ChemComm

Cite this: *Chem. Commun.*, 2012, **48**, 612–614

www.rsc.org/chemcomm

COMMUNICATION

Design of multivalent galactosyl carborane as a targeting specific agent for potential application to boron neutron capture therapy†

Chian-Hui Lai,[a] Yu-Chuan Lin,[b] Fong-In Chou,[b] Chien-Fu Liang,[a] En-Wei Lin,[a] Yung-Jen Chuang[c] and Chun-Cheng Lin*[a]

Received 21st July 2011, Accepted 7th November 2011
DOI: 10.1039/c1cc14447b

A multivalent galactosyl carborane derivative 10 (dendritic glyco-borane, DGB) was synthesized and demonstrated as a potential cell-targeting agent in BNCT with HepG2 cells. DGB 10 improved the delivery of boron to HepG2 cells and neutron irradiation data show DGB 10 with ten-fold improvement at killing the HepG2 cells over BSH.

Boron neutron capture therapy (BNCT) is a binary radiation therapy for cancer that involves the irradiation of non-radioactive ^{10}B-enriched tumors with low energy thermal neutrons to yield high linear energy transfer (LET) particles.[1] Neutron capture results in the formation of excited ^{11}B nuclei that split into highly energetic $^{4}He^{2+}$ (α-particle) and $^{7}Li^{3+}$ ions. Due to the limited penetrable path length (5–9 μm, approximately the radius of a typical cell) of high-LET particles into tissues, the destructive effects of high-LET particles are localized within cells that contain boron atoms,[1] which reduces damage to healthy tissues.

The success of BNCT treatment depends on the amount of ^{10}B inside the tumor cell (15–35 μg of ^{10}B per gram of tumor tissue or 10^9 atoms of ^{10}B per tumor cell) and the differential uptake of boron in tumor and normal cells (greater than 3 : 1).[1] Currently, only two boronated compounds are clinically used for BNCT, sodium borocaptate ($Na_2B_{12}H_{11}SH$ or BSH) and boronophenylalanine (BPA). However, neither agent can selectively target tumor cells, which results in cytotoxicity and limits their application. Thus, a well-designed BNCT agent with a cell-targeting moiety could increase the amount of boron in cancer cells and result in the selective destruction of malignant cells. To enrich cancer cells with ^{10}B atoms for BNCT, carborane cages that contain ten boron atoms are ideal substrates because they possess a large number of boron atoms per molecule and can be readily conjugated with organic molecules or target ligands.[1a] Recently, many carborane-based derivatives categorized as small molecules,[2] polymers,[3] dendrimers,[4] and liposomes[5] have been designed and synthesized.

