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Synthesis, preformulation and liposomal formulation of cholesteryl carborane esters with various fatty chains

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Abstract

The elevated expression of LDL receptor on tumor cells provides one attractive approach for targeted drug delivery to tumor cells. Suitable antitumor compounds, however, need to be synthesized and developed which mimic the native cholesteryl esters (as major constituent of LDL) in chemical structure for targeted delivery to tumor cells through the over-expressed LDL receptors. In the present study, new antitumor compounds were designed containing cholesterol, fatty chain and carborane which is used as the antitumor unit. Three new compounds were synthesized with a three-step reaction scheme. Similar to the native cholesteryl esters, these compounds are extremely hydrophobic and, before any further biological studies, suitable liposomal formulations for these new compounds are required. Various liposomal formulations as well as the preformulation characterization of these new compounds were thus examined. The incorporation efficiency of the compounds in liposomes was found to vary significantly depending on the type of fatty chain attached and the ratio of cholesterol:phospholipid used as the excipients of liposomal formulation.

Keywords: Drug design; Drug targeting; Low density lipoprotein; Liposome; Cholesterol ester; BNCT

1. Introduction

Targeted drug delivery to cancer cells has a great potential to enhance the chemotherapeutical efficacy for the treatment of human malignant cancers. One of the targeting strategies is based on the fact that the rapid dividing cancer cells have high cholesterol demand (Vitols, 1991; Polo et al., 2002; Sarkar et al., 2002). Cancer cells usually grow very aggressively and take up significant amount of exogenous cholesterol (in its ester form) for their proliferation and cell membrane construction. Many types of cancer cells show an elevation in LDL (low density lipoprotein) receptor expression and, thus, higher consumption of LDL (Gueddari et al., 1993; Firstone, 1994; Yen et al., 1995; Maletinska et al., 2000; Chen and Hughes-Fulford, 2001). LDL contains about 1500 molecules of cholesterol esters per LDL particle and functions as the main carrier of cholesteryl esters in blood circulation (Kostner and Laggner, 1989). Therefore, the development of new anti-cancer compounds mimicking the native cholesterol esters presents a potentially effective approach for targeted drug delivery to cancer cells via the elevated LDL receptors (Versluis et al., 1998; Feakes et al., 1999; Ji and Lu, 2001; Sarkar et al., 2002).

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The new compounds can be designed as the derivatives of cholesterol ester containing an antitumor chemical unit. Carborane becomes a good candidate as the antitumor unit because its potential use for boron neutron capture therapy (BNCT) of tumors (Gutman et al., 2000; Ujvary and Nachman, 2001; Tietze et al., 2002). Unlike the utilization of other prodrugs, BNCT does not require the release of the antitumor unit in cells. Fig. 1. shows the general designing strategy for the new compounds which contain cholesterol, carborane and saturated or unsaturated fatty chains with various chain length. The chemical structures mimic that of native cholesterol esters in human (such as cholesteryl esters of stearate and oleate) and the fatty chains are varied to compensate the addition of the antitumor carborane unit. As the new compounds share similar chemical and physical characteristics with native cholesteryl esters, they may interact well with LDL and, thus, utilize the elevated LDL receptor expression on cancer cells for targeted delivery.

Recently, we have synthesized a carborane cholesteryl ester. The compound, cholesteryl 1,12-dicarba-*closo*-dodecaboranel-carboxylate (BCH, see Fig. 1C), is extremely hydrophobic and, thus, was formulated

