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Towards new boron carriers for boron neutron capture therapy: metallacarboranes and their nucleoside conjugates

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Abstract—Thymidine conjugates containing metallacarborane, {8-[5-(N^3-thymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ (**5**) and {8-[5-(O^4-thymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ (**6**) ions and several simple [3-cobalt bis(1,2-dicarbollide)]$^-$ ion (**1**) derivatives have been studied as potential boron carriers for BNCT. Compound **6** and some nonnucleoside derivatives of **1** were not toxic above 100 μM. The partition coefficient for both metallacarborane bearing thymidine conjugates **5** and **6** was more than 500 times higher than that of unmodified nucleoside. The cellular uptake studies showed accumulation of compounds **6** in V79 Chinese hamster cells but not of compound **5**. The low toxicity of conjugate type of **6** together with its high partition coefficient suggest that judicially designed derivatives of metallacarboranes can be considered as potential boron carriers for BNCT.

1. Introduction

Boron neutron capture therapy (BNCT) is a binary system for treatment of cancers, based on absorption of low-energy neutrons by nonradioactive boron-10 (^{10}B) atoms delivered to neoplastic cells in the form of a boron carrying drug.[1–3] The two independent, separately nonlethal constituents of this binary modality are radiosensitiser (boron carrier) containing ^{10}B and nonionising neutron radiation. Capture of neutron by ^{10}B, a stable isotope, results in the formation of excited boron-11 (^{11}B). ^{11}B is unstable and instantly fissions yielding high linear energy transfer (LET) lithium-7 (^{7}Li) and energetic α-particles (^{4}He). The kinetic energy of ^{7}Li and α-particles is about 2.8 million electron volts (eV) of energy (100 million times more than was put in). This, together with high linear energy transfer (LET) makes these particles highly lethal to the cells. It is assumed, that preferential neutron capture of ^{10}B-containing, tumor selective boron carrier in tumor tissue as compared to healthy cells, due to higher accumulation of the carrier in the tumor, will cause preferential killing of cancer cells and induce therapeutic effect (Fig. 1).

For BNCT to be successful, 10^9 ^{10}B atoms must be localised on or preferably within neoplastic cells (20–40 μg of B in one gram of tumor), and a sufficient number of thermal neutrons must be delivered to sustain a lethal ^{10}B (*n*, alpha) lithium-7 reaction. Therefore, the development of highly boron loaded, tumor-selective drugs play an important role if BNCT is to evolve into clinically accepted treatment for cancer. A number of potential boron carriers such as boron-containing porphyrins, amino acids, carbohydrates, nucleic acid bases, nucleosides, polyamines, DNA groove binders, low-density lipoproteins, biopolymers such as peptides, oligophosphates and oligonucleotides have been synthesised and tested.[4–6] The persistent interest in the design and synthesis of

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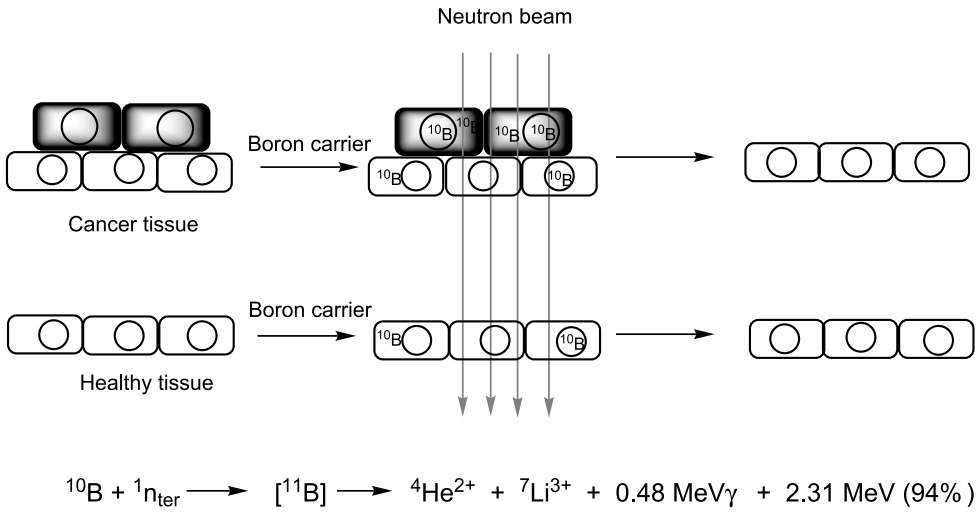

Figure 1. Theoretical mechanism of BNCT of tumors.

boron-containing nucleosides is that such compounds may be selectively accumulated in rapidly multiplying tumor cells, and following their conversion to the corresponding nucleotides, trapped within the cell or ideally, incorporated into nuclear DNA of tumors.[7–11] Regrettably, in spite of the continuous efforts to develop suitable boron caring drugs the clinical applications of boron derivatives are currently limited to two molecules, L-4-(dihydroxyboryl)phenylalanine (BPA) and the sodium salt of thioborane anion ($Na_2B_{12}H_{11}SH$, BSH).