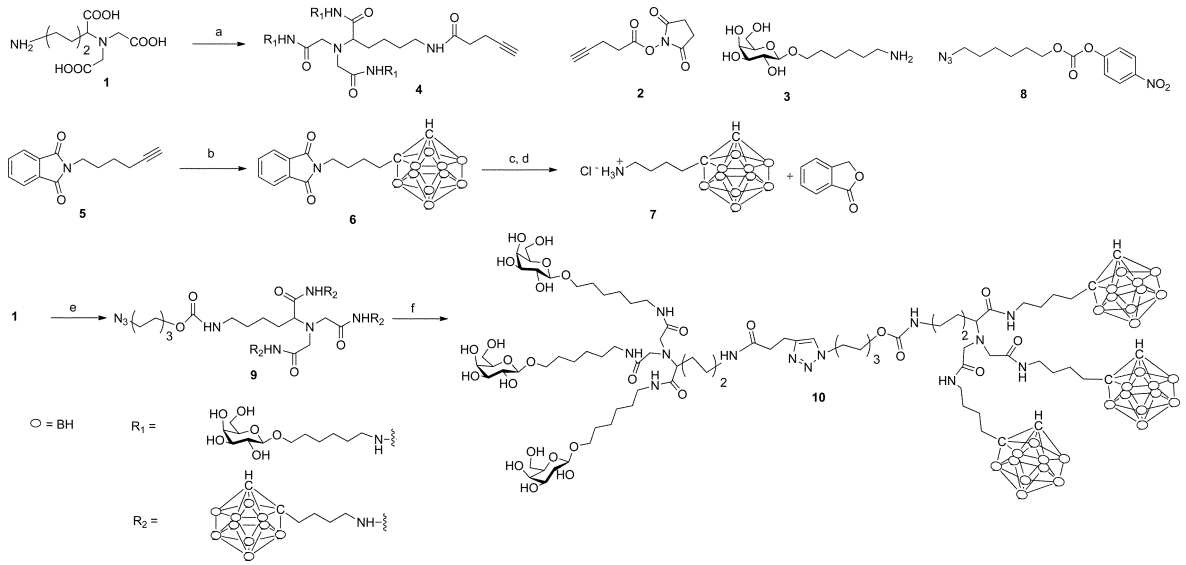
Cell surface carbohydrate–protein interactions are involved in many biological events.[6] For example, asialoglycoprotein receptors (ASGP-Rs) found on the surface of hepatocyte cells ($1–5 \times 10^5$ ASGP-R/cell[7] for HepG2 cells) interact with the nonreducing end of galactose (Gal) or *N*-acetyl-galactosamine (GalNAc) moieties on glycoproteins and trigger the receptor-mediated endocytotic uptake of glycoproteins into the cell.[8] ASGP-R contains two H1 and H2 subunits and exhibits high affinity for triantennary Gal/GalNAc ligands[9] due to the presence of multivalent interactions. The K_d of ASGP-R with mono-, di-, tri-, and tetra-antennary galactosyl oligosaccharides is $\sim10^{-3}$, $\sim10^{-6}$, $\sim5 \times 10^{-9}$, and $\sim10^{-9}$ M, respectively.[9] Previously, we showed that a pre-assembled tri-antennary Gal moiety on fluorescent magnetic nanoparticles (MNP) was a better multivalent ligand for HepG2 cells uptake.[10] Thus, the similar tri-antennary galactosyl structure **4** (Scheme 1) was used as targeting moiety to improve the specificity and water solubility of the boron complex to reduce the cytotoxicity.[2c]

Previously, we applied neutron irradiation to investigate BPA, BSH, and boric acid (BA) as potential boron carriers in the treatment of hepatoma.[11] To further increase the specificity and intracellular uptake of the BNCT agent, we designed a novel, potent BNCT agent, compound **10** (a dendritic glyco-borane, DGB) as shown in Scheme 1. Nearly quantitative yields of DGB **10** were obtained by using Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC)[12] in which the DGB contains two moieties, a trivalent galactose residue for targeting and a trivalent carborane as boron source. Subsequently, the effect of **10** on BNCT was evaluated by irradiating cells with neutrons using the Tsing-Hua Open Pool Reactor (THOR) at the National Tsing-Hua University in Taiwan.

The synthesis of DGB **10** is illustrated in Scheme 1. Trivalent galactosyl alkyne **4** was obtained by reacting *N*,*N*-bis[carboxymethyl]-L-lysine (**1**)[13] with 2,5-dioxopyrrolidin-1-yl pent-4-ynoate (**2**)[14] under basic conditions, followed by coupling with 6-aminohexyl-D-galactopyranoside (**3**)[15] using HATU[16] (46% yield over two steps). Through the modification of literature procedures,[2e,17] decaborane was reacted with *N*-(5-hexynyl) phthalimide (**5**) under reflux to yield **6** (43%). Subsequently, the phthalyl group of **6** was removed by stepwise cleavage,[18] and sodium