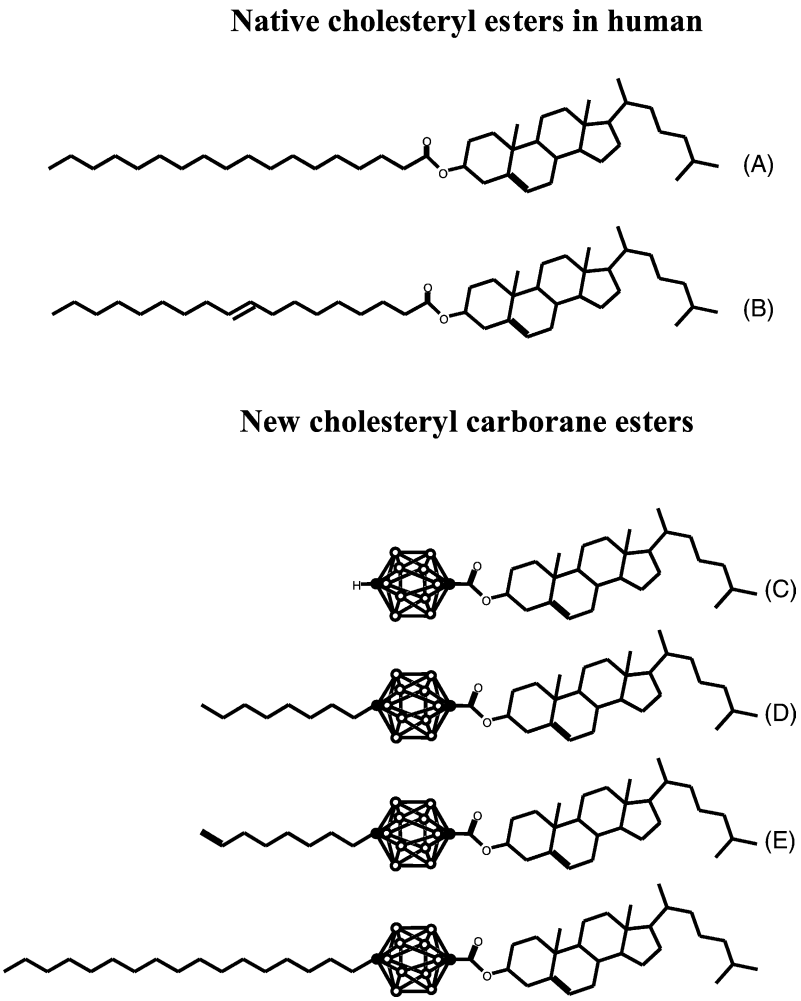


Fig. 1. Designing strategy of new cholesteryl carborane ester compounds. Structure A, B, C, D, E and F are referred as cholesteryl stearate, cholesteryl oleate, BCH, IIIa, IIIb, and IIIc, respectively.

in liposomes for cell culture studies. The cell culture results indicated that BCH, carried by liposomes, resulted in more than adequate cellular uptake. The boron uptake was about 10–11 times higher than the required boron level for successful BNCT (Peacock and Lu, 2001). In the present study, three new carborane-containing cholesterol ester mimics were synthesized. In chemical structure, these compounds are similar to BCH but include various fatty chains (octyl, octenyl and octadecayl) on the second carbon atom of the carborane cage. *p*-Carborane was used because it is boron-rich (containing 10 boron atoms) and it imposes less steric hindrance to further modification on the second carbon atom comparing to the meta or ortho isomers. These compounds are extremely hydrophobic and, before any further biological studies, suitable liposomal formulations for these new compounds are required. Therefore, we further studied preformulation characteristics and liposomal formulations for these new compounds. The effect of different fatty chains (conjugated with the carborane cage) on the drug loading in liposomal formulation was evaluated. Since these new compounds are cholesterol derivatives, the amount of cholesterol used as the excipient of liposomal formulation was also varied to maximize the drug loading.

2. Material and methods

2.1. Reagents and methods

p-Carborane, butylithium, 1-bromooctane, 8-bromo-1-octene, 1-bromooctadecane, phosphomolobdic hydrate, 4-dimethylaminopyridine, chloroform-d and tetrahydrofuran anhydrous were purchased from Aldrich Chem. Co. (Milwaknkee, WI). Cholesterol, dipalmitoyl DL-α-phosphotidylcholine (DPPC), and *N*,*N*-dicyclohexylcarbodimide were purchased from Sigma Chemical Co. (St.Louis, MO).

All reactions used anhydrous solvents and were carried out under dry nitrogen atmosphere. Carbon dioxide (from CO_2 gas cylinder) was dried by bubbling through concentrated sulfuric acid. Chromatographic separations were performed using silica gel (60–200 Mesh) (J.T.Baker). Thin layer chromatography was performed on silica gel 60 F_{254} (E. M. Industries, Inc., Germany). Elemental analysis was provided by Atlantic Microlab (Norcross, GA). Mass spectrometry (ESI) was provided by Mass spectrometry facility in the University of Georgia (Athens, GA). NMR data were recorded on a Brucker-400 AMX spectrometer and the chemical shifts are reported in ppm (δ). Coupling constant were reported in hertz. The abbreviations used are as follows: s, singlet; d, doublet; t, triplet; m, multiplet; dd, double doublet; brs, broad singlet.