Boric acid [$B(OH)_3$] and borane clusters (*closo*-$B_{12}H_{12}^{2-}$ or $C_2B_{10}H_{12}$) are the two main types of boron entities used so far in the synthesis of carrier molecules for BNCT. To a lesser extent boranes (BH^-) and cyanoboranes (BH_2CN^-) are used for synthesis of potential boron caring drugs.[12–15] Herein, we propose a new modifying entity for preparation of potential boron delivering molecules, namely the [8-substituted 3-cobalt bis(1,2-dicarbollides)]$^-$ ion and related species.

Metallacarboranes, discovered by Hawthorne and coworkers[16–18] represent a vast family of metallocene type complexes, consisting of deltahedral heteroborane ligand(s) and one or more metal atoms such as Co, Fe, Ni and a variety of others. Most prominent among them seems to be the [3-cobalt bis(1,2-dicarbollide)]$^-$ ion (**1**) [3-Co-(1,2-$C_2B_9H_{11})_2]^-$ showing remarkable integrity along with an extensive substitution chemistry reviewed recently.[19] But there are other classes of metallacarboranes as well.[20–22] Metallacarboranes, if derived from large carboranes contain about 1.5 times as much boron as BSH and 18 times more boron atoms than boric acid. They are characterized by high lipophilicity, the property which may facilitate transport of the carrier molecules across the blood–brain-barrier (BBB) and improve cellular uptake. The ability of deltahedral ligands to form stable complexes with metals allows incorporation of various metals with different properties into boron carrier molecule and provides an additional advantage to high boron load.

Recently, we developed the first method for the synthesis of metallacarborane/nucleoside conjugates and showed their incorporation into DNA–oligonucleotide.[23] Herein, we present preliminary studies on cytotoxicity of two metallacarborane containing nucleosides, {8-[5-(N^3-thymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ (**5**) and {8-[5-(O^4-thymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ (**6**) ions (Fig. 2); nonnucleoside part of the above conjugate, [8-(5-hydroxy-3-oxa-pentoxy)-3-cobalt bis(1,2-dicarbollide)]$^-$ (**7**), and compare it with natural thymidine (**3**). The partition coefficient of all the compounds has also been measured. The cytotoxicity of several other simple derivatives of [3-cobalt bis(1,2-dicarbollide)]$^-$ ion (**1**) (Fig. 3) has also been determined. In addition, preliminary studies on cellular uptake of two examples metallacarborane/nucleoside conjugates **5** and **6** have been performed.

2. Results

The nomenclature of deltahedral sandwich compounds of the metallacarborane type is very complex and cumbersome. In this article we will exploit the versatility of the semi-trivial nomenclature for *commo*-metalla-bis(icosahedral) sandwich species, coined by the discoverer of this huge realm of sandwich complexes.[16–18] The fundamental ion [3-Co-(1,2-$C_2B_9H_{11})_2]^-$ is designated as [3-cobalt bis(1,2-dicarbollide)]$^-$ (**1**) (Fig. 2, along with the numbering scheme) and its descendants will be considered as its substitution derivatives (also shown in the figures).

In the respective conjugate acids the solvated proton is associated with the most basic moiety, that is, usually the –O–$B_{(8)}$ oxygen. These names will be used throughout this article and, as much as possible, the respective code numbers presented in the above list will be preferred for the sake of brevity and clarity.

{8-[5-(N^3-Thymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ (**5**) and {8-[5-(O^4-thymidine)-3-oxa-pento-

