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Synthesis and Characterisation of a Boron-Rich Symmetric Triazine Bearing a Hypoxia-Targeting Nitroimidazole Moiety

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Abstract: Boron Neutron Capture Therapy (BNCT) is a binary therapy that promises to be suitable in treating many non-curable cancers. To that, the discovery of new boron compounds able to accumulate selectively in the tumour tissue is still required. Hypoxia, a deficiency of oxygen in tumor tissue, is a great challenge in the conventional treatment of cancer, because hypoxic areas are resistant to conventional anticancer treatments. 2-Nitroimidazole derivatives are known to be hypoxia markers due to their enrichment by bioreduction in hypoxic cells. In the present work, 2-nitroimidazole was chosen as the starting point for the synthesis of a new boron-containing compound based on a 1,3,5-triazine skeleton. Two *o*-carborane moieties were inserted to achieve a high ratio of boron on the molecular weight, exploiting a short PEG spacer to enhance the polarity of the compound and outdistance the active part from the core. The compound showed no toxicity on normal human primary fibroblasts, while it showed noteworthy toxicity in multiple myeloma cells together with a consistent intracellular boron accumulation.

Keywords: BNCT; triazines; carboranes; nitroimidazole; boron accumulation

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1. Introduction

Boron Neutron Capture Therapy is a binary treatment in which boron compounds are introduced into cancer cells and then irradiated by thermal neutrons. Neutron capture yields excited ^{11}B which gives nuclear fission generating high-energy alpha particles (^{4}He nuclei) and lithium-7 (^{7}Li) nuclei causing damage to the cell. Due to their high linear-energy transfer (LET), α (^{4}He) and lithium particles deposit their energy within a range of 5–9 µm (approximately the diameter of a cell), limiting their effects mainly to the boron-containing cells [1,2].

Although many compounds have been tested for their suitability for BNCT until today, BSH and BPA are still the only agents clinically used [3]. In the past, most clinical studies focused on the treatment of melanoma and glioblastoma, but BNCT has the potential to treat many types of cancer and even some other diseases like arthritis [4]. The tumor-targeting ability and the selectivity are among the most important requisites for new compounds. In particular, they should be able to accumulate the commonly accepted optimal boron amount for neutron capture therapy, namely $\geq 10^9$ ^{10}B atoms per cell, corresponding to 20 µg boron per g of tumour tissue [3]. Future agents are expected to be designed for specialized tasks and able to offer the use of BNCT to other types of cancer, treating tumour types with very low life expectancy and increase the life quality of patients by eliminating the side effects of conventional cancer therapies.

Hypoxia represents a great obstacle in treating malignant cells. The rapid and uncontrollable tumour growth prevents the build-up of an adequate blood vascular structure causing deprivation of nutrients and oxygen in deeper areas. The resistance of hypoxic areas to both radio- and chemotherapy is the main factor in poor prognosis and cancer recurrence [5,6].

2-Nitroimidazole is known to undergo bioreductive trapping in hypoxic cells, making it a promising starting point for the design of hypoxia-targeting agents [5]. After entering the cell through diffusion, it undergoes single-electron reduction. In non-hypoxic cells, it is oxidized by intracellular oxygen and can leave the cell again. In hypoxic tissue, however, it undergoes further reduction by intracellular nitroreductase enzymes like xanthine oxidase, lipoxygenase, and NADPH oxidase [7]. Therefore, its ability to accumulate in hypoxic cells by bioreductive trapping makes 2-nitroimidazole a promising starting point for the design of hypoxia-targeting agents.

In the last 25 years, various boron-containing 2-nitroimidazole derivatives have been synthesized [8–12]. Recently, we have reported an agent we named B381, which has shown specific accumulation in cancer tissues but, containing only one boron atom per molecule, it has furnished low boron accumulation [12]. To enhance boron molecular mass fractions, clusters like carboranes ($C_2B_{10}H_{12}$) [13] can be used for new 2-nitroimidazole-containing BNCT agents. Modular systems based on triazines are yet described and are combined with *m*-carboranes to boron-rich compounds [14–16]. In this paper, a symmetrical hybrid compound containing two carborane residues and 2-nitroimidazole moiety on a triazine scaffold is described as a potential agent for BNCT.

