Comparison of the capacity of β-cyclodextrin derivatives and cyclophanes to shuttle cholesterol between cells and serum lipoproteins

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Abstract **Previous studies from this laboratory have demonstrated that low concentrations of cyclodextrins (<1.0 mM), when added to serum, act catalytically as cholesterol shuttles to accelerate the exchange of free cholesterol between cells and serum lipoproteins. As cholesterol shuttles, cyclodextrins have the potential to serve as pharmacological agents for modifying cholesterol metabolism. In the present study, we have quantitated the cholesterol-shuttling capacity of a series of newly synthesized β-cyclodextrin derivatives (βCDs), with varying structure, and two double-decker cyclophanes. The general protocol is as follows. [^{3}H]cholesterol-labeled CHOK1 cells are incubated for 2 h with the test compounds alone or together with 5% human serum, and efflux of the cellular [^{3}H]cholesterol is measured. As methyl β-cyclodextrin (MβCD) served as the basis for comparison, initial experiments were conducted that demonstrated there was a dose-dependent stimulation of cell cholesterol efflux as the concentration of MβCD increased, with an EC_{50} that was calculated to be 0.05 mM. To determine the cholesterol-shuttling capacity of the newly synthesized compounds, cell cholesterol efflux is measured when the compounds are present alone, at a concentration of 0.05 mM, or together with 5% human serum. Our results demonstrate that the double-decker cyclophanes are the most efficient cholesterol shuttles. Under our experimental conditions, methyl β-cyclodextrin (MβCD) approximately doubles the efflux of cell cholesterol to serum, whereas one of the double-decker cyclophanes produces a 4-fold stimulation in efflux. Four of the β-cyclodextrin derivatives (βCDs) display shuttling ability similar to that of MβCD. Furthermore, there does not appear to be a structural pattern among the other βCDs which could explain their shuttling capacity.**—Christian, A. E., H-S. Byun, N. Zhong, M. Wanunu, T. Marti, A. Fürer, F. Diederich, R. Bittman, and G. H. Rothblat. **Comparison of the capacity of β-cyclodextrin derivatives and cyclophanes to shuttle cholesterol between cells and serum lipoproteins.** ***J. Lipid Res.*** **1999.** 40: **1475–1482.**

Supplementary key words cholesterol • cyclodextrins • efflux • cyclophanes • tissue culture cells

Reverse cholesterol transport (RCT) is the process by which excess peripheral cell cholesterol is removed by lipoproteins such as high density lipoproteins (HDL) and subsequently returned to the liver to be excreted. The first step in this process is the movement of cholesterol out of the cell plasma membrane and its incorporation into extracellular acceptors such as HDL (1–3). It has been shown that this movement of cholesterol from the plasma membrane of cells to lipoprotein acceptors occurs through a number of mechanisms, one of which is an aqueous diffusion mechanism in which the cholesterol desorbs from the plasma membrane and, after moving through the aqueous phase, is picked up by the acceptor (4–6). A "shuttle-sink" model of cholesterol efflux has been proposed for this process in which different HDL subclasses have been implicated as having a role. It has been suggested that lipid-free apolipoproteins or smaller, phospholipid-rich particles such as pre-β HDL and small α-migrating HDL may serve as the initial acceptors of cell membrane cholesterol and subsequently transport or "shuttle" cholesterol from the cell membrane to lipoproteins that serve as cholesterol "sinks," such as HDL, LDL or VLDL/chylomicrons (7–10). These lipoproteins have the capacity to hold large amounts of cholesterol and

Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; βCDs, β-cyclodextrin derivatives; CD-amine, 6-amino-6-deoxy-CD; CD-Et_2-amine, 6-deoxy-*N*,*N*-diethylamino-CD; CD-mannitol-$(NMe_2)_2$, 6-deoxy-6-*N*-cyclodextrin-*N*,*N*-6′-(dimethylammoniomannityl)-1-*N*,*N*-dimethylamine; CD-Me_2-amine, CD-6-deoxy-6-*N*,*N*-dimethylamine; CDOTs, CD-6-deoxy-6-tosylate; CD-*N*-Xyl-amine, 1,4-diaminoxylylene-6-deoxy-CD; CHOK1, Chinese hamster ovary cells; EMEM, Eagle's minimum essential medium; FBS, fetal bovine serum; hexyl-*S*-CD dimer, 1,6-di-(6^A-thio-β-cyclodextrinyl)-hexane; HS, human serum; 2OHpβCD, 2-hydroxypropyl β-cyclodextrin; MβCD, methyl β-cyclodextrin; *m*-Xyl-*S*-CD dimer, α,α′-di-(6^A-thio-β-cyclodextrinyl)-*m*-xylene; PBS, phosphate-buffered saline; PDIC, 1,4-phenylene diisocyanate.

