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Synthesis of Cholesterol–Carborane Conjugate for Targeted Drug Delivery

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Abstract—The cholesterol–carborane conjugate has been designed and synthesized to selectively deliver boron to tumor cells by means of reconstituted low-density lipoprotein. The chemical stability and cytotoxicity of the new compound have been examined. Several methods have been evaluated for incorporation of the compound into LDL.

Boron Neutron Capture Therapy (BNCT) is a promising treatment for brain tumor or other tumors.[1,2] However, its efficacy depends on the availability of techniques to concentrate boron atoms (^{10}B) in the pre-irradiated tumors. Kahl[3,4] first proposed and studied targeting methods using low-density lipoprotein (LDL) as a boron carrier. Because the amount of new membrane synthesized in rapidly growing cancer cells is considerable, they consume large amounts of cholesterol. The LDL receptors (LDL is the major component of the cholesterol-transport pathway) are much more active on many types of cancer cells than on the corresponding normal cells.[5–9] Thus, LDL and other molecules carried by LDL would be expected to accumulate preferentially in tumor cells.[10]

The method we selected for targeted boron delivery involved synthesizing boronated cholesterol and reconstituting LDL with the boronated cholesterol. Inasmuch as most lipid in LDL is cholesteryl ester (1500 molecules of cholesteryl ester form the lipid core of LDL[11]), boronated cholesterol might conceivably replace some or all of LDL's cholesteryl ester core. Hence, the first step was to boronate cholesterol; that is, to produce a derivative that would mimic native cholesteryl ester and allow the substitution of it in the LDL core. Feakes et al. reported synthesis of boron-containing cholesterol derivatives for incorporation in liposomes.[12] The compound (cholesteryl 1,12-dicarba-closo-dodecaborane-1-carboxylate, BCH; see Fig. 1) we synthesized has a chemical linkage between the cholesterol and the boron cage that differs from the linkages synthesized by Feakes et al. The *para*-carborane cage was selected as the boron source because: (1) it has 10 rather than one boron atoms per molecule; (2) comparing to *ortho*- and *meta*-carborane cage, it imposes less steric hindrance to further modification on the second carbon atom; furthermore, *p*-carborane derivatives cannot form *nido* compounds and hence it would be attractive in terms of boron cage stability; and (3) it increases the lipophilicity of the molecule to which it is bound. The latter advantage might favor cholesteryl carborane ester incorporation into LDL.[13,14] The cholesterol–carborane conjugate was chosen for synthesis for two reasons: its molecular structure resembles that of native cholesteryl esters and its hydrophobic nature facilitates cholesteryl ester replacement (compounds with oleyl and cholesteryl groups are known to reconstitute LDL more readily[15]). In addition, the high hydrophobicity of cholesterol–carborane conjugate might add physical stability to the LDL-boronated cholesterol complex. In this paper, we report the synthesis of the cholesterol–carborane conjugate (BCH), the chemical stability and cytotoxicity of this new compound, and its incorporation into LDL in Tris–HCl buffer solution.

Cholesteryl 1,12-dicarba-closo-dodecaborane-1-carboxylate (BCH), boronated cholesterol intended for replacement of the cholesteryl esters in LDL, was synthesized by a three-step method. The synthesis was initiated by

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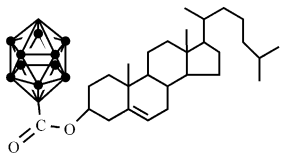


Figure 1. Cholesterol–carborane conjugate (• = BH).