2. Materials and Methods

2.1. Chemical Synthesis

Reagents and solvents were purchased from TCI Europe (Italian distributor: Zentek SRL, Milan, Italy) or Carlo Erba (Milan, Italy) and used without further purifications. *o*-Carborane was purchased from Katchem (Praha, Czech Republic) Celite® 577 from Sigma Aldrich (Milan, Italy). Thin-layer chromatography plates (TLC) equipped with fluorescent indicators were purchased from Merck (Milan, Italy). The visualization was accomplished by UV light (254 and/or 365 nm) and by staining in ceric ammonium molybdate. Palladium chloride was used for carborane derivatives. Solvents were dried with freshly activated molecular sieves (0.3 or 0.4 nm; Sigma Aldrich, Milan, Italy). Reactions were performed under nitrogen atmosphere in oven-dried glassware (when the use of water as reagent or solvent was not specified). Silica gel 230–400 mesh (Merck, Milan, Italy) was used for flash column chromatography.

Infrared spectroscopy was recorded with an FT-IR Thermo-Nicolet Avatar 370. Thermo Finnigan LCQ-Deca XP-plus mass spectrometer equipped with an ESI source and an ion trap detector was used for mass spectroscopy. Orbitrap QExactive PLUS (Thermo Fischer Scientific, Bremen, Germany) was used for the determination of the exact mass of final compound **1**.

A JEOL ECP 300 spectrometer (JEOL Ltd., Tokyo, Japan) was used for NMR analyses. Stuart Scientific SMP3 apparatus were used for the determination of the melting points, which are uncorrected.

1-2-[2-(2-azidoethoxy)ethoxy]ethyl-2-nitro-*1H*-imidazole (5) Compound **2** (290 mg, 1.14 mmol), dissolved in 6.5 mL anhydr. dimethylformamide (DMF) was added under nitrogen atmosphere to a three-necked flask together with Cs_2CO_3 (423 mg, 1.31 mmol) and 2-nitroimidazole **4** (163 mg, 1.43 mmol). Under stirring, N_2 was passed through the solution for 0.25 h, then the reaction was heated to 110 °C for 18 h. After checking the complete conversion of **2** by TLC (EtOAc/cyclohexane 7:3), the solvent was removed under vacuum. The crude was dissolved in EtOAc, filtered through a Celite pad, concentrated, and purified by flash chromatography (EtOAc/cyclohexane 7:3) as eluent. Compound **5** was obtained as a yellow low-viscosity oil in a yield of 72%. MS (ESI) *m/z* Calculated for $C_9H_{14}N_6O_4$: 270.24; found 271.20 $[M + H]^+$. IR: (neat, cm^{-1}) = 2917, 2870 (m, C-H_2), 2102 (s, N_3), 1537 (m, NO_2

asym.), 1484 (s, C-N), 1357 (s, NO_2 sym.), 1115 (s, C-O-C). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm) = 7.22 (s, 1H, Im-H-5), 7.11 (s, 1H, Im-H-4), 4.60 (t, *J* = 4.9 Hz, 2H, Im-NC*H*$_2$CH$_2$O), 3.83 (t, *J* = 4.90 Hz, 2H, NCH$_2$C*H*$_2$O), 3.58 (m, 6H, CH_2), 3.34 (t, *J* = 4.7 Hz, 2H, C*H*$_2$N$_3$). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δ (ppm) = 144.6, 128.1, 127.3, 70.6 (d, 2C), 70.2, 69.5, 50.7, 49.9.

1-2-[2-(2-aminoethoxy)ethoxy]ethyl-2-nitro-*1H*-imidazole (6) To a 25 mL flask containing **5** (380 mg, 1.41 mmol) and 4 mL of 2N HCl, a solution of PPh_3 (423 mg, 2.62 mmol) dissolved in EtOAc (1.2 mL) and Et_2O (2.4 mL) was added dropwise over a period of 45 min under vigorous stirring. After 16 h, still traces of **5** were found in the TLC control (EtOAc/cyclohexane 6:4). Therefore, an additional 0.1 eq. of PPh_3 was added and the solution was stirred for another 24 h. After evaporation of the organic phase, the acidic aqueous phase was extracted 5 times with 5 mL dichloromethane (DCM) to remove Ph_3PO from the solution and the pH was set to 12 by addition of NaOH pellets. The basic aqueous phase was extracted seven times with 5 mL DCM each, the combined organic phases were dried over $MgSO_4$, filtered, and concentrated under vacuum. Compound **6** was obtained in 79% yield as a yellow low-viscosity oil. MS (ESI) *m/z* Calculated for $C_9H_{16}N_4O_4$: 244.25; found 245.10 $[M + H]^+$. IR: (neat, cm^{-1}) = 3379 (w, N-H$_2$), 3117 (w, C-H), 2912, 2869 (C-H), 1536 (m, NO_2 asym.), 1484 (s, C-N), 1357 (s, NO_2 sym.), 1111 (s, C-O-C). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm) = 7.22 (s, 1H, Im-H-5), 7.08 (s, 1H, Im-H-4), 4.59 (t, *J* = 4.9 Hz, 2H, NC*H*$_2$CH$_2$O), 3.81 (t, *J* = 4.90 Hz, 2H, NCH$_2$C*H*$_2$O), 3.52 (m, 6H), 3.42 (t, *J* = 4.7 Hz, 2H, CH_2NH_2), 2.80 (s, 2H, NH_2). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δ (ppm) = 144.9, 128.1, 127.3, 73.0, 70.6, 70.2, 69.4, 49.9, 41.6.