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transport it to the liver for excretion. The transport of the cell-derived cholesterol back to the liver would involve a number of steps, some of which would be mediated by serum factors such as lecithin:cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP), and phospholipid transfer protein (PLTP) (1, 2, 11).

Previous studies have demonstrated that β-cyclodextrins, when added to cell monolayers at high concentrations (10–100 mm) and in the absence of serum or isolated lipoproteins, are able to remove large amounts of cellular cholesterol and retain this cholesterol in the medium as cyclodextrin:cholesterol complexes, thus serving as extracellular cholesterol sinks (12–14). Other studies have demonstrated that low concentrations of β-cyclodextrins (<1.0 mm), when added to culture medium together with serum or serum lipoproteins, act as cholesterol shuttles enhancing the exchange of cholesterol molecules between the cells and the lipoproteins. It was found that methyl β-cyclodextrins (MβCD) were the most effective artificial cholesterol shuttles when compared to other cyclodextrins such as 2-hydroxypropyl (2OHpβCD), carboxymethyl, and tetradecasulfated β-cyclodextrins (7).

Because cyclodextrins have become very useful tools for studying cell cholesterol metabolism (15–17), are presently used as drug-delivery vehicles (18–20), and may in the future serve as pharmacological agents for stimulating RCT, we have screened a number of cyclodextrin-like compounds to determine whether any of these or other synthetic receptors are more efficient cholesterol shuttles than the cyclodextrins that are currently available. For this purpose, a variety of βCDs and double-decker cyclophane compounds (21, 22) were examined for their cholesterol-shuttling capacity via the formation of a water-soluble complex between the macrocycle and cholesterol in the following experimental system. CHOK1 cells, prelabeled with [^{3}H]cholesterol and serving as cholesterol donors, were incubated with 5% human serum ± each cyclodextrin derivative or double-decker cyclophane. The lipoproteins in the human serum acted as cholesterol "sinks" to which the βCDs or double-decker cyclophane compounds would shuttle the labeled cellular cholesterol. The stimulation of efflux in the presence of each compounds at 0.05 mm was compared to the shuttling capability of MβCDs at 0.05 mm, which was found to be the concentration of MβCD at which there was half-maximal stimulation of cholesterol efflux (EC_{50}).

MATERIALS AND METHODS

Materials

β-Cyclodextrins were obtained from Acros (Pittsburgh, PA) and Sigma (St. Louis, MO). MβCD was also kindly provided by Cerestar, USA, Inc. (Hammond, IN). [1,2-^{3}H]cholesterol (51 Ci/mmol) was purchased from DuPont NEN (Boston, MA). Eagle's minimal essential medium (EMEM), EMEM-HEPES media, Ham's F12 media, and PBS were obtained from BIO-Whittaker (Walkersville, MD). Heat-inactivated fetal bovine serum (FBS), gentamicin, cholesterol, and 1,4-diaminoxylene were purchased from Sigma (St. Louis, MO). Pfizer ACAT inhibitor, CP 113,818, was a gift from Pfizer Pharmaceuticals (Groton, CT). Diethylamine, α, α′-dibromo-*m*-xylene and 1,4-phenylene diisocyanate (PDIC) were purchased from Aldrich (Milwaukee,WI). 1,6-Dibromohexane was obtained from Acros. CD-6-deoxy-6-tosylate (CDOTs) was prepared according to Zhong, Byun, and Bittman (23) ^{1}H and ^{1}3C NMR spectra were recorded at 400 and 100 MHz, respectively. MALDI MS were recorded at Texas A&M University. Analytical TLC was carried out on silica gel 60 F254 aluminum-backed plates (EM Separations Technology, Gibbstown, NJ).