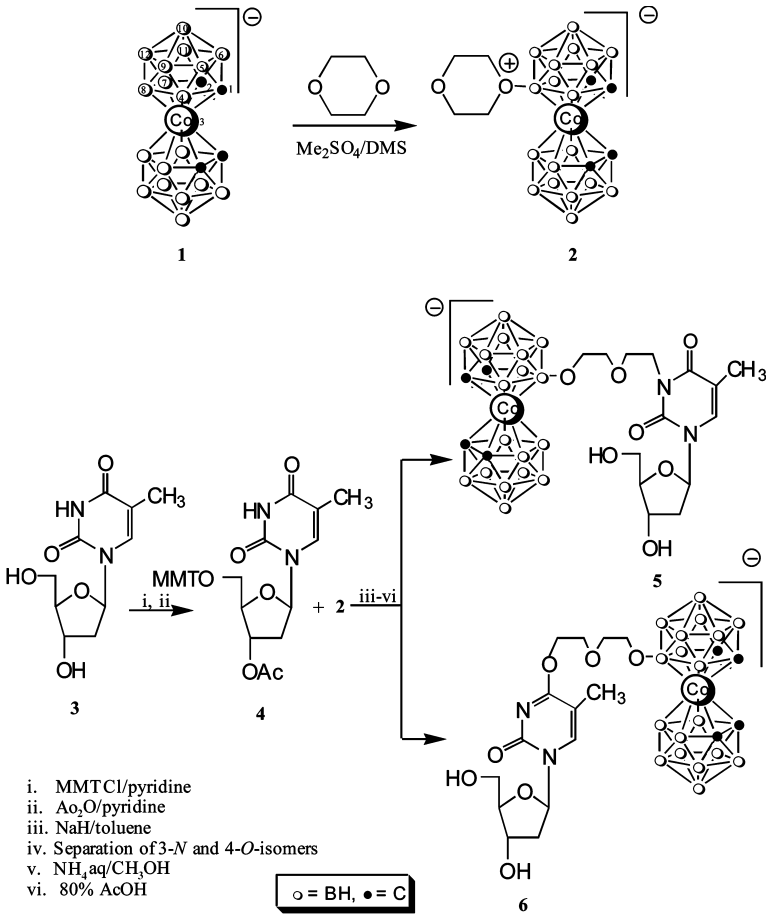

Figure 2. Synthesis of [8-dioxane-3-cobalt bis(dicarbollide)]^0 (**2**) and {8-[5-(N^3-thymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ (**5**) and {8-[5-(O^4-thymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ (**6**) ions.

xy]-3-cobalt bis(1,2-dicarbollide)}$^-$ (**6**) ions were prepared by the alkylation of thymidine at 3-*N*- and 4-*O*-position[23] with [8-dioxane-3-cobalt bis(dicarbollide)]^0 = 8-O(CH_2CH_2)$_2$O-1,2-$C_2B_9H_{10}$-3-Co-1′,2′-$C_2B_9H_{11}$ (**2**).[15] Briefly, alkylation of thymine in fully protected nucleoside, 5′-*O*-monomethoxytrityl-3′-*O*-acetylthymidyne (**4**), was performed via activation of 4-*O* (and competitively 3-*N*) using sodium hydride followed by **2**. The fully protected products of 3-*N* and 4-*O* alkylation were separated by chromatography on silica gel. The deacetylation of 3′-OH group yielded {8-[5-(N^3-5′-*O*-monomethoxytritylthymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ and {8-[5-(O^4-5′-*O*-monomethoxytritylthymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ ions, respectively. The 3′-deprotected derivative was detritylated under acidic conditions leading to deprotected metallacarborane/nucleoside conjugate **5** and **6**.

[8-Dioxane-3-cobalt bis(dicarbollide)]^0 (**2**) was obtained in the reaction of [3-cobalt bis(1,2-dicarbollide)]Cs (**1**)[16] with dioxane in the presence of dimethyl sulfate (Me_2SO_4, DMS) and sulfuric acid.[24] [8-(5-Hydroxy-3-oxa-pentoxy)-3-cobalt bis(1,2-dicarbollide)]$^-$ (**7**) was prepared by heating of **2** with an excess of NaOH in THF/H_2O mixture with stirring at 60 °C. Then, THF was removed under vacuum and next **7** was extracted as the sodium salt into diethyl ether. Removal of diethyl ether in vacuo and drying of the residue to a constant weight yielded **7** quantitatively.[25] For the biological studies **7** obtained as above was additionally purified by RP-HPLC (Econosil RP C18, 5 μm, 4.7 × 250 mm) using triethylammonium carbonate buffer (0.1 M, pH 7.0) containing 60% of acetonitrile. [8-Hydroxy-3-cobalt bis(dicarbollide)]$^-$ ion (**8**) was prepared as described by reductive acetoxylation of [3-cobalt bis(1,2-dicarbollide)]Cs (**1**) followed by hydrolysis of the intermediate[26] and [8-phosphate-3-cobalt bis(dicarbollide)]$^-$ ion (**9**) was prepared in the reaction of **8** with phosphorus oxychloride followed by hydrolysis of the obtained intermediate.[27] [8-(5-Phosphonate-3-oxa-pentoxy)-3-cobalt bis(1,2-dicarbollide)]$^-$ (**10**) was prepared in the nucleophilic dioxane ring opening in **2** with dimethylphosphite anion followed by hydrolysis of the obtained dimethyl ester of phosphonic acid.[28]

