroacetyl)-1H-indol-1-yl)-*N*,*N*-diethylacetamide **7** (1.0 g, 2.78 mmol) and KOH (156 mg, 2.78 mmol) were stirred and refluxed at 95 °C for 4 h in EtOH:H_2O (10 mL, 2:1 (*v/v*)). Upon completion (TLC), the mixture was cooled to ambient temperature and the solvent was removed in vacuo. The reaction mixture was diluted with water and extracted with ethyl acetate. The aqueous layer was cooled to 0 °C and acidified with hydrochloric acid (2 M) to pH 2 to give an oily solid. This solid was then extracted with ethyl acetate, washed with water, brine, dried over anhydrous $MgSO_4$ and rotary evaporated to afford the corresponding carboxylic acid as a light brown solid which was used in the next step without any further purification (533 mg, 70%). This product (400 mg, 1.30 mmol) was dissolved in dried dichloromethane (5 mL) at RT, and DMF (1 drop) was added while stirring. To this solution was added oxalyl chloride (122 μL, 1.43 mmol) dropwise and the mixture stirred for 2 h. The reaction mixture was then concentrated in vacuo. It was then dissolved in dried dichloromethane (10 mL) and cooled to 0 °C. *N*-methylpropargylamine (131 μL, 1.55 mmol) and Et_3N (541 μL, 3.89 mmol) were then added the reaction mixture was stirred at RT for 1 h. Upon completion, the mixture was diluted with dichloromethane (100 mL) and washed with water (100 mL). The aqueous layer was extracted with dichloromethane (2×). Combined organic layer was washed with water, brine, dried over anhydrous magnesium sulfate, and dried in vacuo. The crude product was then purified by column chromatography (eluent: 15–50% ethyl acetate in *n*-hexane) to afford the desired product as a pale-yellow solid (257 mg, 55%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.81 (d, J = 2.4 Hz, 1H), 7.36 (s, 1H), 7.15 (dd, J = 8.7 Hz, J = 1.8 Hz, 1H), 4.70 (s, 2H), 4.31 (d, J = 2.4 Hz, 2H), 3.46–3.37 (m, 4H), 3.19 (s, 3H), 2.31 (t, J = 2.4 Hz, 1H), 1.23 (t, J = 7.1 Hz, 3H), 1.12 ppm (t, J = 7.2 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 166.6, 165.4, 137.2, 132.1, 129.2, 125.9, 122.7, 122.2, 110.6, 109.8, 79.4, 72.8, 47.9, 41.8, 41.1, 14.7, 13.2 ppm.

6-chloro-1-(2-(diethylamino)-2-oxoethyl)-N-methyl-N-(*closo*-1,2-carboran-1-ylmethyl) -1*H*-indole-3-carboxamide (10)

To a solution of *nido*-decaborane (42 mg, 0.342 mmol) and acetonitrile (0.146 mL, 2.787 mmol) in toluene (25 mL), was added 6-chloro-1-(2-(diethylamino)-2-oxoethyl)-N-methyl-N-(prop-2-ynyl)-1H-indole-3-carbox-amide **9** (100 mg, 0.279 mmol). The resulting mixture was stirred under reflux overnight. The solvent was removed under reduced pressure and the residual oil was purified by column chromatography (*n*-hexane-EtOAc solvent gradient system: 100% *v/v* *n*-hexane followed by 20% *v/v* EtOAc, R_f = 0.5) to give the product as an off-white solid (44 mg, 33%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.80 (d, J = 2.4 Hz, 1H), 7.43 (s, 1H), 7.20 (dd, J = 4.5 Hz, J = 2.7 Hz, 1H), 4.84 (s, 2H), 4.42 (s, 2H), 3.45–3.38 (m, 4H), 3.32 (s, 3H), 2.90–1.70 (m, 10H), 1.30 (t, J = 7.0 Hz, 3H), 1.15 ppm (t, J = 7.0 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 166.3, 165.2, 137.5, 131.4, 129.1, 124.1, 121.5, 119.2, 109.6, 104.2, 75.2, 61.1, 47.9, 43.5 41.7, 41.1, 14.6, 13.1 ppm. ^{11}B NMR (128 MHz, $CDCl_3$): δ = −1.15 (1B), −5.11 (1B), −10.81 (2B), −12.16 ppm (6B). ESI-MS $[M+H]^+$ *m/z* 479.2 (calc. 479.1). Anal. Calcd. for $C_{19}H_{32}B_{10}ClN_3O_2$: C 47.74, H 6.75, N 8.79. Found: C 47.51, H 7.11, N 9.07.