2.2. Synthesis procedure

2.2.1. Syntheses of compound Ia, Ib and Ic

An appropriate amount of *p*-carborane dissolved in dry tetrahydrofuran (THF, 30 ml) was treated with 1.6 M *n*-BuLi (equal mmol to *p*-carborane) at −78 °C and under positive flow of dry nitrogen. The mixture was stirred for 1 h, warmed to room temperature, and stirred for additional 20 min. The mixture was cooled to −78 °C and stirred for 15 min. 1-Bromooctane (for Ia), 8-bromo-1-octene (for Ib) or 1-bromooctadecane (for Ic) was added in equal mmol to *p*-carborane over period of 15 min to the reaction mixture. It was stirred for one hour, warmed up to room temperature, and stirred for additional 2 h. The reaction mixture was diluted with ethyl acetate (25 ml), and quenched with 1N HCl solution (25 ml). The aqueous layer was extracted with ethyl acetate (2 × 15 ml). The organic extracts were combined, washed with saturated Nacl solution, dried over Na_2SO_4, filtered and concentrated by rotor evaporator. The residues were purified on silica gel column chromatograph eluted with (0–20%) dichloromethane in petroleum ether to afford compounds Ia, Ib, Ic, containing octyl, octenyl and octadecayl group, respectively.

2.2.2. Syntheses of compound IIa, IIb, and IIc

Compound I (in appropriate amount) was dissolved in dry THF (25 ml). The solution was cooled to −78 °C and *n*-BuLi (1.6 M in hexane, equal mmol to compounds I) was added in dropwise. The mixture was stirred for 1 h, warmed up to room temperature, and stirred for additional 1 h. It was, then, cooled to −78 °C and dry carbon dioxide was bubbled through the mixture for 2 h. The mixture was warmed up to room temperature and stirred overnight while keeping CO_2 bubbling through it. The reaction mixture was diluted with ethyl acetate (25 ml) and quenched

with 1N HCl (15 ml). The aqueous layer was extracted with ethyl acetate (2 × 20 ml). The organic extracts were combined, washed with saturated NaCl, dried over Na_2SO_4, filtered and concentrated by rotor evaporator. The residues were purified on silica gel column chromatography eluted with petroleum ether to afford compounds IIa, IIb, IIc, containing octyl, octenyl and octadecayl group, respectively.

2.2.3. Syntheses of compound IIIa, IIIb, and IIIc

Compound II (an appropriate amount) and cholesterol (equal mmol to compound II) were dissolved in dichloromethane (35 ml). 4-DMAP (5–10% of compound II in weight) was added. The reaction mixture was stirred for 1 h, added with *N,N*-dicyclohexylcarbodimide (equal mmol to compound II), and stirred for 12 h at room temperature. The reaction mixture was concentrated, filtered, and purified on silica gel column chromatography eluted with petroleum ether to afford compounds IIIa, IIIb, and IIIc, containing octyl, octenyl and octadecayl group, respectively. The products were recrystalized using acetone and dichloromethane.

2.3. Preformulation characterization of the new cholesteryl carborane esters

Melting Point was measured using differential scanning calorimeter (Perkin-Elmer Corporation, Norwalk, CT). Optical and polarizing microscopes were used to observe the crystalline status of the new compounds. Solubility of the new compounds in deionized water was conducted at 25 °C and inductively coupled plasma mass spectrometry was used for analysis.