4,6-dichloro-*N*-(2-{2-[2-(2-nitro-*1H*-imidazol-1-yl)ethoxy]ethoxy}ethyl)-1,3,5-triazin-2-amine (8). In a double-necked 10 mL flask, under nitrogen, atmosphere **6** (110 mg, 0.45 mmol) and cyanuric chloride **7** (166 mg, 0.9 mmol) were dissolved in 2 mL of dry THF. To remove the remaining traces of water and oxygen, nitrogen was bubbled through the solution for 15 min. The mixture was cooled to −10 °C, using an ice/salt bath and under vigorous stirring, *N,N*-diisopropylethylamine (DIPEA) (189 µL) was added over 30 min. After 120 min TLC control (EtOAc/cyclohexane 7:3) showed only traces of **6** left. The ice was removed, and the solution was stirred for an additional 30 min at room temperature until all starting material was converted. To remove the precipitate, the reaction was filtered through a pad of Celite and washed with 15 mL of THF. The solvent was evaporated, and the crude product was purified by flash chromatography (EtOAc/cyclohexane 7:3). Compound **8** was obtained in 82% yield as pale viscous oil. MS (ESI) *m/z* Calculated for $C_{12}H_{15}Cl_2N_7O_4$: 391.06; found 392.16 $[M + H]^+$. IR: (neat, cm^{-1}) = 3278 (w, N-H), 2873 (w, C-H), 1358 (s, N-O$_2$ sym.), 1111 (s, C-O-C-). ^{1}H-NMR (300 MHz, $CDCl_3$) (ppm) = 7.22 (s, 1H, Im-H-5), 7.13 (s, 1H, Im-H-4), 6.66 (br. s, 1H, triazine-N*H*), 4.65 (t, *J* = 4.9 Hz, 2H, Im-NC*H*$_2$CH$_2$O), 3.81 (t, *J* = 4.9 Hz, 2H, NCH$_2$C*H*$_2$O), 3.59 (m, 8H, CH_2). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δ (ppm) = 171.0, 170.0, 165.8, 145.0, 128.3, 127.1, 77.4, 70.7, 69.6, 68.9, 49.8, 41.3.

2-*N*,4-*N*-bis({2-[2-(2-azidoethoxy)ethoxy]ethyl})-6-*N*-(2-2-[2-(2-nitro-*1H*-imidazol-1yl) ethoxy]-ethoxyethyl)-1,3,5-triazine-2,4,6-triamine (9) Under a nitrogen atmosphere, 8 (128 mg, 0.33 mmol) was dissolved in 13 mL of dry ACN followed by addition of 3 (163 mg, 0.94 mmol). While stirring, DIPEA (163 µL) was slowly added. After 2 h at room temperature, the reaction was heated to 80 °C for 20 h. When the spot of the disubstituted intermediate had disappeared from the TLC-control, the solvent was removed under vacuum. The purification of the crude product was performed by flash chromatography, using a solvent gradient starting from pure DCM in 100 mL steps to a fraction of 5% MeOH. Compound **9** was obtained in 71% yield as a pale-yellow oil. MS (ESI) *m/z* Calculated for $C_{24}H_{41}N_{15}O_8$: 667.33; found 668.30 $[M + H]^+$. IR: (neat, cm^{-1}) = 3349 (w, N-H), 2917, 2851 (m, C-H), 2103 (s, N_3), 1358 (m, NO_2 sym.), 1097 (s, -C-O-C-). ^{1}H-NMR (300 MHz, $CDCl_3$) (ppm) = 7.26 (s, 1H, Im-*H*-5), 7.10 (s, 1H, Im-*H*-4), 5.62–5.39 (br. s, 3H, triazine-N*H*CH$_2$), 4.60 (t, *J* = 4.9 Hz, 2H, Im-NC*H*$_2$CH$_2$O), 3.81 (t, *J* = 4.90 Hz, 2H, NCH$_2$C*H*$_2$O), 3.60 (m, 28H, CH_2), 3.37 (t, *J* = 4.90 Hz, 4H, C*H*$_2$N$_3$). ^{13}C-NMR (75.4 MHz, $CDCl_3$) (ppm) = 165.9 (br. s, 3C), 144.8, 128.2, 127.5, 77.4, 70.6, 70.4, 70.2, 70.0, 69.4, 50.7, 49.9, 40.4.