Cytotoxicity was established by measurement of 50% inhibition of cell growth (CC_{50}, median cytotoxic concentration) using plaque technique and MTT staining.[29–32] It was found that {8-[5-(O^4-thymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ (**6**) was low in toxicity and had a CC_{50} above 100 μM, {8-[5-(N^3-thymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ (**5**) is characterised by CC_{50} 43–100 μM depending upon the cell system used. It is worthy to point out that extrapolation of the dose-dependent cytotoxicity curves for

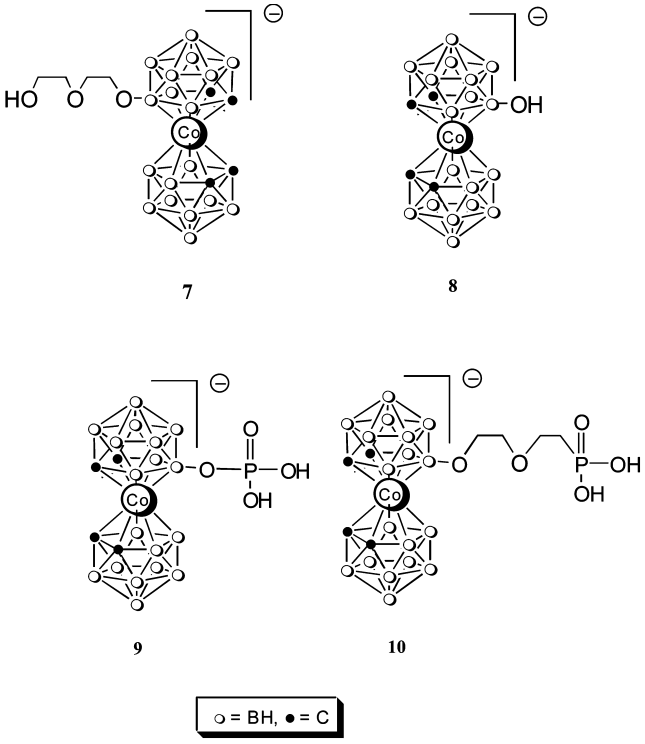

Figure 3. Nonnucleoside derivatives of [3-cobalt bis(1,2-dicarbollide)]$^-$ ion (**1**) substituted at boron-8: [8-(5-hydroxy-3-oxa-pentoxy)-3-cobalt bis(1,2-dicarbollide)]$^-$ (**7**), [8-hydroxy-3-cobalt bis(dicarbollide)]$^-$ ion (**8**), [8-phosphate-3-cobalt bis(dicarbollide)]$^-$ ion (**9**) and [8-(5-phosphonate-3-oxa-pentoxy)-3-cobalt bis(1,2-dicarbollide)]$^-$ (**10**).

compound **6** shown in Figure 4 suggests that this compound is not toxic also at concentrations much higher than 100 μM.

Simple derivatives of [3-cobalt bis(1,2-dicarbollide)]$^-$ ion (**1**) tested in the present study can be divided into two types, one containing neutral ligands such as dioxane (**2**), (5-hydroxy-3-oxa-pentoxy)- (**7**) or OH- (**8**) attached to boron-8 of metallacarborane system, and a second type, containing ionisable phosphate (**9**) or phosphonate (**10**) group (Fig. 3). Compound **2** was nontoxic in PBM and Vero cells showing the CC_{50} above 100 μM in both cases but toxic in CEM cells (CC_{50} = 10 μM). Compound **7** was moderately toxic or toxic in Vero, MRC-5, PBM and CEM cells showing CC_{50} of 68, 42, 14 and 3.3 μM, respectively, but was nontoxic in A549 cell line (CC_{50} above 100 μM). Compounds **8** and **10** did not show toxicity in PBM and Vero cells above 100 μM concentration, and were moderately toxic in CEM cells (CC_{50} = 60–88 μM). Compound **9** was nontoxic in all three cell lines, PBM, CEM and Vero cells at concentrations higher than 100 μM (Fig. 4, Table 1).

Partition coefficient for compounds **3**, **5**–**7** and CDU was established.[33,34] We found that the partition coefficient value for nucleoside conjugates **5** and **6**, bearing metallacarborane complex increased markedly as compared to natural thymidine (**3**), and was 42.65 ± 5.71, 43.13 ± 8.97 and 0.08 ± 0.02, respectively. For comparison, CDU and metallacarborane bearing (5-hydroxy-3-oxa-pentoxy)-linker (**7**) has been characterised by partition coefficient 57.04 ± 11.90 and 46.48 ± 8.19, respectively.