Ethyl 4,6-dichloro-1H-indole-2-carboxylate (12)

To a suspension of 3,5-dichlorophenyl hydrazine (1 g, 4.684 mmol) in ethanol, sodium acetate (385 mg, 4.684 mmol) and ethyl pyruvate (0.52 mL, 4.684 mmol) were added and the mixture was refluxed for 3 h. Upon completion, the mixture was filtered and the residue was washed with hot ethanol (3×). The combined filtrates were evaporated in vacuo to afford the hydrazone which was used in the next step without further purification. Polyphosphoric acid (2.7 mL) was added to the hydrazone and the resulting mixture was heated at 120 °C for 45 min. Upon completion (TLC), the reaction mixture was cooled to ambient temperature and then to 0 °C. The mixture was diluted with water and the pH was adjusted to 10 using KOH. The precipitated solid was filtered off, washed with water, *n*-hexane and air-dried to afford the desired product as pale-yellow solid. The crude compound was crystallized from dichloromethane-hexane to afford the pure compound as a pale-yellow, fluffy solid (1.05 g, 87%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.42 (brs, 1H), 7.35 (t, J = 1.2 Hz, 1H), 7.29 (dd, J = 2.1 Hz, J = 1.2 Hz, 1H), 7.18 (d, J = 1.2 Hz, 1H), 4.47 (q, J = 7.2 Hz, 2H), 1.46 ppm (t, J = 7.2 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 161.8, 137.2, 131.2, 128.6, 128.5, 125.5, 121.4, 110.6, 107.2, 61.7, 14.5 ppm.

Ethyl 4,6-dichloro-1-(4-methoxybenzyl)-1H-indole-2-carboxylate (13)

NaH (60% emulsion in mineral oil, 117 mg, 2.906 mmol) was added to a solution of ethyl 1*H*-indole-2-carboxylate **12** (500 mg, 1.937 mmol) in DMF at 0 °C and the mixture stirred for 30 min. 4-methoxy benzyl chloride (0.394 mL, 2.906 mmol) was then added and the resulting mixture was stirred at ambient temperature for 16 h. Upon completion (TLC), the reaction mixture was diluted with EtOAc and washed with water. The aqueous layer was extracted with EtOAc (3×). The combined organic layer was washed with water (3×), brine, dried over anhydrous $MgSO_4$ and rotary evaporated. The crude compound was purified by column chromatography (ethyl acetate/*n*-hexane, 1:9) to afford the desired product as a colorless solid (650 mg, 88%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.41 (d, J = 0.9 Hz, 1H), 7.28 (d, J = 1.5 Hz, 1H), 7.16 (d, J = 1.5 Hz, 1H), 6.99 (td, J = 8.7 Hz, J = 2.1 Hz, 2H), 6.79 (td, J = 8.7 Hz, J = 2.1 Hz, 2H), 5.71 (s, 2H), 4.35 (q, J = 7.2 Hz, 2H), 1.38 ppm (t, J = 7.2 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 161.9, 159.1, 139.8, 131.1, 129.4, 129.0, 128.6, 127.7, 124.0, 121.4, 114.2, 109.7, 109.4, 61.1, 55.3, 48.0, 14.4 ppm.

4,6-dichloro-1-(4-methoxybenzyl)-N-methyl-N-(prop-2-yn-1-yl)-1H-indole-2-carboxamide (14)

A mixture of ethyl 4,6-dichloro-1-(4-methoxybenzyl)-1H-indole-2-carboxylate **13** (600 mg, 1.586 mmol) ethyl ester, and KOH (267 mg, 4.759 mmol) in ethanol: water (2:1, 10 mL) was heated for 4 h at 100 °C. Upon completion (TLC), the mixture was cooled to room temperature and evaporated in vacuo. The residue was diluted with water and acidified using 2 M HCl. The precipitate that was formed was filtered off and dried in vacuo to afford the de-