2-*N*,4-*N*-bis[2-(2-{2-[4-(1,2-dicarba-*closo*-dodecaboran(12)-1-ylmethyl)-*1H*-1,2,3-triazol-1-yl]ethoxy}ethoxy)ethyl]-6-*N*-(2-{2-[2-(2-nitro-*1H*-imidazol-1-yl)ethoxy]ethoxy}ethyl)-1,3,5-triazine-2,4,6-triamine (1) Compound **9** (155 mg, 0.23 mmol) was dissolved in a mixture of 2 mL *t*-BuOH and 200 µL of water. Compound **10** (100 mg, 0.58 mmol), $Cu(OAc)_2 \cdot H_2O$ (12 mg, 0.058 mmol) and sodium ascorbate (30 mg, 0.15 mmol) were added in this order and the solution was stirred at room temperature for 16 h, until TLC control (DCM/MeOH 93:7) showed full conversion. The reaction was poured into 6 mL of water and washed three times with 10 mL EtOAc each. Purification was carried out by flash chromatography starting with pure DCM (100 mL) using a gradient of MeOH (100 mL 2%, 200 mL 4%, 100 mL 5%). The final product 1 was obtained in 65% yield as yellowish wax. HRMS (ESI) *m/z* $[M + H]^+$ calculated for $C_{34}H_{70}N_{15}O_8B_{20}$: 1036.7393; found 1036.7418. IR: (neat, cm^{-1}) = 3350 (w, -N-H), 2942–2871 (m, C-H-), 2585 (s,-B-H), 1360 (m, $N-O_2$ sym.), 1111 (s, -C-O-C-). ^{1}H-NMR (300 MHz, $CDCl_3$) (ppm) = 7.72 (s, 2H, triazol-C*H*), 7.26 (s, 1H, Im-*H*-5), 7.13 (s, 1H, Im-*H*-4), 5.50 (br. s, 3H, triazine-N*H*CH_2), 4.62 (bt, 2H, Im-NC*H*$_2$$CH_2O$), 4.56 (bt, 4H, triazol-NC*H*$_2$$CH_2$), 3.81 (bs, 8H, NCH_2C*H*$_2$O), 3.60 (m, 28H), 2.1–1.20 (20H, br, BH). ^{13}C-NMR (75.4 MHz, $CDCl_3$) (ppm) = 165.6 (s, 3C, triazine-CNH), 141.4, 128.1, 127.3, 124.6, 70.3, 70.3, 69.4, 59.7, 50, 49.9, 40.4, 33.6. ^{11}B-NMR (96.2 MHz, $CDCl_3$) (ppm) = −3.65, −6.65, −10.45, −13.01.

2.2. Cell Culture

The primary normal adult human fibroblasts (HDFa) (ATCC PSC-201-012; American Type Culture Collection, Manassas, VA, USA) were cultured in fibroblast basal medium completed with 2% fetal bovine serum (FBS, ATCC), 7.5 mM L-glutamine, 5 ng/mL recombinant human fibroblast growth factor (rh-FGF) b, 50 µg/mL ascorbic acid, 1 µg/mL hydrocortisone hemisuccinate, 5 µg/mL rh insulin (ATCC), and cultured in a 37 °C, 5% CO_2 incubator. Cells were grown in 25 cm^2 culture flasks and sub-cultured when they had approximately reached 80% confluence and were actively proliferating. Human multiple myeloma cell line MM.1S (ATCC CRL-2974) was cultured in RPMI-1640 media (Millipore Sigma, St. Louis, MO, USA), enriched with 10% FBS (Gibco, Life Technologies, Grand Island, NY, USA), 2 mmol/L of L-glutamine, 100 U/mL Penicillin and 100 µg/mL Streptomycin (Corning CellGro, Mediatech, Manassas, VA, USA). The cells were incubated at 37 °C (5% CO_2) in NuAire water jacket incubator (Plymouth, MN, USA). Cells were grown in 75 cm^2 culture flasks, scraped and passaged when they reached 70–80% confluence and were actively proliferating; they were used between passage 3–6.