the formation of *p*-carborane carboxylic acid. The *p*-carborane acid was prepared by the method of Grimes[16] with modification (Scheme 1). The reaction conditions were optimized to prepare *p*-carborane monocarboxylic acid with a 95% yield. Two approaches were used to make the cholesterol–carborane conjugate. (1) Carborane acid and cholesterol were combined in the presence of the coupling agent *N,N′*-dicyclohexylcarbodiimide (DCC) and a nucleophilic catalyst 4-(dimethylamino)-pyridine (DMAP) (Scheme 2). (2) Carborane carboxylic acid chloride and cholesterol were combined in the presence of DMAP (Scheme 3). Initially, cholesterol–carborane conjugate was prepared by the first method, but the product yield was very low (about 15%). Changing the esterification conditions, such as temperature and amount of DCC and DMAP, did not significantly increase the yield. The best result was obtained by the second approach (Scheme 3). The carborane carboxylic acid chloride was easily prepared from the acid using thionyl chloride ($SOCl_2$). The reaction of carborane carboxylic acid chloride with cholesterol is clearly the method of choice. The yield of cholesterol–carborane conjugate synthesized by Scheme 3 was 41%, and was much higher than that of the previous method (Scheme 2). The resulting compound, similar to the cholesteryl ester in the core of LDL, is very hydrophobic.

The chemical stability of BCH was studied at pH 5.2, 7.4, and 9.7 at room temperature. Because BCH was extremely water-insoluble, we have used liposomal formulation to solubilize BCH in order to perform the stability studies. Samples were taken for 0, 1, 3, 7, 14, and 28 days. All the samples were analyzed by a high-performance liquid chromatography (HPLC) method specific for BCH. The analysis was performed using a Waters model 2690 separations module equipped with a column heater and a Hewlett-Packard ZORBAX Stable Bond C-18 5m 150×4.6 mm analytical column. The mobile phase used was 50:50 (v:v) methanol–isopropanol and the flow rate was 0.5 mL/min. The column temperature was 40 °C and the UV detector was set at BCH λ_{max} of 202 nm. The retention time for BCH was approximately 11 min. The stability data are shown in Figure 2. It can be seen based on the chromatography analysis that BCH was relatively stable (about 8.3% degradation during the period of 28 days) in the liposomal formulation at the three different pH values.

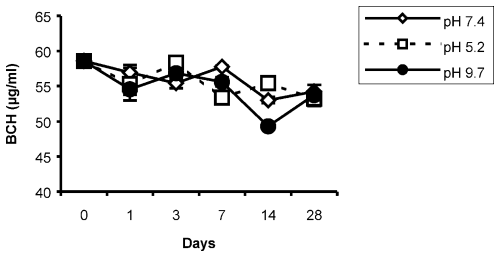
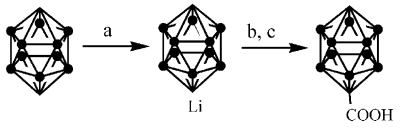


Figure 2. Chemical stability of BCH at pH 5.2, 7.4, and 9.7 ($n = 3$).

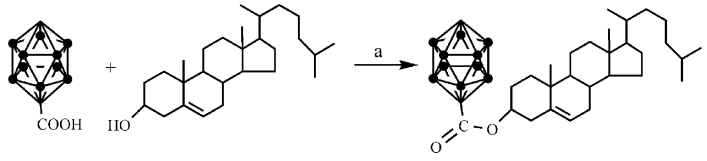
The cytotoxicity of BCH in liposomal formulation was assessed on the SF-763 human glioma cancer cell line (obtained from the tissue bank of the Brain Tumor Research Center at University of California-San Francisco) by determination of percent viable cells relative to control group using MTT assay.[17] Table 1 shows the experiment result indicating that BCH was slightly cytotoxic for the SF-763 cancer cells. It should be emphasized that BCH was designed to mimic native cholesteryl esters for targeted boron delivery to tumor cells. The cytotoxic effect of BNCT occurs when a boron compound interacts with an external neutron beam. Our result indicates that without an external neutron beam,



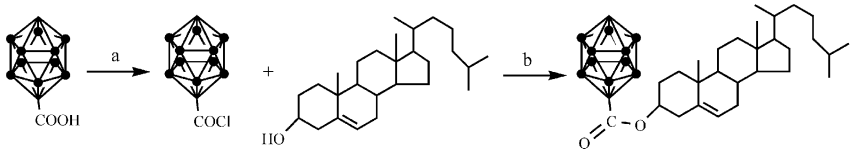
Scheme 1. (a) *n*-BuLi in ether at rt; (b) dry ice at −78 °C; (c) HCl.

