Supplementary Information

Synthesis and cellular uptake of boron-rich pyrazolopyrimidines: Exploitation of the translocator protein for the efficient delivery of boron into human glioma cells

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Contents list

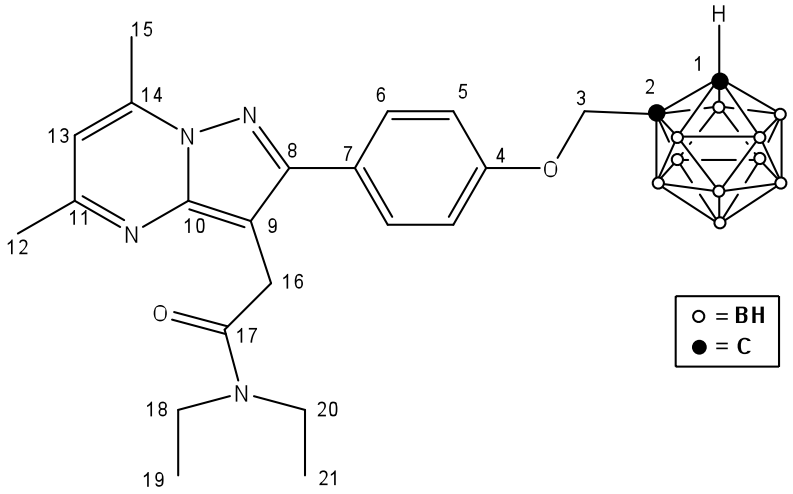
Experimental Details

General

^{1}H, $^{13}C\{^{1}H\}$ and $^{11}B\{^{1}H\}$ NMR spectra were recorded at 300 K on a Bruker AVANCE 400 MHz DRX spectrometer (^{1}H at 400 MHz, ^{13}C at 101 MHz and ^{11}B at 128 MHz). All NMR signals are reported in ppm. ^{1}H NMR spectra were referenced to either TMS (δ 0 ppm) or residual $(CD_3)_2CO$ (δ 2.05). $^{11}B\{^{1}H\}$ NMR spectra were referenced to an external standard $BF_3.OEt_2$ (δ 0 ppm). Coupling constants ($^{n}J_{ij}$) are reported in Hz. Peak multiplicities are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), b (broad). Low resolution ESI-MS data was collected using a Finnigan LCQ detector. Elemental analyses were carried out by Campbell Microanalytical Laboratory, Chemistry Department, The University of Otago, Dunedin, New Zealand. Solvents and reagents were dried according to literature methods.[1] *Nido*-decaborane(14) was purchased from Katchem Pty Ltd (Czech Republic). Compound **3** was synthesised according to the method of Reynolds *et al*.[2]

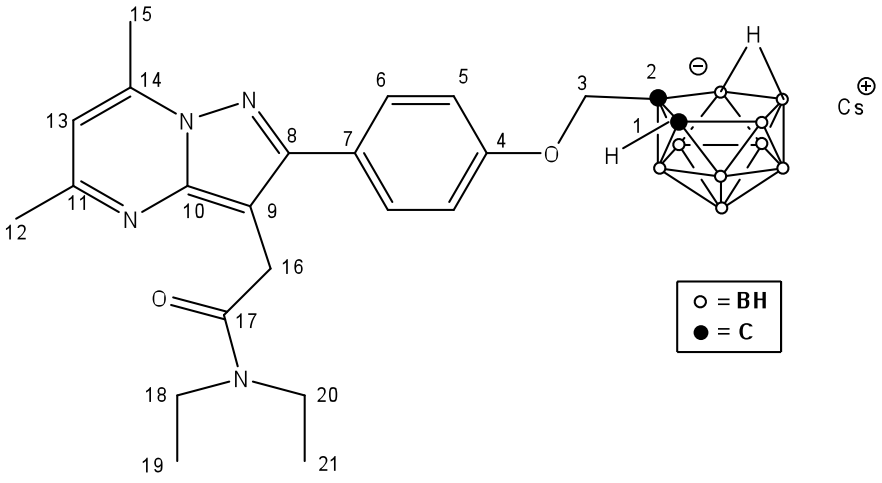
Synthesis

N,N-Diethyl-2-(2-[4-(closo-1,2-carboran-1-ylmethoxy)-phenyl]-5,7-dimethylpyrazolo[1,5-a]pyrimidin-3-yl)-acetamide ***(4)***