2.3. Cell Viability Assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide (MTT) assay, as previously reported [17]. 5×10^3 fibroblast were seeded in 96-well plates in complete medium for 24 h. Attached fibroblasts were treated with increasing concentrations (0.1–100 µg/mL) of test compounds for 24–72 h. MM.1S cells at a concentration of 50,000 cells/well were treated with increasing concentrations (0.1–100 µg/mL) of test compounds for 24–72 h. The percentage of cell viability was calculated as $[100\ (x - y)/(z - y)]$, where x, y, and z were the absorbance read in compound-treated, resting, and compound-untreated cells, respectively. Results are expressed as mean ± SD of at least three experiments run in triplicate.

2.4. Boron Accumulation in Cells Based on ICP-OES

MM.1S cells (2 × 106 cells/1mL media) were treated with compounds for 2 h at 37 °C. Then the cells were washed 4 times with PBS, pellet weight was registered, then resuspended, vortexed, and digested in nitric acid overnight. Samples were diluted with deionized water to a final acid concentration of 5% (v/v), filtered through a 0.22-micron polyethersulfone syringe filter (DiKMA Technologies, Lake Forest, CA, USA), and analyzed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Optima 7300 V series, Perkin Elmer, Waltham, MA, USA). The following day, the compounds were

added and the cells were trypsinized, collected, and proceeded cells after 2, 6, or 24 h. Samples were analyzed for boron content (λ = 249.677 nm) against a calibration curve of boron standards of 0–250 parts per billion (ppb) prepared from 10 parts per million boron standard solution (Inorganic Ventures, Christiansburg, VA, USA).

2.5. Statistical Analysis

Experiments were performed in triplicates and replicated independently at least three times and the results were shown as mean ± standard deviation (S.D.) or mean ± standard error of the mean (S.E.M.). For the statistical significance, one-way ANOVA was used and the values were considered significantly different for * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3. Results and Discussion

3.1. Synthesis of Carborane Hybrid Triazine

Compound **1** (Figure 1), the synthesis of which is reported in the Scheme 1, is composed of three elements: a nitroimidazole subunit, to target hypoxic tumour cells, and two *o*-carboranes as boron moieties, linked to the triazine through triethylene glycol-derived spacers chosen to enhance the solubility of the compound. The spacers were equipped with a primary amine on one side to allow nucleophilic substitution of the chlorine groups of the cyanuric chloride.

The spacer molecules **2** [18] and **3** [19] were prepared according to literature procedures.

Propargyl carborane **10** was prepared from *o*-carborane following our previously reported procedure [20]. The 2-nitroimidazole containing moiety was synthesized starting from spacer **2** by nucleophilic substitution of the mesylate with 2-nitroimidazole in DMF with Cs_2CO_3 as a base, and a reaction time of 18 h at 110 °C [7]. After the workup and flash chromatographic purification, **5** was obtained as a yellow oil in 72% yield. The following aromatic nucleophilic substitution on cyanuric chloride requires the reduction of the azido group of the compound **5** to the corresponding amine **6**, which was achieved by a Staudinger reaction with triphenylphosphine. A two-phase system of Et_2O and HCl was used to allow the easy removal of triphenylphosphine oxide side-product by extraction. Subsequent basification of the aqueous phase allowed the extraction of the desired product **6** with DCM, which was obtained after concentration under vacuum as a pale-yellow oil in a 73% yield and was used directly in the following step.