The cellular uptake experiments showed that compounds **5** and **9** gave boron concentrations within the range of the blank, 31 and 27 pg/10^6 cells, respectively (the blank level of boron was 29 pg/10^6 cells). For compound **6**, accumulation could be confirmed, although at a low level, 60 pg/10^6 cells.

3. Discussion

Despite its attractiveness as a modality for treatment of high grade brain tumors and continuous improvements, BNCT still remains in the stage of clinical experimentation. Conceptually it is the ultimate optimisation of an effective agent (high LET radiation), which aims to deliver selectively to tumor cells. Therefore, to evolve into clinically accepted treatment for cancer both components of binary BNCT system, boron carrier and neutron beam must be considered and optimised. The developments in epithermal neutron beam technology from reactor sources have reached a high level of sophistication, and nonreactor epithermal neutron sources can be made accessible with the availability of additional research and adequate funding in future. However, the obstacles ahead to assure adequate boron uptake into tumor cells are still formidable. This in turn, increases the expectations for a breakthrough in the area of boron delivering drugs. It seems that both currently used BSH and BPA, and other boron modified compounds may not fulfill these expectations. This prompted us to initiate study on metallacarborane derivatives as potential boron carriers for BNCT. Similar idea was conceived recently by Bregadze et al.[35]

To meet the criteria of effectiveness, there must be a significant concentration of boron delivered into tumor tissues by boron caring drug. This requires a high concentration of boron carrier in blood; therefore, the low toxicity is an important criterion and first level of selection of potential boron carriers.

The study on anti-tumor activity of metallacarboranes was originally initiated more than 20 years ago. Toxicity and pharmacokinetics of potassium salt of [3-cobalt bis(1,2-dicarbollide)]$^-$ ion (**1**) in tumor bearing rats[36,37] and mutagenicity in *Salmonella typhimurium* and *Drosophila melanogaster* were tested.[38] Compound **1** was found cytotoxic, but not mutagenic in the system used. The in vitro anti-tumor activity of a tin-*meta*-carborane derivative $\{[(1,7\text{-}C_2B_{10}H_{11}\text{-}1\text{-}COO)Bu_2Sn]_2\}O_2$ has been described.[39] Anti-tumor activity of metallacarboranes other than based on dicarba-*closo*-dodecaboranes has also been described.[22,40] In vitro cytotoxicity of these complexes against selected human tumor cell lines ranged from moderate to high. Detected cytotoxicity of the above compounds makes some of them candidates for further studies as potential anti-tumor agents, but also excludes them as boron carriers for BNCT.

