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Synthesis of a carborane-containing Cholesterol Derivative and Evaluation as Potential Dual Agent for MRI/BNCT Applications

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Abstract.

In this study the synthesis and characterization of a new dual, imaging and therapeutic, agent is proposed with the aim of improving the efficacy of Boron Neutron Capture Therapy (BNCT) in cancer treatment. The agent (Gd-B-AC01) consists of a carborane unit (ten boron atoms) bearing a cholesterol unit on one side (to pursue the incorporation into the liposome bi-layer), and a Gd(III)/1,4,7,10-tetraazacyclododecane monoamide complex on the other side (as MRI reporter to attain the quantification of B/Gd concentration). In order to endow the BNCT agent with specific delivery properties, the liposome embedded with the MRI/BNCT dual probes has been functionalized with a peghilated phospholipid containing a folic acid residue at the end of the PEG chain. The vector allows the binding of the liposome to folate receptors that are overexpressed in many tumors types, and in particular, in human ovarian cancer cells (IGROV-1). In vitro test on IGROV-1 cells demonstrated that Gd-B-AC01 loaded liposomes are efficient carriers for the delivery of the MRI/BNCT probes to the tumor cells. Finally, the BNCT treatment of IGROV-1 cells showed that the number of survived cells was markedly smaller when the cells were irradiated after internalization of the folate-targeted GdB10-AC01/liposomes.

Introduction

Boron Neutron Capture Therapy (BNCT) is a binary treatment that combines low energy neutron irradiation with the presence of boron-containing compounds at the target cells. This methodology has been proposed for several pathologies, although most of the efforts have been focused on cancer.[1-3] BNCT has been under investigation for many years, but it has not entered routine clinical practice yet. Since this treatment is effective only on cells internalizing large amounts of boron (at least 20-30 ppm), the principal cause of its limited success mainly depends on the insufficient delivery of NCT compounds to the target cells. A key-issue deals with the measurement of the local boron concentration that is crucial to determine the optimal neutron irradiation time and to calculate the radiation dose. One of the problems confronting the successful clinical implementation of BNCT is the difficulty of quantitatively mapping the distribution of the boron containing molecules in patients before and during the treatment. To date there is no non invasive way to evaluate boron concentration in diseased and healthy tissues of the subject undergoing the irradiation. Thus, dose calculations are based on boron content values in blood, tumour, and normal tissue obtained from biodistribution studies performed beforehand.[4, 5] Blood samples can be taken just before and even during irradiation to measure the tissue boron concentration, assuming the tumour/blood ratios assessed in previous biodistribution studies. However the boron distribution varies among patients and therefore large uncertainties exist in the tumour-to-blood boron concentration ratio. The recent development of more sensitive imaging techniques and molecular medicine protocols, based on the use of site specific agents able to carry large amounts of both therapeutic and diagnostic agents, can contribute to enhance BNCT efficacy and to become more competitive with the established tumour treatment protocols.[6] Among the compounds developed for BNCT, much attention has been devoted to carboranes, icosahedral cages containing several boron atoms, which could increase the payload of boron in the tumour cells.[7-10] Boron containing compounds can be further functionalized with lipophilic moieties in order to permit their specific and

efficient delivery through the incorporation in targeted nanoparticles. The synthesis of new dual agents for applications in MRI/BNCT where a carborane unit is linked to a lipophilic chain and to a Gd-DOTA complex through amidic bonds has been developed in our laboratory (AT101).[11] According to the synthetic design, the lipophilic probe AT101 binds to LDLs (Low Density Lipoproteins) and accumulates at tumor cells characterized by an up-regulation of LDL transporters. The exploitation of LDL as biological vectors was indeed the key to obtain a high concentration of B atoms/g at the tumor cells together with a good selectivity between tumor and healthy tissues. *In vivo* MR images acquisition showed that the amount of boron taken up in the tumor region was above the threshold required for a successful NCT treatment. More recently, with the purpose of simplifying the synthetic procedure but maintaining, and possibly improving, the favorable properties of AT101, we designed a new structure containing a triazole linker instead of the amidic function (MEA/01).[12] Its adducts were tested "*in vitro*" on B16 melanoma cells and the obtained intracellular Gd/B concentration appears sufficient for further "*in vivo*" BNCT studies.

An alternative promising approach to deliver efficiently boron containing agents to tumors consists of the use of liposomes. In fact, there are many examples in the literature of formulations containing boron compounds encapsulated into the aqueous cavity or, after conjugation with lipids, incorporated into the liposomal bilayer.[13-15] A specific class of liposomes, named "long time circulating" polyethylene glycol (PEG)–coated liposomes, is able to accumulate in solid tumors as a consequence of microvascular permeability and defective lymphatic drainage.[16-18] The extent of passive extravasation is directly dependent on the prolonged residence time of liposomes in the blood stream. As far as the internalization step is concerned one should note that in the absence of a receptor mediated mechanism, the internalization of the intact liposome into tumor cells does not take place. Therefore, in the absence of liposome active targeting the boron containing molecule has first to be released in the tumor extracellular matrix in order to allow its diffusion into cells. Alternatively, the active targeting of liposomes to tumor cells is obviously a promising strategy to improve drug delivery to tumors. To this purpose, liposomes have to be functionalized with specific

ligands able to bind and to internalize through receptors expressed on tumor vasculature or on tumor cells. Recently, Nakamura and co-workers developed liposomes containing mercaptoundecahydrododecaborate (BSH) in the inner aqueous cavity and 10% distearoyl boron lipid (DSBL) incorporated in the phospholipid bilayer.[19, 20] The proposed system appears particularly efficient because the liposome shell itself contains boron in addition to the boron encapsulated in the aqueous cavity. Furthermore, they performed the *in vivo* real-time tracking of 10% DSBL liposomes by MRI using liposomes encapsulating Gd-DOTA, observing a high signal intensity enhancement in the tumor region. The disadvantage of the use of therapeutic and imaging agents as independent molecules, is that they maintain the same biodistribution only if the liposome remains intact. After liposome degradation their biodistribution will be different and dependent on their size, hydrophilic character, residual charges, etc… In order to overcome this problem, in this study, a dual agent in which the imaging (Gd-DOTA) and the BNCT (carborane) components are part of the same molecule, is proposed. The incorporation of the molecule in the liposome bilayer has been pursued by a functionalization with a cholesterol residue. Cholesterol is a standard component of the liposome bi-layer usually introduced in the phospholipid formulation in order to increase the particle stability. On the contrary, complexes bearing only a palmitic chain, as the parent AT101 previously investigated by our group, can not be steadily incorporated in the liposome bi-layer and the complex may be releazed randomly. Synthetic mimics of cholesterol represent an important molecular tool in the fields of bioorganic/medicinal chemistry and chemical biology. Furthermore, carborane cholesterol derivatives were incorporated in unilamellar liposomes showing selective localization in tumours[21, 22] and might be useful vehicles for transporting boron to the tumor site. In this study, the liposomes embedded with MRI/BNCT dual probes have been functionalized with a peghilated phospholipid containing a folic acid residue at the end of the PEG chain, as a specific carrier to transport large amounts of B/Gd dual agents to tumor cells, in particular human ovarian cancer cells, overexpressing folate receptors. Finally, in order to improve the efficacy of the dual agent, the DOTA-monoamide ligand has been complexed with gadolinium

enriched in the ^{157}Gd isotope (95%) that has the largest (255,000b) thermal neutron capture cross section among all the stable isotopes, and it is about 66 times as large as that of ^{10}boron (3838 b).