Table 1. Cytotoxicity of BCH on SF-763 human glioma cells

Experiment group	Control group ($n = 6$)	BCH group ($n = 6$)
Percent viable cells	100±13.7%	98.52±11.5%



Scheme 2. (a) DCC and DMPA.



Scheme 3. (a) Reflux in $SOCl_2$; (b) DMAP in CH_2Cl_2 at rt.

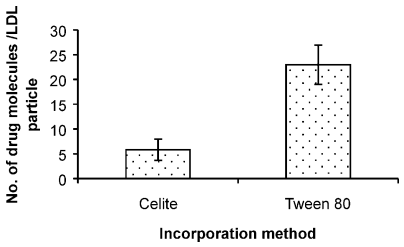


Figure 3. Efficiency of loading cholesterol–carborane conjugate (BCH) into LDL by using Celite with or without Tween 80 (values are means ± SEM of three experiments).

BCH itself does not show severe cytotoxicity and thus meets our design criteria.

The incorporation of cholesterol–carborane conjugate into LDL particles was investigated using a modified Celite transfer system.[18,19] This method allows transfer of relatively large amounts of drug from Celite particle surfaces to LDL without exposing LDL particles to organic solvents. More cholesterol–carborane conjugate was incorporated into LDL using the Celite transfer system with Tween 80 (3.01% of BCH incorporated) than using the Celite transfer system alone (0.904% of BCH incorporated) (see Fig. 3). By either method, incorporation was low. This low efficiency is typical for LDL-loading methods that depend on incubation. It is known that there are many variables (such as temperature, incubation time and surfactants) that may influence the drug entrapment efficiency of the LDL particle.[14] The factors that influence incorporation of cholesterol–carborane conjugate into LDL have to be further explored, especially in the biological environment. Our results show that wetting agents like Tween 80 can enhance the incorporation of cholesterol–carborane conjugate into LDL but the incorporation is low in non-biological fluids.

These results of the studies showed that cholesterol–carborane conjugate could be synthesized in acceptable yields from readily available starting materials. The new compound is stable at different pH at room temperature when it is solubilized in liposomes. It has low cytotoxicity as it is designed to be a radiation inducer for BNCT. Also, the evidence indicates that cholesterol–carborane conjugate can be incorporated into LDL, although the loading methods need to be further developed to enhance the incorporation. Further studies are also needed to examine the transfer of BCH into LDL in biological fluids and the use of long-circulating liposomes, such as a sterically-stabilized liposomal formulation consisting of PEG (polyethylene glycol)-derivatized phospholipids. Chemical reaction conditions for this new compound were explored and optimized. The addition of surfactant (Tween 80) enhanced cholesterol–carborane conjugate incorporation into LDL approximately 4-fold in non-biological fluids. The cholesterol–carborane conjugate and LDL reconstituted with boronated cholesterol appear to be suitable candidate agents for boron delivery and will be tested for their tumor-targeting ability in cultured tumor cells and in a rat model of glioma.