[a] *Department of Chemistry, National Tsing Hua University, 101, Sec. 2, Kuang Fu Rd., Hsinchu 30013, Taiwan. E-mail: cclin66@mx.nthu.edu.tw*
[b] *Nuclear Science and Technology Development Center, and National Tsing Hua University, 101, Sec. 2, Kuang Fu Rd., Hsinchu 30013, Taiwan*
[c] *Department of Medical Science and Institute of Bioinformatics and Structural Biology, National Tsing Hua University, 101, Sec. 2, Kuang Fu Rd., Hsinchu 30013, Taiwan. Fax: +886 3-571-1082*

† Electronic supplementary information (ESI) available: Experimental details, characterization data and NMR spectra for new compounds. See DOI: 10.1039/c1cc14447b



Scheme 1 Synthesis of DGB **10**. Reaction conditions: (a) (1) **2**, DMF/H_2O, NEt_3, 4 °C, 16 h, 90%; (2) **3**, HATU, DMF, NEt_3, 45 °C, 16 h, 51%; (b) $B_{10}H_{14}$, CH_3CN, toluene, reflux, 4 h, 43%; (c) $NaBH_4$, *i*-PrOH, rt, 22 h, 76%; (d) HCl/HOAc, 95 °C, 3 h, 95%; (e) (1) **8**, DMF/H_2O, NEt_3, rt, 16 h, 88%; (2) **7**, HBTU, DMF, 45 °C, 16 h, 53%; (f) **4**, CuI, $CuSO_4$, sodium ascorbate, NEt_3, EtOH/H_2O/tBuOH, rt, 65 h, 98%.

borohydride reduction and acidic hydrolysis were conducted to obtain **7** (72% yield for two steps). Trivalent carborane derivative **9** was synthesized using a procedure that was similar to the method for the synthesis of compound **4**. Compound **1** was treated with activated **8** (Scheme S2 in ESI†) and was coupled with compound **7** under basic conditions. Finally, trivalent galactosyl alkyne **4** was conjugated with trivalent carborane azide **9** by CuAAC[12] to give an almost quantitative yield of DGB **10**. The formation of compound **6** was evidenced by the absence of an alkynyl proton peak and the presence of a carborane C*H* resonance peak at 3.59 ppm in the ^{1}H NMR spectrum ($CDCl_3$). Due to the low solubility of compounds **7** and **10** in $CDCl_3$, the ^{1}H NMR spectra of these compounds were taken in CD_3OD and the carborane C*H* resonance peaks appeared at 4.55 ppm. Moreover, multiple broad peaks appeared between 1.5 and 2.9 ppm, indicating the presence of B*H* protons (see ESI†, the broad B*H* peaks were not integrated in the ^{1}H-NMR spectra). In addition, the ^{13}C NMR spectrum displayed a *C*H carborane resonance peak around 61 ppm and a *C*B carborane resonance peak around 75 ppm. Notably, the ^{11}B NMR spectra of carborane derivatives showed similar peak patterns (see Fig. S1, ESI†). The naturally occurring isotopes of boron (^{10}B, 19.6% and ^{11}B, 80.4%) yield characteristic isotope patterns[19] in the mass spectra of boronyl compounds; thus, mass analysis was also applied to confirm the production of carborane derivative **10** (Fig. S2, ESI†). The reverse HPLC spectrum of compound **10** is shown in Fig. S3 (ESI†). The IR spectra of the boronyl compounds displayed a strong B–H stretch at approximately 2580 cm^{-1} (Fig. S4, ESI†). In addition, the loss of an azido signal at 2100 cm^{-1} in the IR spectrum of **10** indicated the success of CuACC.