RESULTS AND DISCUSSION

Synthesis of B/Gd dual agent. Since the hydroxyl group of cholesterol is sterically hindered and then unreactive, in the first synthetic step it has been functionalized, by a nucleophilic substitution, with a hydroxyl propyl group in order to make easier the linkage to the carborane cage. As shown in, cholesterol was transformed in the corresponding mesylate (1) in quantitative yields, (as assessed by the detection of the signal at 2.97 ppm in the ^{1}H spectrum pertinent to the mesylate CH_3). Intermediate **1** was reacted with propan-1,3-diol in anhydrous dioxane at 120°C overnight. Despite the harsh applied conditions, the product was obtained in 27% yield after chromatographic purification, because of the steric hindrance of the alcoholic group. Alcohol **2** was then oxidized to the corresponding carboxylic acid, following the classical Jones reaction conditions with an overall yield of 78%. The formation of 3-(Cholesten-5-en-3β-yloxy)-propionic acid (**3**,Scheme 1) was confirmed by the detection of a resonance at 170 ppm in the ^{13}C spectrum pertinent to the carbonyl group. The COOH moiety was then coupled to *C*-(2-benzyloxy)-ethyl-*C'*-aminomethyl-*o*-carborane **7** (prepared as previously reported).[11] Firstly, the coupling was attempted according to the procedure proposed by Kamiński,[23] but the desired product was not obtained. A two-step protocol, where the acid was activated with *N*-hydroxysuccinimide (NHS) in the presence of DCC was then applied with the formation of product **4** (64% yield after chromatograhic purification). ^{1}H NMR analysis confirmed the proposed structure. In particular the resonance of the α-methylenic group shifted from 3.39 ppm in the amino derivative **7** to 4.06 ppm in the coupling product.

At this point the functionalization of the other arm of the *o*-carborane has been tackled. As reported in Scheme 2, the benzylic protecting group was selectively removed by Pd/C catalyzed hydrogenation.[24] Different catalyst concentrations and solvents were tested in order to avoid the concomitant hydrogenation of the cholesterol double bond. It was found that 5% of Pd/C in EtOH/CH_2Cl_2 2/1 provide the best conditions, affording after 24 h the desired product **5** in 92% overall yield.

Alcohol **5** was readily oxidized to the corrisponding carboxylic acid **6** by CrO_3 in acetone-sulfuric acid solution (scheme 2).

The structure of derivative **6** was supported by the ^1H and ^{13}C NMR data. The ^1H NMR spectrum showed the disappearance of the signal at 3.75 ppm assigned to the HOCH_2- group, with the concomitant growth in the ^{13}C NMR spectrum, of the signal at 172 ppm corresponding to the carbonyl group.

In order to obtain the molecule containing both the BNCT and the MRI moieties, derivative **6** was reacted with N-*ter*butyl-DOTAMA-C_6-NH_2 using the two-step procedure previously described for the preparation of *C*-(2-benzyloxyethyl)-*C*'-[cholesten-5-en-3β-yloxy-(3-propionacetoamidomethyl)]-*o*-carborane (**4**) (Scheme 2). Carborane **6** was then activated *via* NHS in the presence of DCC and after filtration of DCU, reacted with the suitable amine affording the *C*-[N-*tert*-butylDOTAMA-C_6-)-carbamoylmethyl]-*C*'-[cholesten-5-en-3β-yloxy-(3-propionacetoamidomethyl)-*o*-carborane (**8**) in a 67% overall yield. The structure was completely characterized and confirmed by ESI mass spectrometry with the detection of the characteristic ion at 1327.87 ($M + H^+$). After removal of *tert*-butil ester groups (CF_3CO_2H / CH_2Cl_2), the Gd(III) complex **10** was finally obtained by adding stoichiometric amounts of $GdCl_3$ in an aquous solution of the ligand at pH 6.5 (scheme 3).

Liposome preparation and characterization. Liposomes were prepared by means of the standard thin lipid film hydration method[25] using the following components: POPC, CHOL, Gd-B-AC01, DSPE-PEG2000, DSPE-PEG2000-Folate. Three types of liposomes containing the same amount of POPC (71%) and different amount of cholesterol and Gd-B-AC01, namely 15,5:7.5%, 13:10%, 8:15%, respectively, were prepared. In order to increase the stability and limit their non specific phagocytic uptake, all the liposomes contain 6 % of peghilated phospholipid (DSPE-PEG2000). The millimolar relaxivity (21.5 MHz, 25°C) of Gd-B-AC01incorporated in the liposome formulation was 18.4±0.8 $(mmol/L)^{-1}$ s^{-1}. This value is relatively high as a consequence of both the

decrease of the molecular mobility upon the insertion in the liposome membrane and the relatively fast water exchange rate through the phospholipid bilayer due to the presence in the formulation of a high % of unsaturated phospholipids (POPC). The average liposome hydrodynamic diameter was 125± 10 nm for all investigated formulations as detected by dynamic light scattering measurements. In order to accumulate the Gd-B-AC01 loaded liposome selectively at the tumor cells, 1% of a DSPE phospholipid conjugated to a folate residue has been added to the phospholipid formulation. Overexpression of folate receptors (FRs) is well established in many cancer cells (ovary, brain, kidney, breast and lung)[26, 27] thus identifying this receptor as a potential target for many types of ligand directed cancer therapeutics.[28] FR may be further qualified as a tumor-specific target, since it generally becomes accessible to intravenous administered drugs only after malignant transformation. Folate Receptors (FRs) are normally expressed on cancer cells up to 10^7 FRs/cell but the rate of receptor recycling is relatively low.[29, 30] Folate conjugates bind to folate receptors with high affinity ($K_d \approx 10^{-9}$ M) and they are internalized through receptor-mediated endocytosis. The physicochemical properties of the liposome with 1% folate-phospholipid are very similar to the untargeted control liposome (without folic acid).

NMRD profiles. $1/T_1$ nuclear magnetic resonance dispersion (NMRD) profiles, over a range of Larmor frequencies 0.01< MHz< 70, have been recorded for an aqueous solution of Gd-B-AC01 (0.32 mM) and compared with the profile obtained for Gd-B-AC01 embedded in the liposome membrane (Figure 1). Both profiles show a relaxivity hump at about 30 MHz, typical of slowly moving systems. The relaxivity enhancement observed with respect the free complex is limited by the relatively long exchange time τ_M of the water directly coordinated to the metal ion (inner sphere water) as previously shown for several Gd complexes bearing a DOTA- monoamide ligand structure.[31, 32] The presence of a relaxivity peak between 10 and 60 MHz of the Gd-B-AC01aqeous solution confirms that the complex forms stable micelles. A titration (T_1 vs [Gd-B-AC01]) indicated a cmc lower than 15 μM. The linear dependence of relaxation rates between 300 and 15 μM

indicates that over all this concentration range the complex remains in the micellar form. Lower concentrations are under the detection limit of the relaxometric method.

Uptake experiments on IGROV-1 cells in vitro and MRI analysis. Liposomes were tested in vitro on Human Ovarian Carcinoma IGROV-1 cells to evaluate the amount of Gd taken up by cells after 24 hours incubation by measuring the metal concentration by ICP-MS analysis. These cells express a significant level of FRα (0.5-1 x 10^6 receptors/cell).[33] The liposome stability, as demonstrated by the absence of non specific release of Gd-B-AC01to tumor cells, has been checked by incubating cells, for 24h at 37°C, in the presence of non targeted Gd-B-AC01/liposome containing increasing % of Gd-B-AC01from 7.5 to 15%. The results reported in figure 2A show that the amount of Gd associated non-specifically to IGROV-1 cells is directly proportional to the % loading of Gd-B-AC01in the liposome. This is due to the high affinity of cholesterol for cell membranes that causes the translocation of a small but significative % of the complex from the liposome to the cell membrane. By reducing the % of Gd-B-AC01with respect the other phospholipids, it is possible to reduce the number of released complexes bound aspecifically to the cell surface that can cause the decrease of the ratio between the internalized boron by tumor with respect the surrounding healthy cells. For these reasons the formulation containing the 7.5 % of Gd-B-AC01has been selected to perform the FR targeting study on FR expressing IGROV-1 cells. The Gd moles internalized by IGROV-1 cells measured by ICP-MS after incubation in the presence of increasing concentration of folate-targeted liposomes were compared with those obtained using non-targeted ones. Figure 2B shows that increasing the concentration of targeted and non-targeted liposomes incubated with IGROV-1 cells, the targeted liposomes quickly reach complete saturation of their folate uptake. This behavior demonstrates a high affinity of folate targeted liposomes for the FR receptor. Moreover, it indicates that the non specific Gd-B-AC01 release from non-targeted liposomes does not invalidate the comparison if one works at relatively low liposome concentrations.