2.4. Incorporation of cholesteryl carborane esters in liposomal formulations

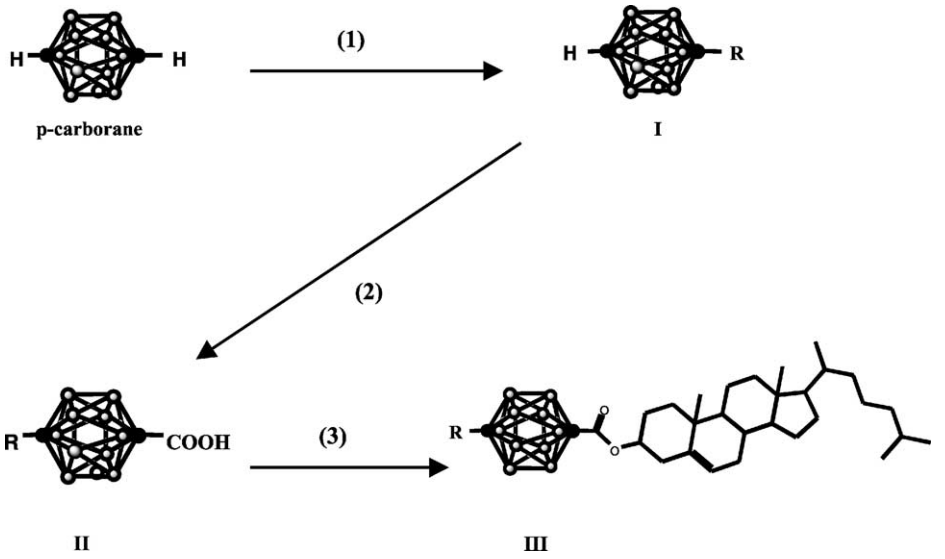
Multilamellar liposomes (MLV's) were prepared by thin film hydration method. Cholesterol, phospholipid and the new compounds were dissolved in a 2:1 mixture of chloroform and methanol in a round bottom flask. The new compounds to lipid ratio was 1:50 (w/w) and total lipid (cholesterol and phospholipid) to water ratio was 1:38 (w/w). Cholesterol and phospholipid were used in three different molar ratios (0:1, 0.33:1, 1:1). The solvent mixture was evaporated under reduced pressure and a thin film of lipid was formed on the bottom of the flask. The film was hydrated at a temperature above the phase transition temperature of the lipid using deionized water and glass beads (5 mm diameter) were added to the dry lipid film to ease the dissolution. The hydration process was performed for several hours using shaking water bath. Liposomes were separated from the glass beads by filtration through a Buchner funnel. The liposomal suspension was centrifuged at 30,000 × *g* for 45 min at 4 °C and the amount of the boron content in liposomal suspension and supernatant was measured by ICP-AES (inductively coupled plasma atomic emission spectroscopy). Phosphorus content was also obtained by ICP-AES and phospholipid content of the liposomal formulations was calculated accordingly. The percentage of the new compounds incorporated in liposomes (the incorporation efficiency or I.E.) was calculated based on boron concentration and phospholipid content.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) was performed using Statgraphics plus two software to compare the mean values of percentage incorporation efficiency for the three different levels of ratio (0:1, 0.33:1, 1:1). Multiple Range Test (Fisher's least significant difference procedure, LSD) was used to determine which means are significantly different from others.

3. Results

Three new carborane conjugates (IIIa, IIIb, IIIc) have been synthesized with a three-step procedure, which is depicted in Scheme 1. In the first step, mono-substitution reactions were achieved by coupling *p*-carborane with the different fatty chains. The overall yield of the reactions was about 80%. Verification of the proposed structures was performed using thin layer chromatography (TLC) and proton nuclear magnetic spectroscopy (NMR). The results were as following: **1-octyl-1, 12-dicarbo-*closo*-dodecaorane (Ia)**: ^{1}H NMR ($CDCL_3$, 400 Hz) δ 2.75 (s, 1H, B-CH), 2.61–1.38 (m, 10H, B-H), 1.35–1.1 (m, 14H, CH_2), 0.86 (t, 3H, $J = 7.31$ Hz).



Scheme 1. A three-step reaction for the synthesis of cholesterol carborane ester compounds. Reagent and solvent: (1) *n*-BuLi in hexanes, anhydrous THF, dry N_2 and bromoalkane or bromoalkene at −78 °C. (2) *n*-BuLi in hexanes, anhydrous THF, dry CO_2 at −78 °C. (3) Dichloromethane, cholesterol, 4-DMAP and *N*,*N*-dicyclohexylcarbomidine.

1-(7-octenyl)-1,12-dicarbo-*closo*-dodecaorane (Ib): ^{1}H NMR ($CDCL_3$, 400 Hz) δ 5.76 (m, 1H, CH_2=$\underline{CH_1}$, 4.96 (dd, 1H, $\underline{CH_2}$=CH_1, J = 1.46 Hz, J = 14.17 Hz), 4.91 (dd, 1H, $\underline{CH_2}$=CH_1, J = 1.05 Hz, J = 9.12 Hz), 2.74 (s, 1H, B-$\underline{C}H_1$), 2.65–1.4 (m, 10H,B-H), 1.40–1.01 (m, 12H, CH_2).