sired carboxylic acid as a colorless solid (520 mg, 93%). To a solution of the carboxylic acid (500 mg, 1.428 mmol) in dichloromethane (10 mL) and dimethylformamide (1 drop, cat.), oxalyl chloride (0.184 mL, 2.142 mmol) was slowly added dropwise and stirred at room temperature for 2 h under a nitrogen atmosphere. The solvent and excess of oxalyl chloride were removed at reduced pressure and the crude material dried under vacuum. To the residue, dissolved in dry dichloromethane (10 mL) and cooled to 0 °C, a mixture of the *N*-methyl propargylamine (0.181 mL, 2.142 mmol) and triethylamine (0.595 mL, 4.283 mmol) in dry dichloromethane (5 mL) was added dropwise. The mixture was stirred at room temperature for 12 h (TLC), and evaporated. The crude residue was dissolved in dichloromethane (50 mL), washed with 1 M HCl, saturated $NaHCO_3$, water, dried over anhydrous $MgSO_4$, and concentrated in vacuo. Purification by column chromatography on silica gel (20 to 30% EtOAc in *n*-hexane) provided the title compound as pale-yellow sticky solid (30 mg, 66%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.30$ (s, 1H), 7.15 (s, 1H), 7.01 (d, $J = 8.4$ Hz, 2H), 6.88–6.74 (m, 3H), 5.37 (s, 2H), 4.11 (s, 2H), 3.74 (s, 3H), 3.04 (s, 3H), 2.31 ppm (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 163.6$, 159.4, 138.4, 132.5, 129.8, 129.5, 128.6, 127.8, 127.3, 124.5, 121.1, 109.4, 103.5, 78.1, 72.1, 55.5, 47.9, 39.8, 34.1 ppm.

4,6-dichloro-1-(4-methoxybenzyl)-N-methyl-N-(*closo*-1,2-carboran-1-ylmethyl)-1H-indole-2-carboxamide (15)

To a solution of *nido*-decaborane (38 mg, 0.306 mmol) and acetonitrile (0.13 mL, 2.492 mmol) in toluene (25 mL) was added 4,6-dichloro-1-(4-methoxybenzyl)-N-methyl-N-(prop-2-yn-1-yl)-1H-indole-2-carboxamide **14** (100 mg, 0.249 mmol). The resulting mixture was stirred under reflux overnight. The solvent was then removed under reduced pressure and the residual oil was purified by column chromatography (*n*-hexane-EtOAc: 100% *v/v* *n*-hexane followed by 20% *v/v* EtOAc, $R_f = 0.45$) to give the product as an off-white solid (42 mg, 31%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.33$–7.31 (m, 1H), 7.22 (d, $J = 1.2$ Hz, 1H), 6.91 (d, $J = 8.0$ Hz, 2H), 6.86–6.81 (m, 3H), 5.43 (s, 2H), 4.21 (s, 2H), 3.80 (s, 3H), 3.19(s, 3H), 2.90–1.72 ppm (m, 10H). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 164.7$, 159.3, 138.5, 130.6, 130.3, 129.0, 127.7, 127.4, 123.8, 121.3, 114.3, 109.1, 104.8, 73.7, 60.3, 55.3, 53.5, 47.5, 39.6 ppm. ^{11}B NMR (128 MHz, $CDCl_3$): $\delta = -1.08$ (1B), −4.89 (1B), −10.39 (2B), −12.64 ppm (6B). ESI-MS $[M+H]^+$ *m/z* 520.6 (calc. 520.5). Anal. Calcd. for $C_{21}H_{28}B_{10}Cl_2N_2O_2$: C 48.55, H 5.43, N 5.39. Found: C 48.65, H 5.71, N 5.62.

Biological assays

Radioligand binding assay

Mitochondria were prepared as previously described,[20] with minor modifications as described below, from kidneys of male Wistar rats killed by cervical dislocation. Kidneys were homogenized in 20 volumes of ice-cold 50 mM Tris/HCl, pH 7.4, 0.32 M sucrose and 1 mM EDTA (buffer A), containing protease inhibitors (160 mg mL^{-1} benzamidine, 200 mg mL^{-1} bacitracine and 20 mg mL^{-1} soybean trypsin inhibitor) with a Teflon pestle in a glass homogenizer and centrifuged at 600×g for 10 min at 4 °C. The resulting supernatant was centrifuged at 10000×g for 10 min at 4 °C. The pellet was then re-suspended in 20 volumes of ice-cold buffer A and centrifuged again at 10000×g for 10 min at 4 °C. The crude mitochondrial pellet was frozen at −20 °C until the time of assay or incubated with 0.6 nM [^{3}H]PK11195 in 50 mM Tris/HCl, pH 7.4 (buffer B), with a range of concentrations of the tested compounds (0.1 nM–10 μM) in a total volume of 0.5 mL for 90 min at 4 °C. The incubation was terminated by dilution to 5 mL with ice-cold buffer B, followed immediately by rapid filtration through glass-fiber filters (Whatman GF/C). The filters were then washed with buffer B (2.5 mL) and the amount of radioactivity retained on the filters was determined using a Packard 1600 TR liquid scintillation counter at 66% efficiency. Non-specific binding was estimated in each case in the presence of 1 mM of unlabelled PK11195. The CC_{50} values were determined and K_i values were derived according to the equation derived previously.[21] Protein concentration was estimated by the method of Lowry et al.[22] with bovine serum albumin (BSA) as standard.