In order to assess whether the amount of Gd internalized in target cells is enough to permit MRI visualization, T_1 weighted images of glass capillaries containing cellular pellets obtained by incubating ca. $1x10^6$ IGROV cells with increasing amounts of targeted and non targeted Gd-B-AC01/liposomes were acquired at 1 and 7 T (Figure 3). At 1 T, there is a dominant effect of the long reorientational motion of the paramagnetic macromolecule that causes a dramatic relaxation rate increase with a consequent signal intensity enhancement increase as shown in the NMRD profiles of figure 1. Clearly, the SI enhancements of labeled cells measured at 1 T (SI=80% after 5μM Gd incubation) were markedly higher than those measured at 7 T (SI=45% after 5uM Gd incubation). The mean SI enhancement (%) of the target tissues was calculated according to the Equation (1):

$$\text{SI \% Enhancement} = ((\text{mean } SI_{POST} - \text{mean } SI_{PRE}) / \text{mean } SI_{PRE}) \times 100$$

The recorded SI of targeted liposome in both cell lines is higher in respect to non-targeted ones. This demonstrates the specific accumulation of folate targeted liposomes in tumor cells with respect to control liposomes. The amount of internalized boron measured by ICP-MS was 38 and 9 ppm for folate-targeted and non-targeted liposomes, respectively. The former amount is significantly higher than the minimum boron concentration necessary to perform BNCT. We can conclude that Gd-B-AC01loaded liposomes are efficient carriers for the delivery of MRI/BNCT probes to tumor cells.

BNCT treatment of IGROV-1 cells.

Figure 4 shows the percent of cells survived to the neutron irradiation. Two cell controls have been used in this experiment. The first did not receive any neutron irradiation and boron and the second control was irradiated without boron. The number of survived cells was significantly lower when cells have been irradiated after folate-targeted GdB10-AC01/liposomes internalization whereas in the absence of boron the effect of neutron irradiation is almost negligible. Furthermore, the

proliferation rate of the boron treated cells survived to the irradiation is significantly lower than that measured on both control cells.

Although the cells have not been completely killed, the purpose of the experiments has been satisfied because these measurements were meant to verify that the new formulations had a potentiality as vectors for BNCT. Further studies with dose escalation are planned.

Conclusions.

In summary, the dual MRI/BNCT probe described in this paper can be exploited to accumulate B and Gd at target tumor cells. The new formulation has shown to have relevant characteristics: 1) ability to selectively concentrate high amounts of B at the tumor cells, 2) possibility to quantify the boron concentration by MRI; 3) the cholesterol versatility to be incorporated in different particles can be exploited to perform a multi-step treatment based on the administration of different drugs targeted to different overexpressed receptors. 4) The amount of internalized B is enough to perform an efficient BNCT treatment and the selective target of B using folate-targeted liposomes is reported to reduce significantly the uptake by healthy cells in the surrounding regions. 5) The use of liposomes as a nanoplatform for the delivery of Gd and B agents will permit the simultaneous delivery of standard anti-tumor drugs (such as doxorubicin) in order to improve the efficacy of the treatment.

EXPERIMENTAL PROCEDURES

General Methods

Flasks and all equipments used for the generation and reaction of moisture-sensitive compounds were dried by electric heater under Ar. THF was distilled from benzophenone ketyl, anhydrous Et_2O was distilled from $LiAlH_4$ and anhydrous CH_2Cl_2 from CaH_2 prior to use. BuLi (1.6 M in hexanes) was obtained from Aldrich. $(Bmim)^+Cl^-$ was purchased from Solvent Innovation GmbH. Decaborane was bought from KATCHEM spol. s r. o. All commercially obtained

reagents and solvents were used as received. Product were purified by preparative column chromatography on Macherey Nagel silica gel for flash chromatography, 0,04-0,063 mm/ 230-400 mesh.

Reactions were monitored by TLC using Silica gel on TLC-PET foils Fluka, 2-25 mm, layer thickness 0.2 mm, medium pore diameter 60 Å. Carboranes and their derivatives were visualized on TLC plates using a 5% $PdCl_2$ aqueous solution in HCl. ^{1}H NMR spectra were recorded at 200 MHz, ^{13}C NMR spectra at 50.2 MHz. Data were reported as follows: chemical shifts in ppm from tetramethylsilane as internal standard, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = double-doublet, m = multiplet, br = broad), coupling constants (Hz), and assignment. ^{13}C NMR spectra were measured with complete proton decoupling. Chemical shifts were reported in ppm from the residual solvent as an internal standard. GC-MS spectra were obtained on a mass selective detector HP 5970 B instrument operating at an ionizing voltage of 70 eV connected to a HP 5890 GC with a cross linked methyl silicone capillary column (25 m X 0.2 mm X 0.33 mm film tickness). ESI MS spectra were obtained on a Waters micromass ZQ spectrometer equipped with ESI ion source. IR spectra were recorded on a Perkin Elmer BX FT-IR. *C*-(2-benzyloxyethyl)-*C'*-aminomethyl-*o*-carborane (**7**) and *N*-*tert*-ButylDOTAMA-C_6-NH_2 were synthesized as described in literature and their spectroscopic data corresponded with those reported.[11]

(3β)-colest-5-en-3-methanesulfonate (1). Cholesterol (12.9 mmol, 5 g) was dissolved in 100 mL of anhydrous Et_2O and cooled to 0 °C in a 250 mL three necked round bottom flask, then Et_3N (1.5 equiv, 19.4 mmol, 2.7 mL) followed by MsCl (19.4 mmol, 1.5 mL) were added. The reaction mixture was stirred for 1 h then quenched with H_2O and extracted with Et_2O (3 x 30 mL). The combined extracts were washed with H_2O (2 x 30 mL), dried and evaporated under reduced pressure leaving 5.9 g (98 %) of a pale yellow oil which was at once used for the following reaction. Found C, 72.37; H, 10.40; S, 6.92. Calc. for $C_{28}H_{48}O_3S$: C, 72.36; H, 10.41; S, 6.90. ν_{max} (neat)/cm^{-1} 2940, 1467, 1353, 1172; δ_H (200 MHz; $CDCl_3$, Me_4Si): 0.65 (3H, s,

C*H*$_3$CH(CH$_2$)$_3$), 0.70-2.05 (40H, m, CH$_3$CH(C*H*$_2$)$_3$, (C*H*$_3$)$_2$CH, C*H*$_3$ ring, C*H*$_2$ ring, C*H* ring), 2.50 (2H, m, C*H*$_2$CH=C), 3.03 (1H, m, C*H*$_3$SO$_2$), 4.50 (1H, m, C*H*O), 5.41 (1H, bd, CH$_2$C*H*=C, *J* = 5.5 Hz) ; δ_c (50.2 MHz; CDCl$_3$, Me$_4$Si): 11.6 (CH$_3$), 18.5 (CH), 18.9 (CH), 20.8 (CH$_2$), 22.4 (CH), 22.6 (CH), 23.6 (CH$_2$), 24.0 (CH$_2$), 27.7 (CH$_3$), 28.0 (CH$_2$), 28.7 (CH$_2$), 31.5 (CH), 31.6(CH$_2$), 35.5 (CH), 35.9 (CH$_2$), 36.1 (CH$_2$), 36.6 (CH), 38.4 (CH$_2$), 38.9 (CH$_2$) , 39.3 (CH$_2$), 39.4 (CH$_2$), 42.1 (Cq), 49.7 (CH$_3$), 55.9 (CH), 56.4 (CH) , 81.7 (CH) , 123.4 (Cq), 138.5 (CH). m/z (ESI+) 465.33 $[M + H]^+$.