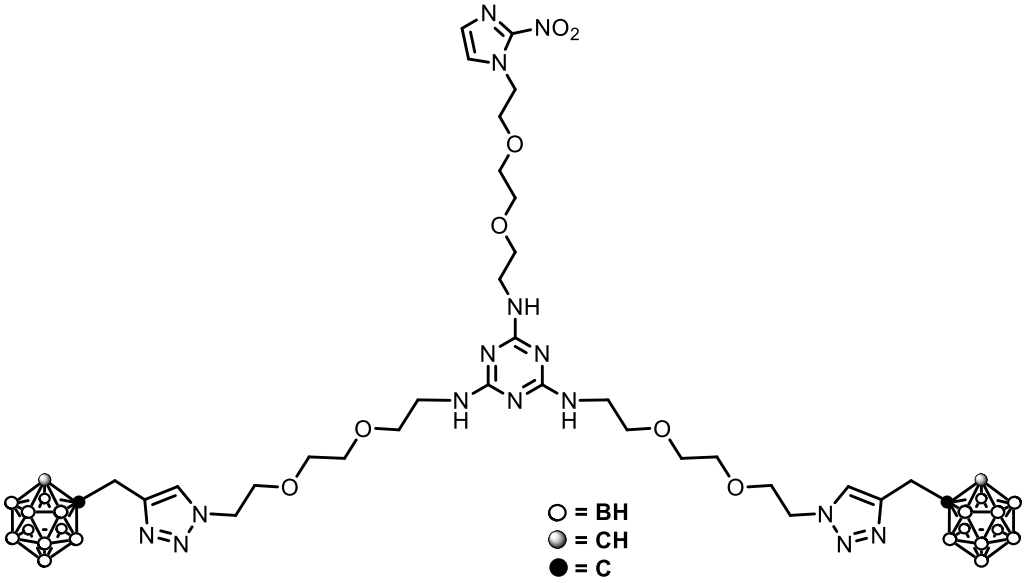
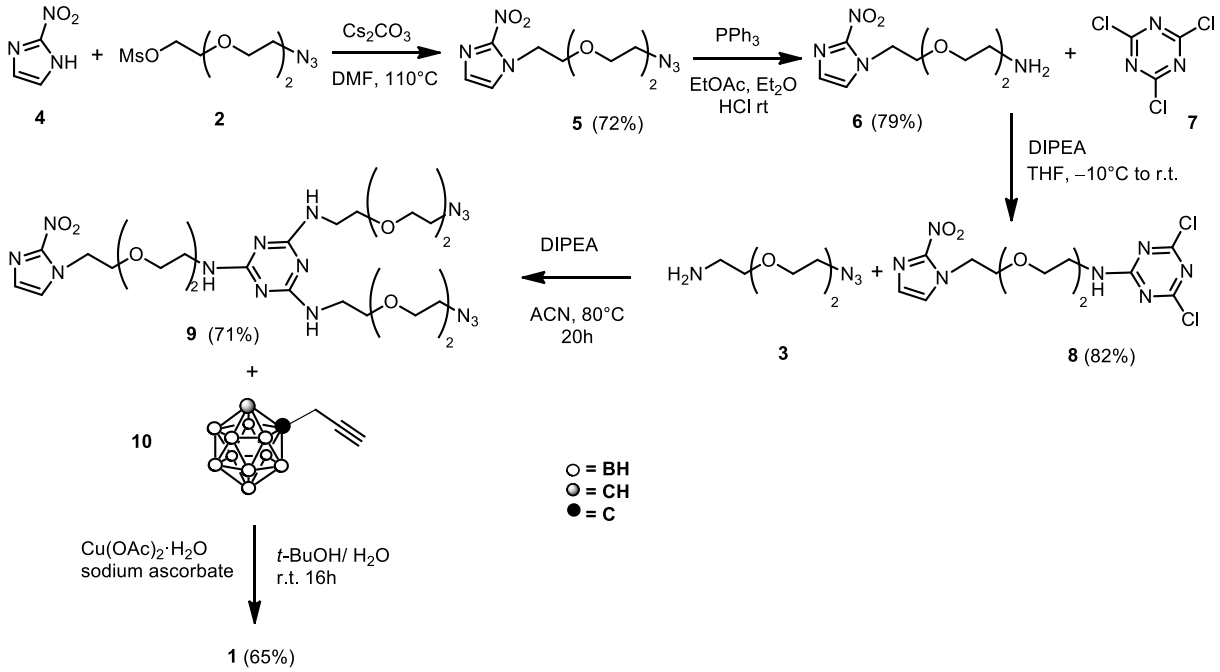


Figure 1. Target molecule **1**, based on a 1,3,5-triazine scaffold.



Scheme 1. Synthesis of the target compound **1**.

The assembly of the substituents on the triazine scaffold was performed in two steps under temperature control, to allow the sequential substitutions of the chlorine atoms. To prevent double or triple substitution [21,22] the first reaction of cyanuric chloride **7** with **6** was performed using an excess of the reagent **7**. In a first attempt, a ratio of 2:1 was used but was reduced to 1.2:1 in the follow-up reaction due to problems in separating the product from the remaining reactant. Under a nitrogen atmosphere, the reactants **6** and **7** were dissolved in dry THF. To keep the reaction under control, the solution was cooled to −10 °C before the dropwise addition of DIPEA. The progression of the reaction was monitored by TLC and stopped immediately when the spot of **6** had disappeared. Flash chromatography of the crude reaction mixture gave **8** as a dense yellow oil in 82% yield. The substitution of the other two chlorine atoms by **3** was started at room temperature. After the slow addition of DIPEA, the reaction was stirred under dry conditions for 90 min and 21 h at 80 °C. By flash chromatography (DCM with solvent gradient MeOH from 0–5%) **10** was obtained as a thick yellow oil (71% yield).