To a solution of *nido*-decaborane(14) (33 mg, 0.27 mmol) and acetonitrile (0.1 mL, 2.2 mmol) in toluene (25 mL), 2-(5,7-dimethyl-2-(4-(prop-2-yn-1-yloxy)phenyl)pyrazolo[1,5-*a*]pyrimidin-3-yl)-*N*,*N*-diethylacetamide **3**[2] (86 mg, 0.22 mmol) was added dropwise. The resulting mixture was stirred at reflux overnight. The solvent was removed under reduced pressure and the residual oil was purified by column chromatography (DCM-methanol solvent gradient system: 100% *v/v* DCM followed by 10% *v/v* MeOH, R_f = 0.6) to give the product as an off-white solid (40 mg, 35%). ^{1}H NMR ($CDCl_3$) δ: 7.82 (d, 2H, $^3J_{HH}$ = 6.8 Hz, PhH_6), 6.92 (d, 2H, $^3J_{HH}$ = 6.8 Hz, PhH_5), 6.52 (s, 1H, H_{13}), 4.45 (s, 2H, H_3), 4.11 (bs, 1H, H_1), 3.91 (s, 2H, H_{16}), 3.52 (q, 2H, $^3J_{HH}$ = 7.2 Hz, H_{20}), 3.39 (q, 2H, $^3J_{HH}$ = 7.2 Hz, H_{18}), 2.74 (s, 2H, H_{15}), 2.54 (s, 2H, H_{12}), 1.21 (t, 3H, $^3J_{HH}$ = 6.8 Hz, H_{21}), 1.10 (t, 3H, $^3J_{HH}$ = 6.8 Hz, H_{19}). ^{13}C NMR ($CDCl_3$) δ: 170.0 (C_{17}), 157.7 (C_{11}), 157.1 (C_4), 154.3 (C_{10}), 147.6 (C_8), 144.7 (C_{14}), 130.3 (C_6), 128.4 (C_7), 114.7 (C_5), 108.4 (C_{13}), 100.9 (C_9), 71.5 (C_2), 69.2 (C_3), 57.7 (C_1), 42.3 (C_{20}), 40.6 (C_{18}), 28.1 (C_{16}), 24.6 (C_{12}), 16.9 (C_{15}), 14.3 (C_{21}), 13.1 (C_{19}).^{11}B NMR ($CDCl_3$) δ: -2.61 (1B), -4.37 (1B), -8.91 (2B), -11.47(2B), -12.79 (4B). ESI-MS $[M+H]^+$ *m/z* 509.4. Anal. Calcd. for $C_{23}H_{36}B_{10}N_4O_2$: C 54.31, H 7.13, N 11.01. Found: C 54.17, H 7.04, N 10.78.

*Cesium ((4-(3-(2-(diethylamino)-2-oxoethyl)-5,7-dimethylpyrazolo[1,5-a]pyrimidin-2-yl)phenoxy)methyl)-7,8-dicarba-nido-undecaboran-7-ate. (Cs·**5**)*



To a solution of **4** (40 mg, 0.08 mmol) in EtOH (15mL), cesium fluoride (36 mg, 0.24 mmol) was added and the mixture stirred at reflux overnight. The solvent was removed under reduced pressure, the residue dissolved in acetone and the insoluble white borate product was removed by filtration. The

acetone was removed under reduced pressure and the residue recrystallised from water/acetone to give a white solid (30 mg, 60%). ^{1}H NMR (d_6-acetone) δ: 7.80 (d, 2H, $^{3}J_{HH}$ = 8 Hz, PhH_6), 6.96 (d, 2H, $^{3}J_{HH}$ = 8 Hz, PhH_5), 6.71 (s, 1H, H_{13}), 4.18 (s, 2H, H_3), 3.92 (s, 2H, H_{16}), 3.65 (m, 2H, H_{20}), 3.41 (m, 2H, H_{18}), 2.74 (s, 2H, H_{15}), 2.54 (s, 2H, H_{12}), 1.21 (m, 3H, H_{21}), 1.10 (m, 3H, H_{19}), -2.48 (bs, 1H, bridging H). ^{11}B NMR (d_6-acetone) δ: -8.9 (1B), -9.7 (1B), -13.9 (1B), -15.5(1B), -16.6(1B), -17.8 (1B), -21.6 (1B), -31.9 (1B), -36.3 (1B). ESI-MS $[M\text{-}Cs]^-$ *m/z* 497.6. Anal. Calcd. for $C_{23}H_{38}B_9N_4O_3Cs$: C 42.58, H 5.90, N 8.64. Found: C 42.88, H 5.85, N 8.29.