3-((3β)-colest-5-en-3-yloxy)-propanol (**2**). According to literature procedure,[25] (3β)-colest-5-en-3-methanesulfonate (**1**) (12.8 mmol, 5.93 g) was dissolved in 100 mL of anhydrous dioxane in a 250 mL three necked round bottom flask. 1,3-Propandiol (319 mmol, 24.3 g) was added and the resulting mixture was stirred overnight at 120 °C. Then the solution was cooled down at room temperature and solvent was removed under reduced pressure. The residue was dissolved in 50 mL of CH_2Cl_2 and washed with H_2O. The organic layer was separated, washed with $NaHCO_3$ (1 X 50 mL), water (1 X 50 mL), and brine (1 X 50 mL), dried over anhydrous K_2CO_3, and evaporated under reduce pressure. The crude was purified on silica gel (petroleum ether/diethyl ether 50/50) giving 1.54 g (27 %) of a white solid. Found C, 81.05; H, 11.80. Calc. for $C_{30}H_{52}O_2$: C, 81.02; H, 11.79. ν_{max} (neat)/cm^{-1} 3434, 2934, 2098, 1642; δ_H (200 MHz; CDCl$_3$, Me$_4$Si): 0.67 (3H, s, C*H*$_3$CH(CH$_2$)$_3$), 0.70-2.49 (42H, m, CH$_3$CH(C*H*$_2$)$_3$, (C*H*$_3$)$_2$CH, C*H*$_3$ ring, C*H*$_2$ ring, C*H* ring), 2.60 (1H, bs, O*H*), 3.19 (1H, tt, *J* = 8.5, 5.2, C*H*O), 3.67 (2H, t, *J* = 5.8 Hz, HOC*H*$_2$CH$_2$O), 3.77 (2H, t, *J* = 5.4 Hz, HOC*H*$_2$CH$_2$O), 5.33 (1H, bd, *J* = 5.2 Hz, CH$_2$C*H*=C); δ_c (50.2 MHz, CDCl$_3$, Me$_4$Si): 11.6 (CH$_3$), 18.5 (CH$_3$), 19.0 (CH$_3$), 20.8 (CH$_2$), 22.3 (CH), 22.5 (CH), 23.8 (CH$_2$), 24.0 (CH$_2$), 27.6 (CH), 28.0 (CH), 31.6 (CH$_2$), 32.3 (CH$_2$), 35.6 (CH), 36.0 (CH$_2$), 36.4 (Cq), 37.0 (CH$_2$), 38.8 (CH$_2$), 39.2 (CH$_2$), 39.5 (CH$_2$), 42.0 (Cq), 49.9 (CH), 56.0(CH) , 56.4 (CH), 60.2 (CH$_2$), 65.8 (CH$_2$), 76.4 (CH), 121.3 (CH), 140.3 (Cq); m/z (ESI+) 467.70 $[M + Na]^+$.

3-(Cholesten-5-en-3β-yloxy)-propionic acid (3). 3-((3β)-colest-5-en-3-oxy)-propanol (**2**) (0.63 g, 1.41 mmol) was dissolved in 21 mL of acetone, then a 3 M solution of CrO_3 (4 equiv, 5.6 mmol, 0.56 g) in H_2SO_4 (7 mL) was added dropwise at 0°C. The reaction mixture was stirred overnight at rt, then quenched with H_2O. The solvent was evaporated under reduced pressure and the mixture was extracted with CH_2Cl_2 (5 × 10 mL), the organic layers were washed once with brine (1 X 10 mL), dried and evaporated giving 0.49 g (76 %) of white solid. Found C, 78.57; H, 11.00. Calc. for $C_{30}H_{50}O_3$: C, 78.55; H, 10.99. ν_{max} (neat)/cm^{-1} 3400, 2938, 1715; δ_H (200 MHz; $CDCl_3$, Me_4Si): 0.68 (3H, s, C*H*$_3$CH(CH$_2$)$_3$), 0.70-2.05 (38H, m, CH$_3$CH(C*H*$_2$)$_3$, (C*H*$_3$)$_2$CH, C*H*$_3$ ring, C*H*$_2$ ring, C*H* ring), 2.05-2.50 (2H, m, C*H*$_2$CH$_2$COOH), 2.62 (2H, t, *J* = 6.4 Hz, C*H*$_2$COOH), 3.21 (1H, m, C*H*O), 3.76 (2H, t, *J* = 6.4 Hz, C*H*$_2$OH), 5.35 (1H, bd, *J* = 5.2 Hz, CH$_2$C*H*=C); δ_c (50.2 MHz, $CDCl_3$, Me_4Si): 11.6 (CH_3), 18.6 (CH_3), 19.1 (CH_3), 20.8 (CH_2), 22.3 (CH_3), 22.6 (CH_3), 23.6 (CH_2), 24.0 (CH_2), 27.8 (CH_2), 28.0 (CH), 31.6 (CH_2), 31.8 (CH), 35.0 (CH_2), 35.6 (CH), 36.0 (CH_2), 36.6 (CH_2), 36.9 (CH_2), 38.7 (CH_2), 39.4 (CH_2), 39.5 (CH_2), 42.1 (Cq), 49.9 (CH), 55.9 (CH), 56.6 (CH), 62.8 (CH_2), 79.3 (CH), 121.5 (CH), 140.4 (Cq), 176.9 (Cq); m/z (ESI+) 459.72 $[M + H]^+$.

***C*-(2-Benzyloxyethyl)-*C*'-[cholesten-5-en-3β-yloxy-(3-propionacetoamidomethyl)]-*o*-carborane (4)**. In a 100 mL three necked round bottom flask, 3-(cholesten-5-en-3β-yloxy)-propionic acid (**3**) (1.27 mmol, 0.58 g) was dissolved in 40 mL of dry CH_2Cl_2 then *C*-(benzyloxy)-ethyl-*C'*-aminomethyl-*o*-carborane (1.26 mmol, 0.38 g) and *N*,*N'*-dicyclohexylcarbodiimide (1.51 mmol, 0.31 g) were added at room temperature. The resulting mixture was stirred at room temperature overnight. The reaction mixture was filtered and was washed with 3% $NaHCO_3$ (1 X 30 mL) and brine (1 X 30 mL). The organic layer was dried, the solvent evaporated under reduced pressure. The crude was purified on silica gel (petroleum ether/ diethyl ether 80/20) giving 0.7 g (73 %) of a white solid.