Cell culture

Media were buffered with sodium bicarbonate and cells were cultured in a humidified incubator at 37°C with 5% CO_2. All media were supplemented with 50 μg of gentamicin/ml. CHOK1 cells were cultured in Ham's F12 media supplemented with 7.5% FBS. Cells were plated in 6-well plates (1.8×10^5 cells/well). One day after plating, the cells were labeled with [^{3}H]cholesterol by incubating for 24 h at 37°C with 2 ml/well Ham's F12 media supplemented with 1% FBS and [^{3}H]cholesterol (2 μCi/ml). The cells were then equilibrated with Ham's F12 media for 18 h at 37°C to allow the labeled cholesterol to distribute among all intracellular pools. During both the labeling and equilibration incubations, Pfizer ACAT inhibitor CP 113,818 (2 μg/ml) was added to the media to inhibit the acyl CoA:cholesterol acyltransferase (ACAT) reaction, thus producing cells in which the labeled cholesterol was only in the free cholesterol (FC) pool.

Human sera and macrocyclic cholesterol shuttles

Samples of sera from normal volunteers were collected (with informed consent), clotted for 1 h at room temperature, pooled, and frozen at −15°C in aliquots as previously described. For each experiment, the appropriate number of aliquots were thawed and used for the preparation of the efflux media. The efflux media were made as follows: 0.1 mm solutions of the cyclodextrins or cyclophanes were made in EMEM-HEPES (pH 7.2) and diluted with EMEM-HEPES to yield 0.05 mm solutions with or without 5% human serum. The synthesis and purification of the β-cyclodextrin derivatives are outlined below. The complete efflux media were made the day before the experiment and stored at 4°C prior to use. The media were warmed to 37°C for 2 h before the start of the experiment.

Measurement of cholesterol efflux

The efflux experiments were initiated when the radiolabeled cell monolayers were washed 3× with EMEM-HEPES (37°C), and 2 ml of EMEM-HEPES, containing the indicated acceptors ± 5% HS, were added to each well. Efflux was measured on cells maintained at 37°C. At the indicated times, 150-μl aliquots of medium were taken to monitor the efflux. These medium samples were filtered through 0.45-μm multiscreen filtration plates (Millipore Corp., Bedford, MA), and 100-μl aliquots of the filtrate were then counted by liquid scintillation to determine the release of labeled cholesterol from the cells. Fractional efflux values were calculated as previously described (12, 13, 25). Briefly, the percentage of the radiolabeled cholesterol that was released was calculated by dividing the total amount of [^{3}H]cholesterol in the incubation medium at each sampling time by the total [^{3}H]cholesterol in the cells at time zero.

Measurement of the cholesterol solubilizing capacity of cyclodextrins and double-decker cyclophanes

The cholesterol solubilizing ability of each of the βCDs and cyclophanes was determined as previously described (26). Briefly, a 5 mm solution of radiolabeled cholesterol (sp. act. = 9.2×10^7 cpm/mmol) in chloroform–methanol 1:1 was dried against the wall of a glass tube under a gentle stream of nitrogen. A 5 mm so-

lution of each cyclodextrin or cyclophane in EMEM-HEPES (pH 7.2) was added. The mixtures were sonicated in a bath sonicator for 3 min and incubated overnight at 37°C in a rotating water bath. After incubation, the solutions were filtered through a 0.45-μm filter to remove any unsolubilized cholesterol. Based on the original specific activity of the radiolabeled cholesterol, the amount of cholesterol that was able to complex with each cyclodextrin derivative or cyclophane was determined.

Chemical syntheses of βCDs

The structures of all of the synthesized cyclodextrin derivatives are illustrated in **Fig. 1**.