Radioligand Binding

Human embryonic kidney (HEK293) cell culture and membrane preparation

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal bovine serum, D-glucose (4500 mg/L), L-glutamine (4 mM), and penicillin/streptomycin (100 U/mL). Cell cultures were maintained at 37˚C in a humidified incubator under 5% CO_2. In order to harvest cells for radioligand binding experiments, cells were first washed with phosphate buffered saline (PBS), and harvested with 0.5% ethylenediaminetetraacetic acid (EDTA) in PBS, before being pelleted at 1000 x rpm for 5 minutes.

The mitochondrial fraction of the cells was obtained by homogenising the cell pellet in three volumes of 50 mM Tris-HCl (pH 7.5), containing 0.33 M sucrose, 1 mM $MgCl_2$, and 25 mM KCl (Solution 1). The homogenate was centrifuged for 10 minutes at 700 x g, at 4˚C. The pellet was then discarded and supernatant centrifuged at 10,000 x g for 10 minutes at 4˚C to yield raw mitochondria. This was purified by discarding the supernatant and resuspending the pellet in 3 volumes of Solution 1, and centrifuging at 20,000 x g for 10 minutes at 4˚C to yield a pellet consisting of pure mitochondria.

Radioligand binding assays

On the day of experimentation, mitochondrial fractions were resuspended in 50 mM Tris-HCl buffer (pH 7.5). Mitochondrial membranes containing a final concentration of approximately 200 μg/mL of protein were incubated with 6 nM [^{3}H]PK11195 in a final reaction volume of 200 μL for 90 min at

4˚C. Incubation occurred in the presence of **4** or Cs·**5** at concentrations of 0.01 nM – 10 μM. Non-specific binding was defined in the presence of 1 μM cold PK11195, and amounted to 5-15% of total binding.

After incubation, assays were terminated by rapid filtration through a 96-well filter plate in ice-cold incubation buffer (50 mM Tris-HCl, pH 7.5), and washed 6 times with 200 μL of ice-cold incubation buffer, using a Brandel 96-sample vacuum harvester. The base of the filter plate was then sealed off and approximately 20 μL scintillation cocktail was added to each well. The top of the plate was sealed and filters were soaked in scintillation cocktail overnight at room temperature. Bound radioactivity was obtained as counts per minute (CPM), as measured using a TriLux MicroBeta scintillation counter (PerkinElmer), with a counting time of 1 min per well. At least three independent experiments were carried out in duplicate.

Curve fitting and K_i values were generated using GraphPad Prism 5.0 (Version 5.04). Briefly, the percentage of specifically bound radioligand was logarithmically transformed using the equation X = log(X), where X is the concentration of cold ligand. Logarithmically transformed data was then analyzed using nonlinear regression, plotting the specifically bound radioligand against the cold ligand concentration. The curve of best fit was determined by using the extra sum-of-squares F-test, with the generation of a *p*-value to denote significance.

Cytotoxicity and Cell Uptake Studies

Preparation of BPA-fr

BPA was converted to its more soluble BPA-fructose (BPA-fr) complex by weighing L-BPA and D-(–)-fructose (1:1) into a 5 mL volumetric flask then adding 2 mL milliQ water. An aqueous solution of NaOH (1 M) was added with gentle swirling until complete dissolution of the solids was achieved, at which time the pH of the solution was equal to 8.81. The pH was adjusted to 7.45 with HCl. The concentration of the solution was adjusted to 20 mM with milliQ water, and the solution was passed through a 0.22 μm filter.

Cell line

T98G human glioblastoma multiforme cells were purchased from ATCC. The cells were maintained as a monolayer in minimum essential medium (MEM), supplemented with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 g/mL) and L-glutamine (2.5 mM), at 37 ^{o}C in a humidified 5% CO_2 atmosphere.