1-octadecyl-1,12-dicarbo-*closo*-dodecaorane (Ic): ^{1}H NMR ($CDCL_3$, 400 Hz) δ 2.75 (s, 1H, B-CH), 2.65–1.52 (m, 10H, B-H), 1.43–1.03 (m, 34H, CH_2), 0.88 (t, 3H, J = 6.62 Hz).

In the second step, compounds I were carboxylated with CO_2 to afford the carboxylic acid derivatives, the intermediate of the target compounds. The overall yield was about 50%. Verification of the proposed structures was performed using thin layer chromatography, proton nuclear magnetic spectroscopy and mass spectroscopy. The results were as following: **12-octyl-1, 12-dicarbo-*closo*-dodecaorane-1-carboxylic acid (IIa)**: ^{1}H NMR ($CDCL_3$, 400 Hz) δ 9.01 (brs, 1H, COOH), 3.1–1.4 (m, 10H, B-H), 1.3–0.9 (m, 14H, CH_2), 0.86 (t, 3H, J = 7.3 1 Hz). MS (ESI) *m/z* calculated for $C_{11}H_{28}$ $B_{10}O_2$ [M^+]: 300, found [M^+]: 300; [M^+-1-CO_2]: 255; [$2M^+$]: 600.

12-(1-octenyl)-1,12-dicarbo-*closo*-dodecaorane-carboxylic acid (IIb): ^{1}H NMR ($CDCL_3$, 400 Hz) δ 8.5 (brs, 1H, COOH), 5.78 (m, 1H, CH_2=$\underline{CH_1}$), 4.97 (dd, 1H, $\underline{CH_2}$=CH_1, J = 1.69 Hz, J = 15.48 Hz), 4.92 (dd, 1H, $\underline{CH_2}$=CH_1, J = 0.84 Hz, J = 9.33 Hz), 3.1–1.5 (m, 10H, B-H), 1.4–0.75 (m, 12H, CH_2). MS (ESI) *m/z* calculated for $C_{11}H_{26}B_{10}O_2$ [M^+]: 298, found [M^+]: 298, [$2M^+$] = 596.

12-octadecyl-1, 12-dicarbo-*closo*-dodecaorane-1-carboxylic acid (IIc): ^{1}H NMR ($CDCL_3$, 400 Hz) δ 3.2–2.9 (brs, 1H, COOH), 2.8–1.5 (m, 10H, B-H), 1.4–1.05 (m, 34H, CH_2), 0.88 (t, 3H, J = 6.56 Hz). MS (ESI) *m/z* calculated for $C_{21}H_{48}B_{10}O_2$ [M^+]: 440, found [M^+]: 440, [$2M^+$] = 880, [$3M^+ + 1$] = 1321.

In the third step, coupling the carboxylic acid derivatives (compounds II) with cholesterol was achieve using 4-dimethylaminopyridine as catalyst and dicyclohexylcarbodiimide as dehydrating reagent. The overall yield was about 30%. Structural verification of the final target compounds was performed using thin layer chromatography, proton and ^{13}C nuclear magnetic spectroscopy, differential scanning calorimeter and elemental analysis. The results were as following: **Cholesteryl-3β-12-octyl-1, 12-dicarbo-*closo*-dodecaorane-1-carboxylate (IIIa)**: ^{1}H NMR ($CDCL_3$, 400 Hz) δ 5.45 (s, 1H, Chol-6), 4.48 (s, 1H, Chol-3) 2.85–1.48 (m), 1.45–1.05 (m), 0.98 (s, 3H,

C–$\underline{CH_3}$), 0.90 (d, 3H, CH_2–$\underline{CH_3}$, $J = 6.48$ Hz), 0.87 (d, 3H, CH–$\underline{CH_3}$ $J = 1.76$ Hz), 0.85 (d, 6H, CH–$\underline{CH_3}$, $J = 1.72$ Hz), 0.65 (s, 3H, C–$\underline{CH_3}$). ^{13}C NMR ($CDCL_3$) δ 23.5, 57.35, 56.5, 50.3, 42.68, 40.07, 39.9, 37.75, 36.87, 32.19, 29.7, 29.47, 28.6, 28.4, 27.52, 24.21, 23.21, 22.95, 21.4,19.66, 19.10, 14.46, 12.23.