As last step **10** was connected to **9** exploiting the copper(I) catalysed variant of the azide-alkyne Huisgen cycloaddition [23]. This reaction allows the regioselective synthesis of 1,4-disubstituted triazoles at room temperature in aq. solutions catalyzed by Cu(I). Finally, *t*-BuOH/H_2O (9:1) was used as the solvent. After 16 h the reaction was stopped and after purification by flash chromatography (DCM with 0–5% MeOH) compound **1** was obtained in 65% yield.

3.2. Viability Test on Human Primary Fibroblasts

To evaluate the effect of compound **1** and BSH on cell viability human primary fibroblasts were used as representative cells, being fibroblasts present in many tissues and organs. Human primary fibroblast viability was assessed after cell treatment (24–72 h) with increasing concentrations (0.1–100 μM) of each compound, by MTT assay. No evident reduction of cell viability was measured at any concentrations and times considered (Figure 2) on human primary fibroblasts.

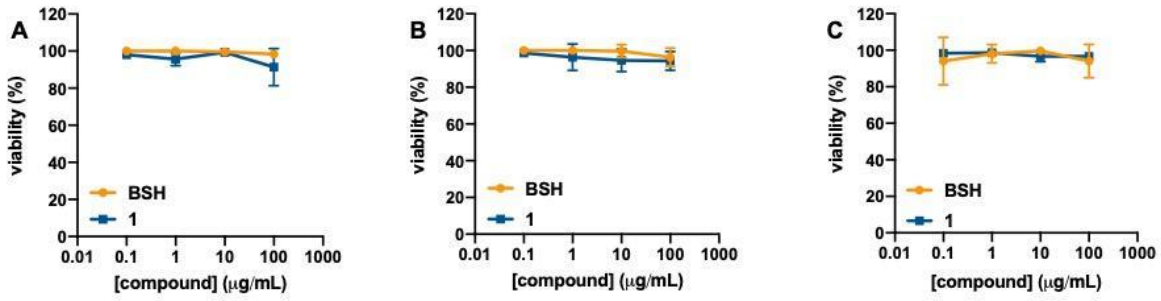


Figure 2. Effects of compound **1**, and sodium borocaptate (BSH) on cell viability. The effect of tested compounds on human primary fibroblast viability were assessed by MTT assay after 24 h (**A**), 48 h (**B**), and 72 h (**C**) treatment with increasing concentrations (0.1–100 μg/mL) in each compound. The concentration-response curves show the percentage of cell viability in comparison with controls (untreated cells). The data represent mean ± SD of at least three independent experiments run in triplicate.

3.3. Viability Test and Intracellular Boron Content on Human Multiple Myeloma Cell Line

Next, we tested the effect of the compound on cell viability of a cancer cell line derived from multiple myeloma (MM) patients. MM.1S cells were treated (24–72 h) with increasing concentrations of compound **1** (0.1–100 μg/mL), as well as known MM chemotherapy including Ixazomib (0.1–1 ng/mL) and Pomalidomide (0.1–100 μg/mL) for 72 h, and cell viability was measured by MTT assay. Figure 3A demonstrates that Ixazomib in ng range is very toxic and MM.1S cells are very sensitive, Pomalidomide is less toxic and the compound **1** is moderate to MM.1S cells in μg range, implying that the compound **1** is being moderately toxic to MM.1S cells during longer exposures. Therefore, we tested the accumulation of the compound at 2 h based on boron content using ICP-OES. We demonstrate that MM.1S cancer cells accumulate boron at the highest concentration already at 2 h post-exposure (Figure 3B).