Carbamoyl-CD dimer and carbamoyl-CD. To a solution of 1.5 g (1.33 mmol) of CD in 95 ml of anhydrous pyridine was added 100 mg (0.66 mmol) of PDIC over 30 min at 75°C. After the reaction mixture was stirred for 12 h at 80°C, the product was purified by preparative TLC on silica gel GF plates (1-mm thick, Analtech, Newark, DE) eluted with 1-PrOH:H_2O:EtOAc:conc. NH_4OH (5:3:1:1 by volume, solvent system A). Two UV-active bands were scraped from the plate and eluted with solvent system A. The solvents were removed by evaporation under reduced pressure. Suspended silica gel that co-eluted with each product was removed by precipitating the product dissolved in a minimal volume of H_2O by addition of 2-propanol. On lyophilization, there was obtained 1.25 g (yield, 78%) of the carbamoyl-CD dimer, $CDO_2CNHC_6H_4NHCO_2CD$, R_f 0.18. Also obtained was 82.8 mg (yield, 9.6%) of the mono-coupled analog, carbamoyl-CD, $CDO_2CNHC_6H_4NHCO_2H_2$, R_f 0.45. **Carbamoyl-CD:** ^{1}H NMR (in D_2O) δ 7.15 (b), 4.96–4.99 (m), 3.31–3.88 (m); ^{13}C NMR (in D_2O) δ 180.60, 144.6, 128.0, 125.62, 101.76 (m), 80.40, 72.84, 72.53, 71.80, 71.63, 71.40, 70.85, 63.80, 59.34. **Carbamoyl-CD dimer:** ^{1}H NMR (in D_2O) δ 7.50 (b), 7.32 (b), 4.95 (b), 3.53-3.91 (m); ^{13}C NMR (in D_2O) δ 180.92 (C = O), 177.65 (C = O), 122.0, 102.22, 81.29, 73.37, 72.32, 72.11, 64.52, 60.43.

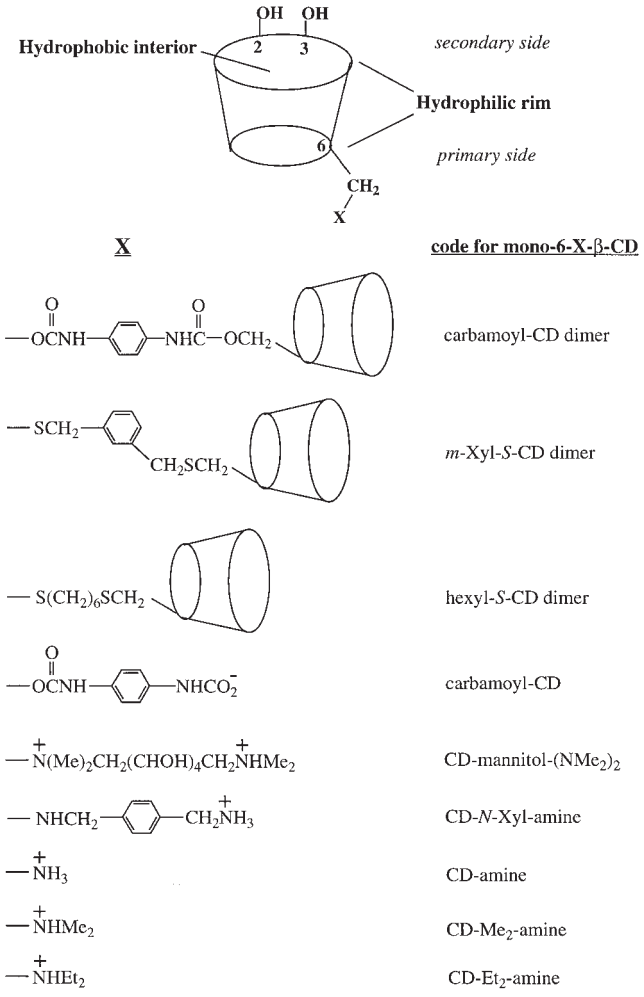

Fig. 1. Representation of the structures of the primary side modified β-cyclodextrins. One of the hydroxyl groups at C-6 was replaced as shown.

6-Deoxy-6-*N*,*N*-diethylamino-CD, CD-Et_2-amine. CDOTs (5.1 g, 4.2 mmol) was dissolved in diethylamine (43.6 ml, 0.42 mol). The solution was stirred for 4 d at room temperature. The excess diethylamine was removed under vacuum. The product was obtained by crystallization from H_2O, yielding 3.1 g (63%) as white needles; TLC (solvent system A), R_f 0.50. MS, $C_{46}H_{79}O_{34}N$, calculated 1189.43, found 1190.35 (M + H)$^+$, ^{1}H NMR (in D_2O/DMSO-d_6 1:1) δ 104.62, 84.30, 75.85, 75.57, 74.69, 63.8, 49.22, 41.04 (m).