The cytotoxicity of compound **10** was evaluated by conducting MTT assays on HepG2 cells with ASGP-R and Hela cells without ASGP-R. The results (Fig. S5, ESI†) revealed that compound **10** had a concentration dependent cytotoxic effect on HepG2 cells after 6 h of incubation. Alternatively, compound **10** did not have cytotoxic effects on Hela cells, indicating that uptake occurs *via* ASGP-R mediated endocytosis, not passive diffusion. The incubation time of compound **10** with HepG2 cells (72 h, Fig. S6 (ESI†)) was increased, and enhanced cytotoxicity was observed, further confirming the uptake of **10** by HepG2 cells. Notably, when cells were incubated with low concentrations of boron-containing compounds ($\leq$50 ppm) for 6 h, no significant cytotoxicity was observed. For comparison, HepG2 cells were incubated with BSH at boron concentrations of 25 and 50 ppm for 6h, and cytotoxicity was not detected (Fig. S7, ESI†). In addition, soft-agar colony formation assays were employed to show that compound **10** (at boron concentrations of 12.5 and 50 ppm) did not affect cell proliferation (Fig. S8, ESI†).

The amount of compound **10** taken up by HepG2 cells was determined by inductively coupled plasma–mass spectrometry (ICP-MS). HepG2 cells were seeded and then cultured in DMEM overnight. The cells were incubated in a solution of DMEM containing compound **10** (boron concentration = 12.5, 25, and 50 ppm) and BSH (boron concentration = 50 ppm), respectively. After 6 h of incubation, the cells were washed three times with PBS buffer and were treated with a strong oxidant and strong acid to determine the boron concentration by ICP-MS. As illustrated in Fig. 1, HepG2 cells showed higher uptake of compound **10**. Under all of the studied conditions, more than 10^{10} boron atoms were delivered per HepG2 tumor cell. Although only 19.6% of the boron in the carborane cage was ^{10}B, this amount is sufficient for the success of BNCT. Using equal boron concentration, the uptake efficiency of DGB **10** and BSH into HepG2 cells was compared, and DGB **10** showed 20-fold greater boron uptake than that of BSH. Overall, DGB **10** showed selective targeting capability and highly efficient boron delivery, which are requirements for potential BNCT agents.

Subsequently, the use of DGB **10** as a potential BNCT agent for hepatoma was evaluated. HepG2 cells were treated with DGB **10** at boron concentrations of 50 ppm for 6 h and were irradiated with thermal neutrons at a neutron flux of 1.1×10^{11} n cm^{-2} s^{-1} in the THOR. To compare the cell killing

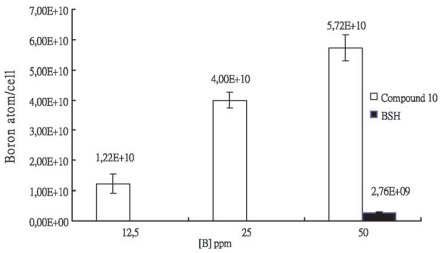


Fig. 1 Average boron concentration obtained by ICP-MS per HepG2 cell after incubation with compound **10** or BSH for 6 hours.

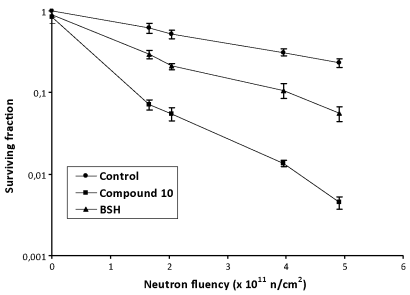


Fig. 2 The fraction of HepG2 cells that survived after irradiation with thermal neutrons. HepG2 cells that were not treated with a boron-containing compound (●), treated with compound **10** at a boron concentration of 50 ppm for 6 h (■), and treated with BSH at a boron concentration of 50 ppm for 6 h (▲).