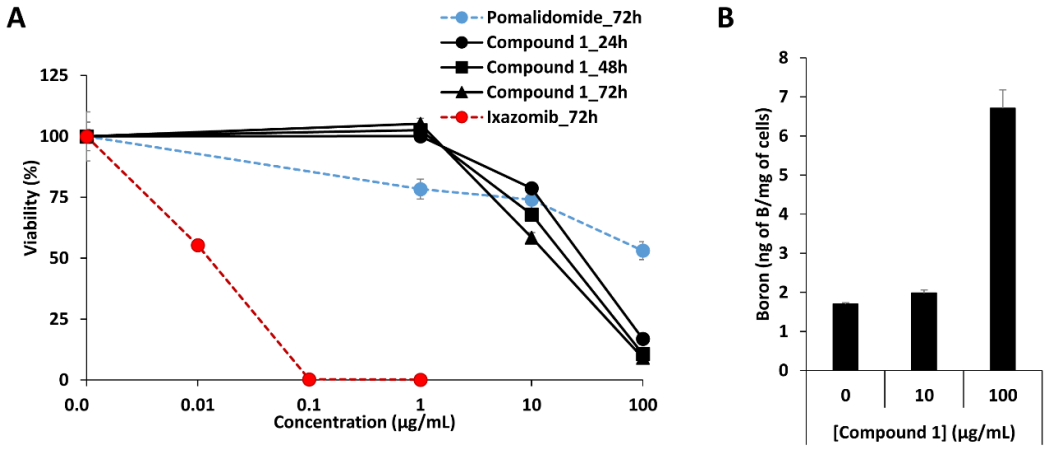


Figure 3. Effects of compound **1,** Ixazomib and Pomalidomide on cell viability and intracellular content of boron on MM.1S cells post compound **1** exposure. Human cancer cells (MM.1S) were treated with increasing concentrations (0.1–100 μg/mL) for 24 h, 48 h, and 72 h, as well as Ixazomib (0.1–1 ng/mL) and Pomalidomide (0.1–100 μg/mL) for 72 h (**A**). Cell viability was assessed by MTT assay. The concentration-response curves show the percentage of cell viability in comparison with controls (untreated cells). The data represent the mean ± SD of at least three independent experiments run in quadruplets. One-way ANOVA was performed showing significance between 24 vs. 48 h ($p < 0.001$), 24 vs. 72 h ($p < 0.001$) and 48 vs. 72 h ($p = 0.1222$, non-significant) MM.1S were treated with increasing concentrations (1–100 μg/mL) of compound **1** for 2 h and the boron content was measured by ICP-OES. The data represent the mean ± SD of three independent experiments run in triplicates. Student *t*-test was performed showing significance between 0 vs. 10 μg/mL ($p = 0.0047$), 0 vs. 100 μg/mL ($p < 0.001$) and 10 vs. 100 μg/mL ($p < 0.001$) (**B**).

4. Conclusions

As stated in the introduction, we have previously reported a hypoxia-targeted BNCT agent B381, based on 2-nitroimidazole, which has shown specific accumulation in cancer tissues in vitro and in vivo due to the specific reduction of the 2-nitroimidazole and conjugation to intracellular proteins [12]. However, because B381 compound included only one boron atom in each molecule (per 2-nitroimidazole), it resulted in relatively low boron tissue accumulation. In this study, and to improve the low boron accumulation observed with B318, we proposed the use of highly boronated 2-nitroimidazole derivative. Herein we have reported the synthesis, stability evaluation, and preliminary biological evaluation of a triazine-based derivatives containing 2-nitroimidazole and two symmetrical *o*-carborane units, **1** (20 boron atoms per molecule). Compound **1** showed no toxicity on normal human primary fibroblasts (representing normal tissue), while it showed significant toxicity in multiple myeloma (cancer) cells. The results revealed that at 100 ug/mL no toxicity was observed in normal cells, while it abolished the cancer cells with over 90% killing, demonstrating an outstanding therapeutic index. The cellular toxicity was driven by intracellular boron accumulation that was observed even within 2 h of exposure. These results were demonstrated in tissue cultures with normal oxygen; in the future we will investigate the biology of the compound and its derivatives in a deeper manner, especially exploring the mechanism of its accumulation in hypoxic tumour cells in vitro and its accumulation in cancer models in vivo, where tumour hypoxia is expected to develop.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-8994/13/2/202/s1, Figure S1: ^{1}H NMR spectrum of **5** in $CDCl_3$; Figure S2: ^{13}C NMR spectrum of **5** in $CDCl_3$: Figure S3: ^{1}H NMR spectrum of **6** in $CDCl_3$; Figure S4: ^{13}C NMR spectrum of **6** in $CDCl_3$; Figure S5: ^{1}H NMR spectrum of **8** in $CDCl_3$; Figure S6: ^{13}C NMR spectrum of **8** in $CDCl_3$; Figure S7: ^{1}H NMR spectrum of **9** in $CDCl_3$; Figure S8: ^{13}C NMR spectrum of **9** in $CDCl_3$; Figure S9: ^{1}H NMR spectrum of **1** in $CDCl_3$; Figure S10: ^{13}C NMR spectrum of **1** in $CDCl_3$; Figure S11: ^{11}B NMR spectrum of **1** in CD_3OD; Figure S12: HRMS of **1**; Figure S13: HRMS MS/MS of **1**.

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