M.p.= 76-79 °C. Found C, 67.45; H, 9.84; S. Calc. for $C_{42}H_{73}B_{10}NO_3$: C, 67.43; H, 9.83. ν_{max} (neat)/cm^{-1} 3332, 2581, 1660; δ_H (200 MHz; $CDCl_3$, Me_4Si): 0.68 (3H, s, C*H*$_3$CH(CH$_2$)$_3$), 0.70-2.49 (40H + 10H, m, CH$_3$CH(C*H*$_2$)$_3$, (C*H*$_3$)$_2$CH, C*H*$_3$ ring, C*H*$_2$ ring, C*H* ring, B*H*), 2.40 (2H, t, *J* = 5.2 Hz, C*H*$_2$CH$_2$CONH), 2.69 (2H, t, *J* = 6.0 Hz, PhCH$_2$OCH$_2$C*H*$_2$), 3.23 (1H, m, C*H*O), 3.68 (4H, m, PhCH$_2$OC*H*$_2$CH$_2$, OC*H*2(CH$_2$)$_2$CONH), 4.07 (2H, d, *J* = 6.4 Hz, CONHC*H*$_2$), 4.51 (2H, s, PhC*H*$_2$O), 5.38 (1H, bd, *J* = 4.8 Hz, CH$_2$C*H*=C), 7.31 (1H, t, *J* = 6.0 Hz, CON*H*), 7.39 (5H, bs, Ph); δ_c (50.2 MHz; $CDCl_3$, Me_4Si): 11.6 (CH_3), 18.6 (CH_3), 19.2 (CH_3), 20.9 (CH_2), 22.4 (CH_3), 22.7 (CH_3), 23.7 (CH_2), 24.1 (CH_2), 27.8 (CH), 28.1 (CH_2), 31.7 (CH), 31.8 (CH_2), 35.1 (CH_2), 35.6 (CH_2), 36.0 (CH), 36.7 (CH_2), 36.8 (Cq), 36.9 (CH_2), 38.8 (CH_2), 39.3 (CH_2), 39.6 (CH_2), 41.2 (CH_2), 42.1 (Cq), 50.0 (CH), 56.0 (CH), 56.6 (CH), 63.2 (CH_2), 68.2 (CH_2), 72.9 (CH_2), 78.4 (Cq), 79.1 (Cq), 79.4 (CH), 121.9 (CH), 127.6 (CH), 127.7 (CH), 128.3 (CH), 137.3 (Cq), 140.1 (Cq), 171.2 (Cq); m/z (ESI+) 749.15 $[M + H]^+$.

_C_-(Hydroxyethyl)-_C_'-[cholesten-5-en-3β-yloxy-(3-propionacetoamidomethyl)]-_o_-carborane (5)

In a 50 mL two necked round bottom flask, *C*-(2-Benzyloxyethyl)-*C*'-[cholesten-5-en-3β-yloxy-(3-propionacetoamidomethyl)]-*o*-carborane (**4**) (0.18 g, 0.24 mmol) was dissolved in 20 mL of a mixture of EtOH-CH_2Cl_2 (50-50), then Pd/C (10%) was wet with few drops of water and added. The reaction mixture was stirred overnight at rt in a H_2 saturated atmosphere, then filtered and the solvent was evaporated under reduced pressure giving 0.14 g (88 %) of a white solid. M.p.= 103-106 °C. Found C, 63.90; H, 10.25; S. Calc. for $C_{35}H_{67}B_{10}NO_3$: C, 63.88; H, 10.26; ν_{max} (neat)/cm^{-1} 3433, 2583, 1655, 1587; δ_H (200 MHz; $CDCl_3$, Me_4Si): 0.70 (3H, s, C*H*$_3$CH(CH$_2$)$_3$), 0.80-2.40 (41H + 10H, m, CH$_3$CH(C*H*$_2$)$_3$, (C*H*$_3$)$_2$CH, C*H*$_3$ ring, C*H*$_2$ ring, C*H* ring, O*H*, B*H*), 2.50 (2H, t, *J* = 5.0 Hz, HOCH$_2$C*H*$_2$), 2.63 (2H, t, *J* = 6.2 Hz, C*H*$_2$CH$_2$CONH), 3.24 (1H, m, C*H*O), 3.73 (2H, t, *J* = 6.4 Hz, OC*H*$_2$(CH$_2$)$_2$CONH), 3.80 (2H, t, *J* = 5.2 Hz, HOC*H*$_2$CH$_2$), 4.11 (2H, d, *J* = 6.6 Hz, CONHC*H*$_2$), 5.39 (1H, bs, CH$_2$C*H*=C), 7.36 (1H, bt, CON*H*); δ_c (50.2 MHz; $CDCl_3$, Me_4Si): 11.6 (CH_3), 18.5 (CH_3), 19.2 (CH_3), 20.9 (CH_2), 22.4 (CH_3), 22.6 (CH_3), 23.6 (CH_2), 24.1 (CH_2),

27.8 (CH), 28.0 (CH_2), 31.7 (CH), 31.8 (CH_2), 33.7 (CH), 35.6 (CH_2), 36.0 (CH), 36.6 (CH_2), 36.8 (Cq), 37.4 (CH_2), 38.7 (CH_2), 39.3 (CH_2), 39.5 (CH_2), 41.4 (CH_2), 42.1 (Cq), 49.9 (CH), 55.9 (CH), 60.8 (CH_2), 63.1 (CH_2), 78.1 (Cq), 79.0 (Cq), 79.6 (CH), 122.0 (CH), 140.0 (Cq), 171.7 (Cq); m/z (ESI+) 659 $[M + H]^+$.

[Cholesten-5-en-3β-yloxy-(3-propionacetoamidomethyl)-*o*-carboranyl]-acetic acid (6) *C*-(Hydroxyethyl)-*C*'-[cholesten-5-en-3β-yloxy-(3-propionacetoamidomethyl)]-*o*-carborane (**5**) (0.48 g, 0.73 mmol) was dissolved in 21 mL of acetone, then a 3 M solution of CrO_3 (4 equiv, 2.9 mmol, 0.29 g) in H_2SO_4 (7 mL) was added carefully at 0 °C. The reaction mixture was left overnight at rt, then quenched with H_2O. The solvent was evaporated under reduced pressure and the mixture was extracted with CH_2Cl_2 (5 × 10 mL), the organic layers were washed once with brine (1 × 10 mL), dried and evaporated giving 0.42 g (85%) of pale yellow oil. Found C, 62.53; H, 9.76; N, 2.09. Calc. for $C_{35}H_{65}B_{10}NO_4$: C, 62.56; H, 9.75, N, 2.08; ν_{max} (neat)/cm^{-1} 3316, 2932, 2613, 2577, 1739, 1586, 1552; δ_H (200 MHz; $CDCl_3$, Me_4Si): 0.70 (3H, s, C*H*$_3$CH$(CH_2)_3$), 0.80-2.45 (41H + 10H, m, CH_3CH(C*H*$_2)_3$, (C*H*$_3)_2$CH, C*H*$_3$ ring, C*H*$_2$ ring, C*H* ring, B*H*), 2.63 (2H, t, *J* = 5.6 Hz, C*H*$_2$$CH_2$CONH), 3.24 (1H, m, C*H*O), 3.79 (2H, t, *J* = 5.6 Hz, OC*H*2$(CH_2)_2$CONH), 4.11 (2H, d, *J* = 4.4 Hz, CONHC*H*$_2$), 5.39 (1H, bs, CH_2C*H*=C δ_c (50.2 MHz; d_6DMSO, Me_4Si): 12.3 (CH_3), 19.0 (CH_3), 19.6 (CH_3), 22.9 (CH_2), 23.1 (CH_3), 23.2 (CH_3), 24.5 (CH_2), 25.9 (CH_2), 28.0 (CH), 29.7 (CH_2), 32.0 (CH), 33.9 (CH_2), 35.8 (CH), 36.2 (CH_2), 36.3 (CH), 36.9 (CH_2), 37.0 (Cq), 39.0 (CH_2), 40.6 (CH_2), 41.6 (CH_2), 42.4 (CH_2), 42.7 (CH_2), 43.1 (Cq), 48.1 (CH), 50.2 (CH), 56.2 (CH_2), 56.8 (CH_2), 76.0 (Cq), 78.7 (Cq), 81.6 (CH), 121.5 (CH), 141.0 (Cq), 168.9 (Cq); 171.3 (Cq); m/z (ESI+) 696 $[M + Na + H]^+$.