Cholesteryl-3β-12-(1-octenyl) -1,12 -dicarbo-*clo-so*-dodecaorane-1-carboxylate (IIIb): ^{1}H NMR ($CDCL_3$, 400 Hz) δ 5.79 (m, 1H, CH_2=$\underline{CH_1}$), 5.3 (m, 1H, Chol-6), 4.97 (dd, 1H, $J = 1.65$, $J = 15.52$), 4.93 (dd, 1H, $J = 3.99$), 4.42 (m, 1H, Chol-3) 3.2–1.6 (m), 1.6–1.1 (m), 0.98 (s, 3H, 3H, C–$\underline{CH_1}$), 0.9 (d, 3H, CH_1–$\underline{CH_3}$, $J = 6.48$ Hz), 0.86 (d, 6H, CH_1–$\underline{CH_3}$, $J = 4.93$ Hz), 0.66 (s, 3H, C–$\underline{CH_3}$); ^{13}C NMR ($CDCL_3$) δ 139.39, 139.33, 123.52, 114.73, 57.05, 56.52, 50.32, 42.70, 39.92, 377, 36.58, 36.19, 34.05, 32.28, 29.30, 29.08, 29.03, 28.63, 28.43, 27.54, 23.24, 22.98, 21.42, 19.69, 19.12, 12.253.

Cholesteryl-3β-12-octadecyl-1,12-dicarbo-*closo*-dodecaorane-1-carboxylate (IIIc): ^{1}H NMR ($CDCL_3$, 400 Hz) δ 5.31 (s, 1H, Chol-6), 4.43 (s, 1H, Chol-3), 2.2–1.4 (m), 1.3–1.1 (m), 0.99 (s, 3H, *C*–$\underline{CH_3}$), 0.89 (d, 3H, CH_2–$\underline{CH_3}$, $J = 6.2$ Hz), 0.87 (d, 3H, CH_1–$\underline{CH_3}$, 1.75 Hz), 0.85 (d, 6H, CH_1–$\underline{CH_3}$, $J = 1.74$ Hz), 0.66 (s, 3H, C–CH_3); ^{13}C NMR ($CDCL_3$) δ 138.95, 123.09, 56.63, 56.09, 49.89, 42.28, 39.5, 37.35, 31.93, 31.85, 29.69, 29.66, 29.58, 29.53, 29.41, 29.36, 29.14, 28.21, 28.01, 22.82, 22.70, 22.55, 19.26, 11.83.

The elemental analysis results of these new compounds are shown in Table 1 and the results indicate that the actual percentage of C and H found in the final products is almost exact to that of calculated ones. The results from the preformulation characterization of the new compounds are shown in Table 2. These compound are extremely hydrophobic and poorly soluble in water. Conjugating side chain (IIIa, C_8) to BCH resulted in increased melting point from 235 °C to 251 °C. Also, changing from saturated (IIIa, C_8) to unsaturated side chain (IIIb, C_8) led to an increase in melting point by 10 °C. Further increase in the number of carbon of the saturated fatty chain from 8 to 18 (IIIc) decreased the melting point from 251 to 210 °C. Under optical and polarizing microscopy, all the compounds were found to be in crystalline form. The size and shape of these crystalline forms were not uniform, respectively.

Table 1
Elemental analysis of the final cholesteryl carborane ester compounds

Compound	C (%)		H (%)	
	Theoretical	Founded	Theoretical	Founded
IIIa	68.152	68.25	10.76	10.92
IIIb	68.42	68.59	10.58	10.69
IIIc	71.23	71.31	11.45	11.61

The incorporation efficiency of BCH and the three new compounds in liposomal formulation was evaluated based on the drug:lipid ratio of 1:50 (w/w). The ratio of total lipid (cholesterol and phospholipid) to water was kept constant at value of 2.6%. The ratio of cholesterol and phospholipid, however, were used in three different molar values (0:1, 0.33:1, 1:1). The experimental results are shown in Fig. 2. The incorporation of BCH in liposomal formulations was increased (statistically significant difference at 95% confidence level) with an increase in cholesterol/phospholipid ratio from 0:1 to 0.33:1 and further increase of this ratio to 1:1 led to a decrease in I.E (statistically insignificant). For compound IIIa, an increase in the cholesterol/phospholipid ratio from 0:1 to 0.33:1 was accompanied with a decrease in I.E (statistically