Monocarboxylic *p*-Carborane Acid

p-Carborane was purchased from Aldrich Chem. Co. (Milwaukee, WI, USA). *n*-BuLi (1.1 mL, 1.66 mmol, 1.6 M in hexane) was slowly added at 0 °C to a stirred solution of *p*-carborane (200 mg, 1.38 mmol) in ether (80 mL) in a flask fitted with a reflux condenser. The reaction mixture was warmed to room temperature and stirred for 3 h. The reflux condenser was removed and the reaction mixture was cooled to −78 °C (dry ice/acetone). Dry ice (CO_2) was added to the reaction mixture under a positive flow of nitrogen. The reaction mixture was warmed to room temperature and excess ether was removed by vacuum. The residue was dissolved in 10 mL of water and extracted with ether (2×5 mL). The aqueous layer was acidified with 5 M HCl solution to pH 1 and the product extracted with ethyl acetate. Proton NMR and ^{13}C NMR were used to confirm the structure of product. ^{1}H NMR (400 Hz, $CDCl_3$) δ 1.6–3.2 (10H, B–H); ^{13}C NMR (400 Hz, DMSO-d_6) δ 166.14, 67.15, 65.34.

Cholesteryl 1,12-Dicarba-closo-dodecaborane-1-carboxylate

Eighty milligrams of *p*-carborane carboxylic acid (0.425 mmol) was placed in a 25-mL flask. Thionyl chloride (5 mL) were added to the flask, which was quickly attached to a reflux condenser protected by a drying tube. The assembly was mounted in an oil bath set at 78 °C, and the reaction was allowed to proceed for 4 h. Excess SO_2Cl was removed by vacuum. Cholesterol (165 mg, 0.426 mmol) was dissolved in 3 mL of methylene chloride (containing 50 μL of pyridine). The reaction mixture was stirred under nitrogen for 48 h. The excess solvent was removed and residue was subjected to silica gel column chromatography. The product cholesterol–carborane conjugate was a white solid with mp: 234–236 °C and IR spectra exhibiting strong B–H stretches at 2545 cm^{-1}. Proton NMR and ^{13}C NMR were used to confirm the structure of the product. ^{1}H NMR (400 Hz, $CDCl_3$) δ 1.6–5.1 (10H, B–H), 5.34 (1H, Chol 6), 4.43 (1H, Chol 3); ^{13}C NMR ($CDCl_3$) δ 61.72 (acid 1), 139.24 (Chol 5), 122.71 (Chol 6). Anal. calcd for $C_{30}H_{56}O_2B_{10}$: C, 64.76; H, 10.06. Found: C, 64.87; H, 10.14.

Cytotoxicity Study of BCH Using the MTT Assay

Briefly, the cells were seeded into a 96-well plate at 10^5 cells per well and incubated for 24 h. The cells were then incubated for 16 h with 10 μL BCH in liposomal formulation (154.5 μg BCH/mL). To determine cell viability MTT was dissolved in phosphate buffered saline at a concentration of 5 mg/mL and filtered through a 0.2 μm membrane filter. Ten microliters of MTT solution were added to each well and the cells were incubated at 37 °C for 4 h. After the incubation time, acid isopropanol solution was added to the wells and mixed thoroughly to dissolve the dark blue crystals and then the plates were incubated for 10 min at room temperature. The absorbance of the samples was

determined at 595 nm using a Multiskan Ascent V1.22 microplate reader. The cytotoxicity of BCH was examined based on the percent viable cells relative to control group.

Loading of Cholesterol–Carborane Conjugate into LDL

In this procedure, 500 μg of cholesterol–carborane conjugate dissolved in chloroform were added to a tube containing a Celite dispersion. After evaporating the chloroform under N_2, LDL (0.63 mg of LDL protein) in Tris–HCl buffer was added to the tube. The mixture was incubated at 37 °C in a shaking water bath for 24 h. After incubation, the Celite was removed by centrifugation at $1500 \times g$ for 30 min, and then the LDL solution was dialyzed thoroughly against PBS. The concentration of cholesterol–carborane conjugate was determined by means of the inductively coupled plasma–mass spectrometer (ICP–MS).

In later experiments, a wetting agent, Tween 80 (0.05% of the final volume), was added to the above-mentioned chloroform solution of cholesterol–carborane conjugate and Celite. The purpose was to test these agents as loading enhancers. The experiment for BCH–LDL incorporation was repeated three times. LDL (human) was purchased from Sigma Co.

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