***C*-[N-*tert*-ButylDOTAMA-C_6-)-carbamoylmethyl]-*C*'-[cholesten-5-en-3β-yloxy-(3-propionacetoamidomethyl)-*o*-carborane (8).** According to the previous procedure described for

compound **3,** [cholesten-5-en-3β-yloxy-(3-propionacetoamidomethyl)-*o*-carboranyl]-acetic acid (**6**) (0.2 g, 0.29 mmol) was reacted with *N*-*tert*-butylDOTAMA-C_6-NH_2 (0.19 g, 0.29 mmol). The crude was purified on silica gel (eluant: CH_2Cl_2-MeOH 96-4, then CH_2Cl_2-MeOH 80-20) affording 0.15 g (42%) of pale yellow oil. Found C, 62.57; H, 9.82; N, 7.41. Calc. for $C_{69}H_{129}B_{10}N_7O_{10}$: C, 62.55; H, 9.81, N, 7.40; ν_{max} (neat)/cm^{-1} 3294, 2933, 2575, 1730, 1665, 1552; δ_H (200 MHz; $CDCl_3$, Me_4Si): 0.70 (3H, s, C*H*$_3$CH(CH$_2$)$_3$), 0.80-2.50 (78H + 10H, m, CH$_3$CH(C*H*$_2$)$_3$, (C*H*$_3$)$_2$CH, C*H*$_3$ ring, C*H*$_2$ ring, C*H* ring, NHC*H*$_2$C*H*$_2$NH, NHCOCH$_2$(C*H*$_2$)$_4$CH$_2$NHCO, B*H*), 1.50 (27H, s, COO*tBu*), 3.10-3.80 (13H, m, C*H*$_2$COOtBu, NHCO*CH*$_2$(CH$_2$)$_4$C*H*$_2$NHCO, C*H*O), 3.79 (2H, bt, OC*H*2(CH$_2$)$_2$CONH), 4.11 (2H, d, *J* = 6.2 Hz, CONHC*H*$_2$), 5.39 (1H, bd, CH$_2$C*H*=C), 8.20 (1H, bs, N*H*CO), 8.70 (2H, m, 2 X N*H*CO); δ_c (50.2 MHz; $CDCl_3$, Me_4Si): 11.6 (CH_3), 18.5 (CH_3), 19.3 (CH_3), 20.9 (CH_2), 22.4 (CH_3), 22.7 (CH_3), 23.7 (CH_2), 24.1 (CH_2), 25.2 (CH_2), 27.8 (9 X CH_3), 28.4 (CH_2), 28.4 (CH_2), 28.5 (CH_2), 29.5 (CH_2), 31.6 (CH), 31.7 (CH_2), 32.9 (CH_2), 35.6 (CH), 36.0 (CH_2), 36.9 (Cq), 37.1 (CH_2), 38.1 (CH_2), 38.5 (CH_2), 38.9 (CH_2), 39.3 (CH_2), 39.6 (CH_2), 41.4 (CH_2), 41.6 (CH_2), 42.0 (2 X CH_2), 46.0 – 54.0 (8 × CH_2), 50.0 (CH), 55.4 (2 × CH_2), 56.0 (CH), 56.6 (CH), 57.0 (Cq), 63.8 (2 X CH_2), 65.8 (Cq), 75.5 (Cq), 79.0 (2 X CH), 80.0 (Cq), 81.7 (Cq), 81.8 (Cq), 121.7 (CH), 140.7 (Cq), 166.3 (Cq), 171.1 (Cq), 171.3 (Cq), 171.4 (Cq), 171.9 (Cq), 172.2, (Cq); m/z (ESI+) 1350 $[M + Na + H]^+$.

***C*-[DOTAMA-C_6-)-carbamoylmethyl]-*C*'-[cholesten-5-en-3β-yloxy-(3-propionacetoamidomethyl)-*o*-carborane (9).** In a 25 mL round bottom flask, 247 mg (0.186 mmol) of *C*-[N-*tert*-ButylDOTAMA-C_6-)-carbamoylmethyl]-*C*'-[cholesten-5-en-3β-yloxy-(3-propionacetoamidomethyl)-*o*-carborane (**8**) were cooled to 0 °C, dissolved in 5 mL of a mixture of CF_3COOH-CH_2Cl_2 (50-50) and stirred for 4 h at rt. After evaporation of CF_3COOH-CH_2Cl_2, 204 mg (1.99 mmol) of viscous colorless oil were obtained (95%). Found C, 59.21; H, 9.17; N, 8.49. Calc. for $C_{57}H_{105}B_{10}N_7O_2$: C, 59.19; H, 9.15, N, 8.48; ν_{max} (neat)/cm^{-1} 3390, 2931, 2581, 1741, 1676, 1586; δ_H (400 MHz; CD_3SOCD_3, Me_4Si): 0.65 (3H, s, C*H*$_3$CH(CH$_2$)$_3$), 0.80-2.40 (51H

+ 10H, m, $CH_3CH(CH_2)_3$, $(CH_3)_2CH$, CH_3 ring, CH_2 ring, CH ring, $NHCH_2CH_2NH$, $NHCOCH_2(CH_2)_4CH_2NHCO$, BH), 3.10-3.80 (13H, m, $CH_2COOtBu$, $NHCOCH_2(CH_2)_4CH_2NHCO$, CHO), 3.79 (2H, bt, $OCH2(CH_2)_2CONH$), 4.00 (2H, d, *J* = 6.2 Hz, $CONHCH_2$), 5.30 (1H, bs, CH_2CH=C), 7.30 (1H, m, NHCO), 8.60 (2H, m, 2 X NHCO); δ_c (100.4 MHz; CD_3SOCD_3, Me_4Si): 11.6 (CH_3), 18.5 (CH_3), 19.3 (CH_3), 20.8 (CH_2), 22.4 (CH_3), 22.7 (CH_3), 23.1 (CH_2), 23.8 (CH_2), 24.7 (CH_2), 28.4 (CH_2), 28.4 (CH_2), 28.5 (CH_2), 29.5 (CH_2), 31.6 (CH), 31.7 (CH_2), 32.9 (CH_2), 35.6 (CH), 36.0 (CH_2), 36.9 (Cq), 37.1 (CH_2), 38.1 (CH_2), 38.5 (CH_2), 38.9 (CH_2), 39.3 (CH_2), 39.6 (CH_2), 41.4 (CH_2), 41.6 (CH_2), 42.0 (2 X CH_2), 46.0 – 54.0 (8 × CH_2), 50.0 (CH), 55.4 (2 × CH_2), 56.0 (CH), 56.6 (CH), 57.0 (Cq), 63.8 (2 X CH_2), 65.8 (Cq), 75.5 (Cq), 79.0 (2 X CH), 80.0 (Cq), 121.7 (CH), 140.7 (Cq), 166.3 (Cq), 171.1 (Cq), 171.3 (Cq), 171.4 (Cq), 171.9 (Cq), 172.2, (Cq); m/z (ESI+) 1350 $[M + Na + H]^+$.

Gd(III)-*C*-[*N*-(DOTAMA-C_6)carbamoylmethyl]-*C*'-2-[1,3-bis(hexadecyloxy)propan-2-yloxy]acetoamidomethyl-*o*-caborane complex (10). In a 100 mL round bottom flask, 70 mg of *C*-[DOTAMA-C_6-)-carbamoylmethyl]-*C*'-[cholesten-5-en-3β-yloxy-(3-propionacetoamidomethyl)-*o*-carborane (**9**) (0.055 mmol) and 1 equivalent of $GdCl_3$ were dissolved in water at rt. The pH solution was checked and maintained to 6.5 by 1 M NaOH aqueous solution. The solution was stirred 24h, then the pH was adjusted to 8.5 by 1 M NaOH aqueous solution and the mixture was stirred for 2 h, then filtered over 0.2 mm syringe filter, the pH was adjusted to 7 by a 1 M HCl aqueous solution and the solvent evaporated. Inorganic salts were removed by gel filtration on Sephadex® G10 column. The solvent was removed affording 63 mg of complex (0.043 mmol, 40%). Found C, 46.68; H, 7.88; S. Calc. for $C_{12}H_{24}B_{10}O_2$: C, 46.73; H, 7.84; m/z (ESI+) 1451.90 $[M + H]^+$.