efficiency of the boron agents in BNCT, HepG2 cells treated with DGB **10** or BSH at a boron concentration of 50 ppm for 6 h, as well as untreated control cells were irradiated with thermal neutrons. Fig. 2 shows the surviving fraction of neutron-irradiated HepG2 cells. In addition, the surviving fraction is also plotted as a function of the neutron fluency. As demonstrated in Fig. 2 and Fig. S8 (ESI†), after irradiation with neutrons, compound **10** killed more cells than BSH, a commonly used BNCT agent in clinics (morphological differences in the colony formation of HepG2 cells treated with compound **10** or BSH as a function of the neutron dosage are shown in Fig. S9 (ESI†)). As the intensity of neutron irradiation increased, an enhancement in the killing effect on HepG2 cells treated with DBG **10** and BSH was observed. Although cells not treated with boron also died after irradiation, the cell killing efficiency of DBG **10** was ten-fold greater than that of BSH-exposed cells at neutron fluxes of 3.96×10^{11} and 4.90×10^{11} n cm^{-2} s^{-1}, respectively. Notably, when the dose of irradiated thermal neutrons is increased, the cell lethal effect is correspondingly increased, which is no dose contribution from the BNCT.[11] Compared to previous reports,[11] DBG **10** provided superior cytotoxic effect at low irradiation flux (1.66×10^{11} n cm^{-2} s^{-1}). Furthermore, upon irradiation at an intensity of 4.90×10^{11} n cm^{-2}, the surviving fraction of cells treated with DBG **10** was only 0.45%. Thus, the results clearly demonstrated that DBG **10** provides superior *in vitro* cytotoxic effects and indicated that targeting is of great importance in the development of BNCT agents.

The success of BNCT treatment ultimately depends on its *in vivo* tumor-localizing property and its ability to enrich boron atoms in tumors. In the present study, we demonstrated the usefulness of DGB **10** as a potential boron delivery agent. The di-dendrimer structure of **10** provides multivalent ligand receptor interactions for selective cell targeting and enhances the delivery of boron into cells. In addition, the use of galactose as a targeting moiety increases the water solubility of the carborane cage.

This work was financially supported by Academia Sinica, the National Tsing Hua University, and the National Science Council, Taiwan.