Cell uptake studies

Human glioblastoma multiforme (T98G) cells and human glial cells (SVGp12) were purchased from the ATCC. The cells were maintained as monolayers in minimum essential medium (MEM), supplemented with 10% fetal bovine serum, penicillin (100 units mL^{-1}), streptomycin (100 g mL^{-1}) and L-glutamine (2.5 mM), at 37 °C in a humidified 5% CO_2 atmosphere. Stock solutions of **10** and **15** (10 mM in DMSO) were freshly prepared. The T98G cells were separately cultured as a monolayer in 75 cm^2 flasks to 70–80% confluence then incubated with the drug-containing culture medium (final concentration 100 μM) for 72 h at 37 °C in a humidified 5% CO_2 atmosphere. The medium was removed and the cells washed once with PBS (2 mL). Cells were harvested by 5 min incubation with trypsin (3 mL) followed by washing with further PBS (3 mL). Harvested cells were pipetted to a single cell suspension then divided into aliquots (100 μL) for protein analysis. The remaining cells were sedimented by centrifugation at 2000 rpm for 3 min, then the supernatant was removed and the cell pellet was analysed for boron content. Microwave digestion and measurement of boron concentration by ICP-OES, according to previously established procedures.[3] The cell pellets were transferred to pre-cleaned TFM vessels with MQ-water (2×0.25 mL) then dried for 40 min at 100 °C in a fan forced oven and cooled to room temperature. The cell pellets were suspended in HNO_3 (70%, 7.5 mL) and H_3PO_4 (85%, 2.5 mL) and subjected to two high-pressure microwave digestion cycles using a Milestone Ethos Plus microwave lab station. The microwave digestion program involved heating to 200 °C over 10 min, holding for 10 min then heating to 240 °C over 5 min and sustaining this temperature for a further 20 min. Between program cycles, the closed sample vessels were cooled to 150-100 °C. At the end of the digestion process, the closed vessels were cooled to room temperature to minimize volatile analyte losses, and the resulting homogeneous solutions diluted with MQ-water to a final volume of 30 mL. ICP-OES analyses were performed in triplicate by using a PerkinElmer Optima DV7300 ICPOES at a plasma gas flow of 15 L min^{-1}, auxillary gas flow of 0.5 L min^{-1}, nebuliser gas flow of 0.7 L min^{-1} using a Burgener PEEK Mira Mist nebuliser with cyclonic spray chamber and pump rate of 0.5 L min^{-1}. Yttrium (1 ppm) was used as an internal standard by online injection to correct for loss of analyte during sample preparation or sample inlet. Standard solutions of boric acid (0, 0.05, 0.1, 0.5, 1 and 5 ppm diluted from certified 1000 mg L^{-1} boric acid) were used to prepare a calibration plot. To account for any variations in total cell number, the measured boron content was normalized to cell protein measured in the corresponding cell preparations and expressed in units of μg B mg^{-1} protein. The analysis of protein content in cell pellets is described below.

Protein analyses

The bicinchoninic acid (BCA) protein assay was used to determine protein concentration, as described previously.[3,18] A BSA protein

standard curve was prepared each time the assay was performed. Lysis of cells was achieved using three snap freeze-thaw cycles and cell debris was sedimented by centrifugation at 13 300 rcf for 5 min. The supernatant solution was then analyzed for protein content by taking repeated 15 μL samples (n = 3) of blank, 1 mg mL^{-1} BSA protein standard (200, 400, 800 and 1000 μg mL^{-1}, made up to volume with MQ-water) or boron-containing compound treated protein samples and depositing these into a 96-well plate format. Next, a freshly prepared solution of commercially-sourced BCA and $CuSO_4 \cdot 5H_2O$ (50:1, 285 μL) was added to each well and the mixture incubated at 37 °C for 45 min. Absorbance was then measured at 560 nm using an VictorX multi-wavelength plate reader (PerkinElmer) and protein content was determined by comparison to the BSA standard curve.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: boron neutron capture therapy · carborane · glioblastoma · indoles · translocator protein

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