Liposome preparation and characterization.

All the phospholipids used in the liposome preparation (POPC: 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine, DSPE-PEG 2000 Methoxy: 1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-N[Methoxy(Polyethylene Glycol)-2000] Ammonium Salt, DSPE-PEG 2000-Folate {1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000] ammonium salt} were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol) was purchased from Sigma-Aldrich (St. Louis, MO). Gd-AC101 was synthesized according to the above procedure. Liposomes were prepared by following the thin lipid film hydration method.[34, 35] The following liposomes formulation were prepared using POPC:Chol:Gd-B-AC01:DSPE-PEG2000 Methoxy:DSPE-PEG2000-Folate; Lipo-Ctrl 1:71:8:15:6:0 molar ratio. Lipo-Ctrl 2: 71:13:10:6:0 molar ratio. Lipo-Ctrl 3: 71:15,5:7,5:6:0 molar ratio. Liposomes with Folate (Lipo-Folate) were prepared adding 1% molar ratio of DSPE-PEG 2000 Folate in all previous described formulations; moreover DSPE-PEG 2000 was added in 5% molar ratio instead of 6%. Briefly, all lipids were dissolved in chloroform and the organic solution was slowly evaporated for removing the solvent until a thin film was formed. The film was then hydrated at 55°C with 1.5 ml of a saline buffer containing 5mM Hepes and 150mM NaCl, pH=7.4 (HBS). The suspension of multilamellar vesicles (MLV) was extruded (Lipex extruder, Northern Lipids Inc., Canada) four times on polycarbonate filters with a pore diameter of 400 nm, four times on a 200 nm and three times on 100 nm filter. The final liposome suspension was dialyzed two times at 4°C against a 100-fold volume of TBS. The amount of Gd-B-AC01incorporated in the liposome was determined by ^{1}H NMR T_1 measurement at 21.5 MHz, 25°C (Stelar Spinmaster, Mede, Italy) of the mineralized complex solution (in HCl 6M at 120 °C for 16 h). The hydrated mean diameter of liposomes was determined using a Malvern dynamic light-scattering spectrophotometer (Malvern Instruments, Malvern, UK). All samples were analyzed at 25°C in filtered (cutoff= 30 nm) PBS buffer (pH=7). The polydispersity index (PDI) for all the liposomes prepared in this work was smaller than 0.2. The liposomes were used within two weeks from the preparation and stored at 4°C under argon.

^{1}H Relaxation rate measurements. The longitudinal water proton relaxation rates were measured on the Stelar Spinmaster spectrometer (Stelar, Mede, Italy) operating at 21.5 MHz by means of the standard inversion recovery technique (16 experiments, 2 scans). A typical 90j pulse width was 3.5 ms and the reproducibility of the T1 data was ± 0.5%. The 1/T1 nuclear magnetic relaxation dispersion (NMRD) profiles of water protons were measured over a continuum of magnetic field strength from 0.00024 to 0.5 T (corresponding to 0.01–20 MHz proton Larmor frequency) on the fast field-cycling relaxometer (Stelar Spinmaster FFC 2000; Stelar) equipped with a silver magnet. The relaxometer operates under complete computer control with an absolute uncertainty in the 1/T1 values of ± 1%. The typical field sequences were used the nonpolarized sequence between 40 and 8 MHz and prepolarized sequence between 8 and 0.01 MHz. The observation field was set at 13 MHz. Sixteen experiments of two scans each were used for the T1 determination for each field.

Cell culture and uptake experiments. Human ovarian carcinoma cell line (IGROV-1) cell line was kindly provided by Dr. Claudia Cabella (Bracco Imaging, Colleretto Giacosa TO). IGROV-1 cells were cultured in RPMI (Lonza) supplemented with 10% (v/v) FBS, 2 mM glutamine, 100 U ml^{-1} penicillin, and 100 U ml^{-1} streptomycin. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO2. At 80% confluence, cells were detached with 0,02% EDTA (Lonza). For the in vitro uptake experiments, about $4{,}5\times10^{5}$ of IGROV were seeded in 6 cm diameter culture dishes. After 6 h, medium was removed and it was replaced with RPMI w/o Folate. After 24h, cells were incubated for 24 h with Lipo-Folate or Lipo-Ctrl. At the end of incubation, cells were washed three times with 5 ml ice-cold PBS, detached with 0.05% trypsin and 0.02% EDTA in PBS and transferred into glass capillaries for MRI analysis (see below). Gd content of IGROV-1 cells was determined using inductively coupled plasma mass spectrometry (ICP-MS) (Element-2; Thermo-Finnigan, Rodano, Milan, Italy). Sample digestion was performed with 2 ml of concentrated HNO_3 (70%) under microwawe heating (Milestone MicroSYNTH Microwave labstation equipped with an optical fiber temperature control and HPR-1000/6 M six position high pressure reactor, Bergamo,

Italy). After digestion, sample volumes were brought to 3 ml with ultrapure water and samples were analyzed by ICP-MS. Protein concentration was determined from cell lysates by the Bradford assay, using bovine serum albumin as standard.

MRI. MR images were acquired at 1 and 7 T field. MR images at 1 T were acquired with an Aspect M2 High Performance MRI System (Aspect Magnet Technologies Ltd, Netanya, Israel) consisting of a NdFeB magnet, equipped with a 35 mm solenoid Tx/Tr coil of inner diameter 35 mm. This system is equipped with fast gradient coils (gradient strength, 450 mT m−1 at 60 A; ramp time, 250 μs at 160 V) with a field homogeneity of 0.2–0.5 gauss. MR images at 7T were acquired on a Bruker Avance300 spectrometer equipped with a Micro 2.5 microimaging probe (Bruker BioSpin, Ettlingen, Germany) using two birdcage resonators with 30 and 10 mm inner diameter.

For recording MR images in vitro, cells were centrifuged at the bottom of glass capillaries placed in a phantom embedded of high gelling agarose gel (1% in PBS). MR images were acquired using a standard T1weighted multislice spin echo sequence, using the following parameters: TR/TE/NEX 250/4/6, resolution 78 μm, slice thickness 1 mm at 7 T and TR/TE/NEX 250/7/16, resolution 78 μm, slice thickness 1 mm at 1 T, respectively. At both field strengths a standard saturation recovery protocol was used to measure T1 relaxation times.

Cells irradiation

Seven flasks, four with IGROV-1 cells previously incubated for 24h in presence of folate targeted GdAC01/liposomes, at a 10μM Gd concentration, and three with non-treated control cells were irradiated in the thermal column of the TRIGA Mark II reactor at University of Pavia, Italy. The cells cultured in presence of liposomes were washed with cold PBS before irradiation. At the end of the irradiation, the medium was removed, it was replaced with RPMI and flasks were placed at 37 °C in a humidified atmosphere of 5% CO_2.

The irradiation position had been previously characterized from the point of view of neutron flux distribution by means of thin activation foils.[35] At a reactor power of 250 kW the thermal neutron flux in air in that position is $(1.17\pm0.03)\cdot10^{10}$cm^{-2} s^{-1}, while the epithermal and fast components are at least two orders of magnitude lower. The flux is roughly constant (less than 1%) along the vertical direction thus the flasks were superposed and irradiated at the same time. The irradiation time has been fixed at 15 minutes at a reactor power of 30 kW, corresponding to a thermal neutron fluence of $1.26\cdot10^{12}$ cm^{-2}. The radiation dose absorbed by cells treated with 10 μM was 6.2 Gy and the dose absorbed by control cells was 0.6 Gy.