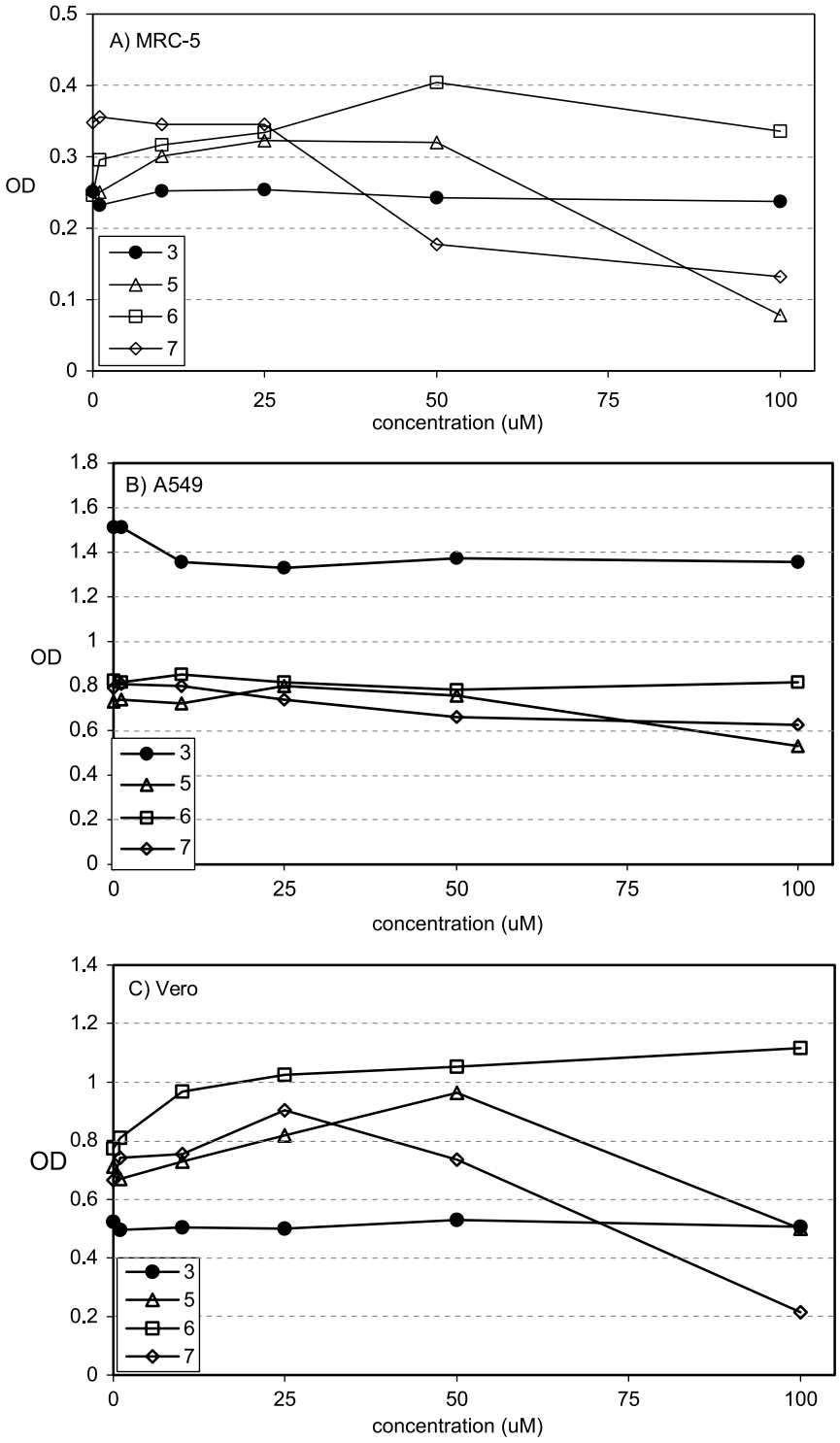

Figure 4. Dose-dependent cytotoxicity curves in MRC-5, A549 and Vero cells for thymidine (**3**), [8-(5-hydroxy-3-oxa-pentoxy)-3-cobalt bis(1,2-dicarbollide)]$^-$ (**7**) and their conjugates {8-[5-(N^3-thymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ (**5**) and {8-[5-(O^4-thymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ (**6**).

In contrast, we have found that conjugates of metallacarborane complex type of [3-cobalt bis(1,2-dicarbollide)]$^-$ ion (**1**) with nucleoside unit, and in some cases simple nonnucleoside derivatives are characterised by low cytotoxicity. Thus, compounds **6** and **9** are nontoxic up to 100 μM in Vero, A549, MRC-5, PBM and CEM cells, similarly to natural thymidine **3**. Metallacarborane/thymidine conjugate containing metallacarborane modification attached at 3-*N* (**5**) and nonnucleoside metallacarborane derivative **8** and **10** show moderate toxicity at higher concentrations. For comparison, for 5-carboranyl-2′-deoxyuridine (CDU)

Table 1. Cytotoxicity of [8-dioxane-3-cobalt bis(dicarbollide)]^0 (**2**), thymidine (**3**), {8-[5-(N^3-thymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ (**5**) and {8-[5-(O^4-thymidine)-3-oxa-pentoxy]-3-cobalt bis(1,2-dicarbollide)}$^-$ (**6**) and their components: [8-(5-hydroxy-3-oxa-pentoxy)-3-cobalt bis(1,2-dicarbollide)]$^-$ (**7**), [8-hydroxy-3-cobalt bis(dicarbollide)]$^-$ ion (**8**), [8-phosphate-3-cobalt bis(dicarbollide)]$^-$ ion (**9**) and [8-(5-phosphonate-3-oxa-pentoxy)-3-cobalt bis(1,2-dicarbollide)]$^-$ (**10**)

Compound	Cell system, CC_{50} (μM)				
	Vero	A549	MRC-5	PBM	CEM
2	>100	ND	ND	>100	10
3	>100	>100	>100	ND	ND
5	94	82	68	43	>100
6	>100	>100	>100	>100	>100
7	68	>100	42	14	3.3
8	>100	ND	ND	>100	60
9	>100	ND	ND	>100	100
10	>100	ND	ND	>100	88

CC_{50} was 17 μM as established in Vero cell line but it was not toxic up to 100 μM to primary human lymphocytes and the glioma cell lines U253 and 9L.[41,42] The toxicity of BPA used clinically in BNCT is much lower and was assigned as $8.4–8.6 \times 10^{-3}$ M in B-16 and TIG-1–20 cell lines.[43]