Notes and references

1 (*a*) A. H. Soloway, W. Tjarks, B. A. Barnum, F. G. Rong, R. F. Barth, I. M. Codogni and J. G. Wilson, *Chem. Rev.*, 1998, **98**, 1515–1562; (*b*) M. F. Hawthorne and A. Maderna, *Chem. Rev.*, 1999, **99**, 3421–3434; (*c*) R. F. Barth, J. A. Coderre, M. G. H. Vicente and T. E. Blue, *Clin. Cancer Res.*, 2005, **11**, 3987–4002.
2 (*a*) G. Calabrese, A. C. N. M. Gomes, E. Barbu, T. G. Nevell and J. Tsibouklis, *J. Mater. Chem.*, 2008, **18**, 4864–4871; (*b*) C. Thimon, L. Panza and C. Morin, *Synlett*, 2003, 1399–1402; (*c*) L. F. Tietze, U. Griesbach, U. Bothe, H. Nakamura and Y. Yamamoto, *ChemBioChem*, 2002, **3**, 219–225; (*d*) A. Maderna, R. Huertas, M. F. Hawthorne, R. Luguya and M. G. H. Vicente, *Chem. Commun.*, 2002, 1784–1785; (*e*) A. Crivello, C. Nervi, R. Gobetto, S. G. Crich, I. Szabo, A. Barge, A. Toppino, A. Deagostino, P. Venturello and S. Aime, *JBIC, J. Biol. Inorg. Chem.*, 2009, **14**, 883–890; (*f*) L. F. Tietze, U. Bothe, U. Griesbach, M. Nakaichi, T. Hasegawa, H. Nakamura and Y. Yamamoto, *ChemBioChem*, 2001, **2**, 326–334.
3 (*a*) T. B. Yisgedu, X. Chen, S. Schricker, J. Parquette, E. A. Meyers and S. G. Shore, *Chem.–Eur. J.*, 2009, **15**, 2190–2199; (*b*) S. B. Kahl and J. Li, *Inorg. Chem.*, 1996, **35**, 3878–3880; (*c*) H. Li, F. R. Fronczek and M. G. H. Vicente, *J. Organomet. Chem.*, 2009, **694**, 1607–1611; (*d*) H. Li, F. R. Fronczek and M. G. H. Vicente, *Tetrahedron Lett.*, 2008, **49**, 4828–4830.
4 (*a*) M. C. Parrott, E. B. Marchington, J. F. Valliant and A. Adronov, *J. Am. Chem. Soc.*, 2005, **127**, 12081–12089; (*b*) B. P. Dash, R. Satapathy, J. A. Maguire and N. S. Hosmane, *Org. Lett.*, 2008, **10**, 2247–2250.
5 (*a*) X. Q. Pan, H. Wang, S. Shukla, M. Sekido, D. M. Adams, W. Tjarks, R. F. Barth and R. J. Lee, *Bioconjugate Chem.*, 2002, **13**, 435–442; (*b*) J.-D. Lee, M. Ueno, Y. Miyajima and H. Nakamura, *Org. Lett.*, 2007, **9**, 323–326; (*c*) S. Altieri, M. Balzi, S. Bortolussi, P. Bruschi, L. Ciani, A. M. Clerici, P. Faraoni, C. Ferrari, M. A. Gadan, L. Panza, D. Pietrangeli, G. Ricciardi and S. Ristori, *J. Med. Chem.*, 2009, **52**, 7829–7835.
6 A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart and M. E. Etxler, *Essentials of glycobiology*, Cold Spring Harbor Laboratory Press, New York, 2nd edn, 2009.
7 (*a*) P. H. Weigel and J. A. Oka, *J. Biol. Chem.*, 1983, **258**, 5095–5102; (*b*) P. H. Weigel and J. H. N. Yik, *Biochim. Biophys. Acta, Gen. Subj.*, 2002, **1572**, 341–363.
8 D. T. Connolly, R. R. Townsend, K. Kawaguchi, W. R. Bell and Y. C. Lee, *J. Biol. Chem.*, 1982, **257**, 939–945.
9 (*a*) Y. C. Lee, R. R. Townsend, M. R. Hardy, J. Lönngren, J. Arnarp, M. Haraldsson and H. Lönn, *J. Biol. Chem.*, 1983, **258**, 199–202; (*b*) Y. C. Lee and R. T. Lee, *Acc. Chem. Res.*, 1995, **28**, 321–327.
10 C.-H. Lai, C.-Y. Lin, H.-T. Wu, H.-S. Chan, Y.-J. Chuang, C.-T. Chen and C.-C. Lin, *Adv. Funct. Mater.*, 2010, **20**, 3948–3958.
11 F. I. Chou, H. P. Chung, H. M. Liu, C. W. Chi and W. Y. Lui, *Appl. Radiat. Isot.*, 2009, **67**, S105–S108.
12 (*a*) V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596–2599; (*b*) W. H. Binder and R. Sachsenhofer, *Macromol. Rapid Commun.*, 2007, **28**, 15–54; (*c*) R. K. V. Lim and Q. Lin, *Chem. Commun.*, 2010, **46**, 1589–1600.
13 L. Schmitt, C. Dietrich and R. Tampe, *J. Am. Chem. Soc.*, 1994, **116**, 8485–8491.
14 C. D. Hein, X.-M. Liu, F. Chen, D. M. Cullen and D. Wang, *Macromol. Biosci.*, 2010, **10**, 1544–1556.
15 X. Wu, C. C. Ling and D. R. Bundle, *Org. Lett.*, 2004, **6**, 4407–4410.
16 S.-Y. Han and Y.-A. Kim, *Tetrahedron*, 2004, **60**, 2447–2467.
17 J. G. Wilson, A. K. M. Anisuzzaman, F. Alam and A. H. Soloway, *Inorg. Chem.*, 1992, **31**, 1955–1958.
18 J. O. Osby, M. G. Martin and B. Ganem, *Tetrahedron Lett.*, 1984, **25**, 2093–2096.
19 A. F. Armstrong and J. F. Valliant, *Dalton Trans.*, 2007, 4240–4251.