Proliferation assay. The day after irradiation cells were detached with 0,02% EDTA and trypan blue exclusion test of cell viability was performed. Then, around $6x10^5$ IGROV-1 cells from each differently treated flask were seeded in 10 cm diameter culture dishes. After 1, 2, 3 and 4 days, cells were washed with PBS, detached with 0.05% trypsin and 0.02% EDTA in PBS and transferred into falcon tubes. Then, cells were sonicated for 30" at 30% power in ice and protein concentration from cell lysates was determined by the Bradford method, using bovine serum albumin as standard.

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Supporting Information description, ^{1}H NMR, ^{13}C NMR and DEPT spectra of products 1-5 and 8. This material is available free of charge via the Internet at http://pubs.acs.org."

Footnotes

Author Contributions. D.Alberti and A.Toppino contributed equally.

The authors declare no competing financial interest.

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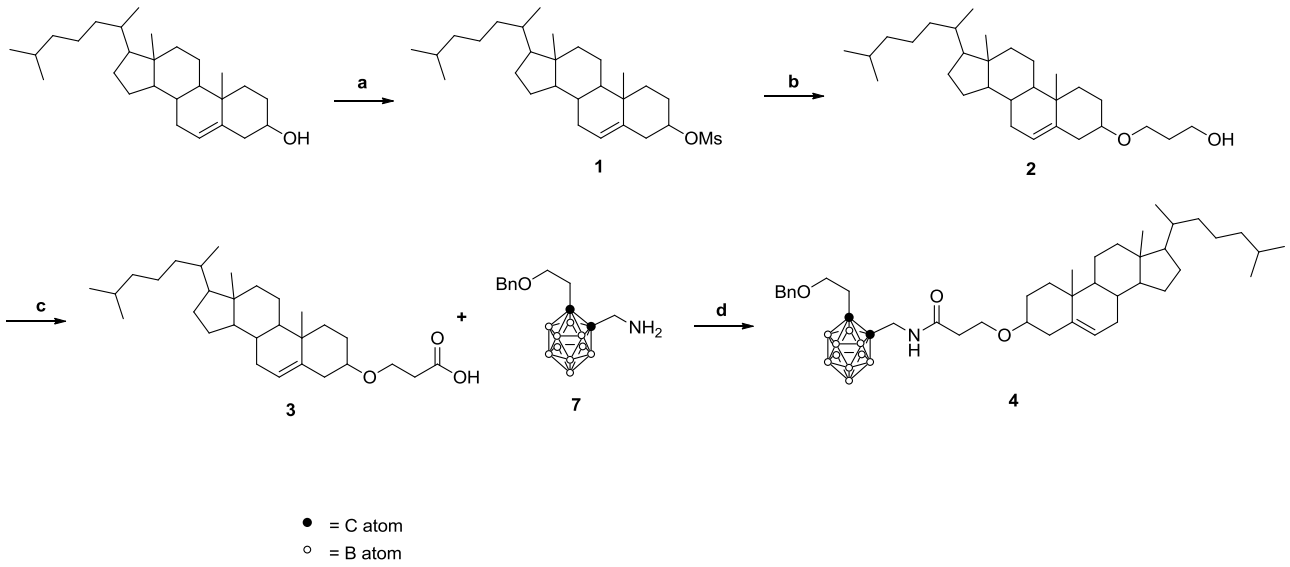
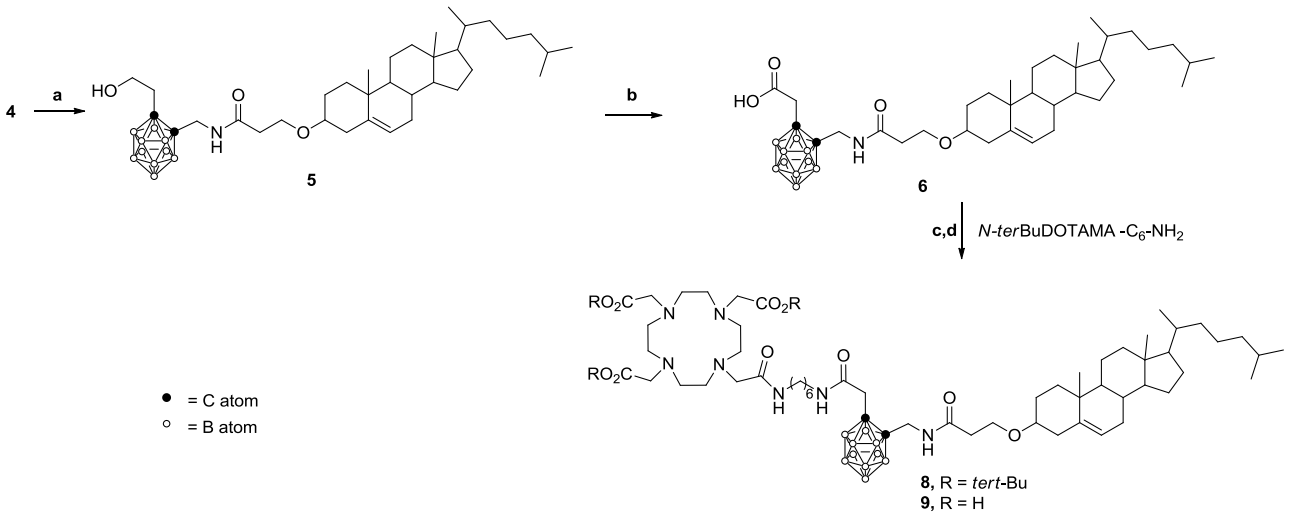
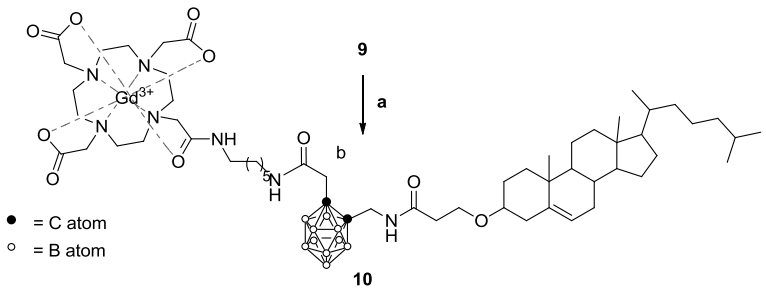


Scheme 1 *C*-(2-Benzyloxyethyl)-*C*'-[cholesten-5-en-3β-yloxy-(3-propionacetoamidomethyl)]-*o*-carborane (4). *Reaction conditions and yields*: a) MsCl, Et_3N, Et_2O, 0 °C → r.t (> 99%); b) $HO(CH_2)_3OH$, dioxane, 120 °C (27%); c) 3M CrO_3 in H_2SO_4, acetone, r. t. (80%); d) Dicyclohexylcarbodiimide, CH_2Cl_2, rt (79%) or *N*-hydroxysuccinimide, diclohexylcarbodiimide, CH_2Cl_2, rt (64%); e).



Scheme 2 Synthesis of *C*-[DOTAMA-C_6-)-carbamoylmethyl]-*C*'-[cholesten-5-en-3β-yloxy-(3-propionacetoamidomethyl)-*o*-carborane (9). *Reaction conditions and yields*: a) H_2, Pd/C, EtOH, CH_2Cl_2, rt (92%); b) 3M CrO_3 in H_2SO_4, acetone, r. t. (80%); c) *N*-hydroxysuccinimide, Dicyclohexylcarbodiimide, *i*-$PrEt_2N$, CH_2Cl_2, rt (42%); d) CF_3COOH, CH_2Cl_2, r. t., (95%).



Scheme 3 Synthesis of Gd(III)-*C*-[*N*-(DOTAMA-C_6)carbamoylmethyl]-*C*'-2-[1,3-bis(hexadecyloxy)propan-2-yloxy]acetoamidomethyl-*o*-caborane complex (10, Gd-B-AC01). a) $GdCl_3$, H_2O/MeOH