Cytotoxicity assays

Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[3] Cells were harvested with trypsin (0.1% *v/v*) and cell pellets were isolated by centrifugation. Cells were then re-suspended to single cells suspension, cell numbers counted using a Haemocytometer counter (Weber) and then seeded at a density of 1×10^4 cells/well in 96-well plates by using 100 μL growth medium and allowed to adhere overnight at 37^{o}C. Cells were incubated at 37^{o}C in a humidified atmosphere of 5% CO_2 in the presence of compounds **4**, Cs·**5**, BPA-fr, BSH and vehicle (control). Serial dilutions (1:2 and 1:10) of compound solution in cell medium were added to wells in triplicate. Compounds were tested at a concentration range prescribed by the limits of compound solubility and that produced a sigmoidal cytotoxic response. After 72 h of drug exposure, MTT solution in PBS (20 μL, 0.25% *w/v*) was added to each well and the incubation continued for 4 h. Culture medium and excess MTT solution were removed and the resulting reduced formazan crystals dissolved by the addition of 150 μL DMSO.

Cell viability was determined by measuring the absorbance of the purple formazan solution at 600 nm using a Victor$_3$V microplate reader (Perkin Elmer). All readings were corrected for absorbance from negative control and wells containing medium alone. The level of purple intensity was expressed relative to the corresponding control as percent viability. Corresponding IC_{50} values for each of the compounds tested were then determined at the dose required to induce a 50% decrease in cell viability from sigmoidal dose-response curves produced in GraphPad Prism 5. Experiments were repeated at least three times for each compound and all IC_{50} values are reported with standard errors.

Cell uptake studies

Stock solutions of **4** and Cs·**5** (20 mM in EtOH/DMSO (3:1)), BPA-fr (20 mM in water) and BSH (20 mM in water) were prepared. Warm (37°C) culture medium was treated with the stock boron solutions to final concentrations of 96.9, 110, 200 and 100 μM, respectively. The T98G cells were cultured as a monolayer in 75 cm^2 flasks to 70-80% confluence then incubated with the boron-containing culture medium at the respective concentrations 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). PBS (4 mL) was added to the culture flask and then cells were harvested with a rubber policeman, rinsing with further PBS (2 + 1 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 analyzed for boron content.

Microwave digestion and measurement of boron concentration by ICP-OES

As certified boron reference materials based on the carborane chemical class are not commercially available, quality control in boron recovery after microwave digestion was performed on analytically weighed samples of Cs·**5**, BPA and BSH. For closed-system digestions, the determined averaged recoveries were 74.4 ± 8.9% (N = 3), 90.9 ± 2.5% (N = 3), 96.3 ± 1.6% (N = 3) for Cs·**5**, BPA and BSH, respectively. The losses of boron can be attributed to volatilization and/or surface adsorption.

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), as described previously,[4] 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 Perkin Elmer Optima DV7300 ICPOES at a plasma gas flow of 15 L/min, auxillary gas flow of 0.5 L/min, nebuliser gas flow of 0.7 L/min using a Burgener PEEK Mira Mist nebuliser with cyclonic spray chamber and pump rate of 0.5 L/min. 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 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 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.[5] This assay relies on the reduction of alkaline Cu(II) by proteins. 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 13300 rcf for 5 min. The supernatant solution was then analyzed for protein content by taking repeated 15μL samples (n = 3) of blank, 1 mg/mL BSA protein standard (200, 400, 800 and 1000 μg/mL, made up to volume with MQ-water) or boron-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^{o}C for 45 min. Absorbance was then measured at 595 nm using an Ultramark multi-wavelength plate reader (BioRad, Australia) and protein determined by comparison to the BSA standard curve.

Table 1. Cytotoxicity (IC_{50}) values and ICP-OES determination of boron uptake in human glioblastoma cells (T98G) treated with compounds **4,** Cs·**5,** BPA-fr and BSH.

Entry	Compound	IC_{50} (μM) (±SE, N)	Dose (μM)	μg B / mg protein (±SE, N)
1	**4**	219.7 (±35.9, *N* = 4)	96.9	19.75 (±1.13, *N* = 9)
2	Cs·**5**	220.9 (±40.3, *N* = 4)	110	11.28 (±0.28, *N* = 9)
3	BPA-fr	>800 (*N* = 3)	200	1.38 (±0.24, *N* = 6)
4	BSH	>800 (*N* = 3)	100	1.49 ±0.26, *N* = 6)

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