The carboranyl cage is characterised by extremely high lipophilicity. This feature is recently being exploited in the application of some borane clusters as hydrophobic component (pharmacophore) in biologically active molecules providing them with capacity to interact hydrophobically with other molecules such as proteins or lipids of cellular membranes. Carborane pharmacophores may also improve the ability of modified molecules to penetrate lipid bilayers and potentially increase the cellular uptake.[44] [3-Cobalt bis(1,2-dicarbollide)]$^-$ ion (**1**) and its derivatives inherit part of carborane cluster lipophilicity combined with high affinity towards inorganic and organic cations. This unique property is being presently used for selective extraction of selected cations from diluted water solutions into organic solvents.[19,45]

The lipophilic property of metallacarborane function and its ability to affect lipophilicity of a carrier molecule is clearly illustrated by the high partition coefficient of nucleoside/metallacarborane conjugates. The partition coefficient of conjugates **5** and **6** is several hundred times higher than unmodified thymidine and is comparable with partition coefficient of metallacarborane bearing (5-hydroxy-3-oxa-pentoxy)-linker (**7**) and carborane bearing nucleoside, CDU.

The low level of accumulation of compounds **5** and **6** as it has been shown by cellular uptake studies in V79 Chinese hamster cells is somewhat disappointing. One should take into consideration, however, that accumulation of boron is cell type dependent and differs even among subpopulations of malignant glioma cells. Therefore, studies in other cell types and with different metallacarborane/nucleoside conjugates are needed to select conjugate with optimised properties.

4. Conclusions

Derivatives of metallacarboranes type of [3-cobalt bis(1,2-dicarbollide)]$^-$ ion (**1**) are proposed as modifying entity potentially useful in construction of novel boron carriers for BNCT. Nucleoside/metallacarborane conjugate and some simple metallacarborane derivatives substituted at boron-8 are characterised by low toxicity and high lipophilicity, an advantageous and preferred property for potential boron delivering drugs.

Although only various cobaltacarborane derivatives are the subject of this paper, we are well aware that a variety of their ferra- or metalla-analogues could be tailored as well. We focused on the cobaltacarboranes as on the models because they are diamagnetic (in contrast to, e.g., the Fe^{III} analogue) and can be characterised by multinuclear NMR spectroscopy. However, we recognize that other metal-centres might bring about new dimension to the subject. The work in this direction is ongoing in our laboratories.

5. Experimental

5.1. Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), L-glutamine, penicillin G and streptomycin were bought from Sigma–Aldrich Chemie GmbH (Germany); dimethylformamide (DMF) was from Riedel-de Haë (Germany) and sodium dodecylsulfate (SDS) was from Fluka Chemie GmbH (Switzerland). Dimethylsulfoxide (DMSO) was bought from Sigma–Aldrich Chemie GmbH (Germany) and foetal bovine serum (FBS) was from Gibco BRL (Germany). Eagle's minimal essential medium (EMEM) was from IIET (Institute of Immunology and Experimental Therapy) PAS (Poland). CellTiter 96 Aqueous One Solution cell proliferation assay was bought from Promega (Madison, WI).

5.2. Methods

5.2.1. Cells. The human MRC-5 cells (ATCC CCL-171) and monkey Vero cells (ATCC CCL 81) were grown in EMEM. Human cell line A549 (ATCC CCL 185) was propagated in Dulbecco's modified Eagle's medium. All culture media were supplemented with 10% FBS, 2 mM L-glutamine and 100 units/mL penicillin G—100 μg/mL streptomycin.

5.2.2. Cytotoxicity of compounds 3, 5–7 in MRC-5, A549 and Vero cells. The compounds (Figs. 2 and 3) were suspended in DMSO or in distilled water and then in Eagle's minimal essential medium (EMEM) supplemented with 2% of foetal bovine serum. The final concentration of DMSO in the culture medium was 0.1%. The cells were seeded at 1×10^5 cells/well in 96-well tissue culture plates and allowed to proliferate at 39 °C for 24 h in growth medium. The medium was then replaced by new maintenance medium containing different concentrations of the test compounds (three wells for

each concentration). After a 2-day incubation at 37 °C in 5% CO_2, the number of viable cells was determined by the formazan method (see below). Cytotoxicity of the compounds is expressed as the 50% cytotoxic concentration (CC_{50}), which is the concentration required to reduce cell growth by 50% of the (untreated) control.