Table 2
Preformulation properties of cholesteryl carborane ester compounds

Compound	R	Molecular weight	Mp °C	Internal structural	Aqueous solubility[a]
BCH	H	556.93	235	Crystalline	–
IIIa	$–(CH_2)_7CH_3$	669.085	251	Crystalline	–
IIIb	$–(CH_2)_6CH=CH_2$	667.07	260	Crystalline	–
IIIc	$–(CH_2)_{17}CH_3$	809.354	210	Crystalline	–

[a] Aqueous solubility at 25 °C is less than 50 ng/1 ml of water.

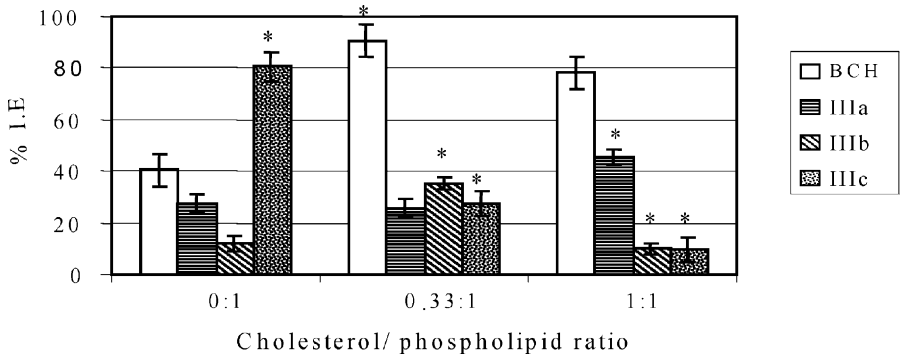


Fig. 2. Incorporation efficiency of BCH and the three new compounds in various liposomal formulations. (*) indicates statistical significant difference at 95% level of confidence.

insignificant) and the increment in the ratio from 0.33:1 to 1:1 led to increase in I.E (statistically insignificant). For compound IIIb, I.E. was increased (statistically significant) from 0:1 to 0.33:1 and I.E. was decreased (statistically significant) from 0.33:1 to 1:1. For compound IIIc, the increase in the cholesterol/phospholipid ratio resulted in a statistically significant decrease in the I.E.

4. Discussion

Boron neutron capture therapy of cancer is based on the irradiation of boron (^{10}B) compound with low-energy thermal neutrons to produce cell-destroying alpha particles (i.e. helium nucleus) (Barth et al., 1996; Chen et al., 1997). However, to minimize the destruction of the neighboring normal cells and maintain the BNCT effectiveness, it is very important to selectively deliver sufficient amount (20–25 μg of boron per gram of cell) of boron to tumor cells. Recently, attempts have been made in our laboratory and others to synthesize boron-containing cholesteryl esters and other hydrophobic boron compounds to facilitate the interactions to LDL for targeted drug delivery. Ji and Lu (2001) synthesized a cholesteryl ester linked to a hydrophobic *p*-carborane cage (BCH). Feakes et al. (1999) constructed a series of boron compounds by linking *o*-carborane to cholesterol with various fatty spacers between them. Laster et al. (1991) synthesized carborane carboxylic acid esters of fatty alcohols to replace the cholesterol ester in the core of LDL. Cell cultures studies indicated that some of these compounds, including BCH, were taken up by cancer cells to reach the cellular boron concentration up to 280 μg boron/g cells (more than 10 times higher than that required for successful BNCT) (Laster et al., 1991; Peacock and Lu, 2001). Because of their highly hydrophobic nature, liposomal formulations or other type of lipid carriers are generally required to solubilize these types of boron compounds.