5.2.3. Formazan method.[30] Colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) method was used. This method is based on the fact that the yellow tetrazolium salt MTT is converted to dark blue formazan by living cells but not by dead cells or culture medium. MTT was diluted in PBS at a concentration of 5 mg/mL. To measure cell killing or growth, 25 μL of the MTT dye solution was added to each well of the 96-well microplate with cell culture. Next, the plates were incubated for 2 h at 37 °C in 5% CO_2. Then, 100 μL of the solvent solution containing: 1/45 mL of DMF, 2/13.5 g of SDS and 3/55 mL of distilled water, were added to each well. After overnight incubation at 37 °C, optical density at 550 nm was measured in ELISA reader Multiscan RC (Labsystems, Finland).

5.2.4. Cytotoxicity of compounds 2, 8–10 in PBM, CEM and Vero cells.[31] PBM, CEM and Vero cells (5×10^4 cells per well) were seeded in 96-well plates in the presence of increasing concentrations of the test compounds (Figs. 2 and 3) and incubated at 37 °C with 5% CO_2. After a 5-day incubation, cell viability defined as CC_{50} was measured using the CellTiter 96 Aqueous One Solution cell proliferation assay.

5.2.5. Partition coefficient (*K*) measurement.[33] To 100 μM solution of compounds **3**, **5**–**7** and CDU in deionised H_2O containing 5% of CH_3OH (1 mL) in 2 mL Eppendorf tube octanol (1 mL) was added. The resulting mixture was shaken vigorously at room temperature (20–22 °C) for 1 h to ensure that the compound's transfer between the two phases was at equilibrium and then the mixture was left standing for 1 h to separate the phases. Each sample was subsequently centrifuged for 5 min at 13,000 rpm then 0.9 mL of H_2O or organic solution was transferred to 1 mL UV cell and the UV absorption at $\lambda = 260$ (compound **3**) or 310 nm (compounds **5**–**7**) was measured. The partition coefficient is defined as the ratio of the amount of the compound present in the organic phase to that present in the aqueous phase.

5.2.6. Cellular uptake.[46] Cellular uptake was studied using a filtration technique where the cells were freed from surrounding medium by centrifugation through an oil layer. Instead of trichloroacetic acid (TCA) used in original method, half-concentrated HNO_3 was applied as lysing medium in modified procedure, as precipitation was seen with TCA, which might lead to loss of boron. V79 Chinese hamster cells were used for the measurement of uptake of boron in cells. The cells were grown in F10 medium containing 5% newborn calf serum at 37 °C in 5% CO_2. For the uptake experiments, about 1×10^6 cells were seeded into 10 cm diameter Petri dishes, 24 h before the experiment. The compounds were dissolved in DMSO at concentrations of 77 mM (compounds **5** and **6**) and 93 mM (compound **9**), and 10 μL was diluted into 10 mL medium to final concentrations of 77 μM (compounds **5** and **6**) and 93 μM (compound **9**), respectively. After incubation for 3 h, the cells were scraped off the dish with a sterile cell wiper and suspended in the incubation medium by vigorous pipetting through a Pasteur pipette. An aliquot of the cell suspension was used to determine the number of cell per mL. The cells were sedimented by centrifugation at 1500 rpm for 8 min, the major part of the supernatant was removed, and the cells were resuspended in the remaining incubation solution (about 0.5 mL). The cell suspension was layered, in an Eppendorf 2 mL centrifuge cup, on top of a bottom layer (0.7 mL) of 20% aqueous HNO_3, onto which a mixture of nine parts silicon oil (Fluka, AP100, density 1.06 g/mL) and one part mineral oil (Fluka, density 0.85 g/mL) had been pipetted. The resulting three-layer ensemble was centrifuged for 3 min at 1500 rpm, whereupon the cells had passed from the incubation medium through the oil into the bottom layer and lysed. A sample of the bottom layer was withdrawn by piercing the centrifuge cup with a hypodermic needle, and analysed for boron. Removal of cells from the dish, centrifugation and withdrawal of the bottom layer was achieved within 20 min. The sample of the bottom layer was diluted 1:20 with bidistilled water and filtered through a 0.2 μm disposable filter. Boron analysis was carried out in a ThermoElectron Element 2 ICP-MS apparatus, measuring both boron-10 and boron-11. Samples were diluted with water to concentration ranges appropriate for the sensitivity of the apparatus. Concentrations were determined by the increment method, comparing the increase of boron signal after the addition of known concentrations of boric acid over that of the blank solution. Concentrations are expressed as pg boron/10^6 cells (= ppb, assuming a cell volume of 10^{-9} mL per cell). A blank was obtained by incubating cells in medium not containing any boron compound, and harvesting and analysing them in the same way.

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