In this paper, three new boron compounds were synthesized. Attempt was made initially to synthesize these new compounds directly from BCH by substituting the hydrogen atom (linking to the second carbon on carborane cage) with various fatty chains. However, the reaction was not successful because BCH (specifically, the ester bond between carborane cage and cholesterol) was not stable in the reaction condition with extremely strong base (*n*-butylthium). The reaction step was redesign to first link *p*-carborane to various fatty chains and then to couple them with cholesterol. In the first step, monosubstitution (Ia, Ib and Ic) of fatty chains on one side of carborane was achieved by reducing the temperature to −78 °C, using the *n*-BuLi and fatty chains in equivocal molar to *p*-carborane and adding the reagents slowly. Cholesterol was coupled with the carboxylic acid derivatives, IIa, IIb and IIc, using dicyclohexylcarbodiimide and 4-dimethylaminopyridine.

Similar to BCH, these new compounds are extremely hydrophobic and poorly soluble in aqueous solution. Melting points of these compounds follow the similar pattern as those of native cholesterol esters: the short chain esters have higher crystalline melting temperatures and extension of the fatty chain shows

a general trend to lower crystalline melting temperatures (Ginsburg et al., 1984). With the increase in the number of carbons, the crystal becomes less stable and less ordered leading to a decrease in the melting point. Presence of double bond in the fatty chain of compound IIIc resulted in an increase in the melting point, apparently due to the stronger intermolecular interactions.

Owing to the poor water solubility, liposomal formulation was used to solublize BCH for biological studies and our previous study showed that liposomal formulation was a suitable vehicle for this type of cholesteryl carborane ester compounds. However, the incorporation of new compounds in liposomes needs to be investigated before further biological studies. The present study used three liposomal formulations containing different cholesterol:phospholipid ratios and examined the incorporation of four cholesteryl carborane ester compounds.

The type of fatty chains attached to carborane had significant effect on the incorporation of these new compounds in liposomal formulation. For BCH and compound IIIb, the highest I.E.s ($90.65 \pm 6.33\%$ and $35.35 \pm 2.64\%$, respectively) were achieved at 0.33:1 cholesterol:phospholipid ratio. For compound IIIa, the highest I.E. ($45.39 \pm 3.15\%$) was achieved at 1:1 cholesterol:phospholipid ratio. For compound IIIc, the highest I.E. ($80.73 \pm 5.64\%$) was achieved at 0:1 cholesterol:phospholipid ratio. The results are in agreement with the literature data that the incorporation of hydrophobic drugs in liposomal formulation depends on the size and spatial structure of the drug molecule and the presence of certain function groups (Samedro et al., 1993). Perez-Soler and Preier (1990) have shown that presence of iodine in position 2 or demethoxylation in position 4 of anthracyclines significantly enhanced the entrapment of the compounds in multilamellar liposomes. Although the addition of various fatty chains to carborane unit changed the incorporation efficiency in our study, no clear trend can be found directly related to the size of the fatty chains.

Changes in cholesterol:phospholipid ratio as the excipients of liposomal formulation also affected the incorporation efficiencies in our experiments. Cholesterol and these hydrophobic compounds are located in the phospholipid bilayer of liposomes. It is known that the incorporation of cholesterol in phospholipid membrane should not exceed 50% in molar fraction (Lai et al., 1985). New (1990) showed that the increase of cholesterol beyond certain concentration reduced the incorporation efficiency due to the disruption of linear structure in liposomal membrane resulting in the reduction in intramolecular interaction. Lai et al. (1985) concluded that the formation of a cholesterol–phospholipid complex did not required the presence of 3β-OH and cholesteryl hemisuccinate had similar effect as cholesterol on the thermotropic properties of dipalmitoyl phosphotidylcholine. These observations suggest that the amount of cholesterol affect the physical stability of phospholipid bilayer and, thus, the co-existence of cholesterol and cholesteryl esters in liposomal membrane. Our experiment confirmed that the incorporation efficiencies for various cholesteryl carborane esters depended on the amount of cholesterol used in liposomal formulation. Based on the studies, selection can be made to use different liposomal formulations in the future biological studies involving these new compounds.

In conclusion, the addition of various fatty chains to the cholesteryl carborane ester can be achieved through a three-step chemical reaction. Similar to BCH, these new compounds are extremely hydrophobic and present in crystalline forms. The melting points, however, are ranged from 210 to 260 °C among these compounds. The incorporation efficiencies of these compounds in liposomes vary significantly depending on the type of fatty chain attached and the ratio of cholesterol:phospholipid used as the excipients of liposomal formulation.

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