1,4-Diaminoxylylene-6-deoxy-CD, CD-*N*-Xyl-amine. CDOTs (1.3 g, 1 mmol), NaI (0.15 g, 1 mmol) and 1,4-diaminoxylene (68 mg, 0.5 mmol) were dissolved in 100 ml of pyridine. After the solution was stirred at 60°C for 2 d, pyridine was removed by vacuum evaporation. Column chromatography on CM-Sephadex C-25 using a gradient of 0 to 0.4 M NH_4HCO_3 followed by lyophilization gave 300 mg (47%) of the product as a light brown powder; TLC (solvent system A), R_f 0.22. ^{1}H NMR (D_2O) δ 8.83–8.84 (d), 8.07–8.10 (m), 5.02–5.20 (m), 3.38–4.03 (m), 2.85–2.88 (d), 2.56–2.59 (d); ^{13}C NMR (D_2O) δ 146.54, 144.98, 128.10, 126.88, 126.63, 101.56, 82.51, 81.44, 80.83, 72.79, 72.58, 71.72, 71.14, 70.46, 61.50, 60.55, 59.95, 58.95, 44.77, 44.44.

6-Amino-6-deoxy-CD, CD-amine. CDOTs (2.8 g, 2.17 mmol), NaI (0.15 g, 1 mmol) and NaN_3 (1.44 g, 21.7 mmol) were dissolved in 9 ml of DMF. The reaction mixture was stirred at 65°C for 2 d, then passed through a column of Amberlite MB-3 (elution with H_2O), giving 6-azido-6-deoxy-CD. The latter was reduced with triphenylphosphine (2.65 g, 10 mmol) and conc. NH_4OH (17.5 ml) in DMF (10 ml). The reaction mixture was stirred at room temperature for 12 h, then passed through an Amberlite MB-3 column using H_2O as the eluent, yielding 0.5 g (20%) of the product as a white powder; TLC (solvent system A), R_f 0.31.; lit. R_f 0.6 (HOAc, $CHCl_3$, H_2O 8:1:1) ^{1}H NMR (D_2O) δ 4.85–4.91 (m), δ 3.31-3.80 (m), 3.06 (b); ^{13}C NMR (D_2O) δ 102.50, 82.13 (m), 81.64, 74.01, 73.81, 73.35, 72.91, 72.85, 72.72, 72.09 (m), 68.75, 61.19, 40.98.

CD-6-deoxy-6-*N*,*N*-dimethylamine, CD-Me_2-amine. CDOTs (4.31 g, 3.35 mmol) and dimethylamine hydrogen chloride (2.76 g, 33.5 mmol) were dissolved in 100 ml of dry pyridine. After the mixture was stirred at 65°C for 2 d, it was passed through a column of CM-Sephadex C-25 using a gradient of 0 to 0.4 m NH_4HCO_3. After lyophilization, a light brown powder (0.38 g, 9.8%) was obtained; TLC (solvent system A), R_f 0.54. MS, $C_{44}H_{75}O_{34}N$, calculated 1161.42, found 1162.38 (M + H)$^+$, ^{1}H NMR (D_2O) δ 4.94–5.26 (m), 3.16–4.09 (m), 2.80 (s); ^{13}C NMR (D_2O) δ 101.82, 82.03, 81.42, 81.30, 73.04, 72.67, 72.12, 71.82, 70.86, 60.10, 59.98, 38.15 (m).

α,α′-Di-(6^A-thio-β-cyclodextrinyl)-*m*-xylene, *m*-Xyl-*S*-CD dimer. A suspension of α,α′-dibromo-*m*-xylene (66 mg, 0.25 mmol), potassium thioacetate (70 mg, 0.61 mmol) and 18-crown-6 (6.6 mg, 0.025 mmol) in 25 ml of dry THF was stirred overnight. The reaction mixture was diluted with 50 ml of hexane and filtered through a pad of silica gel, which was washed with 100 ml of hexane–EtOAc 10:1. The filtrate was concentrated under reduced pressure and the residue was dried on high vacuum for 2 h. The residue was dissolved in 25 ml of dry DMF, 1 ml of MeOH and K_2CO_3 (138 mg, 1.0 mmol) and CDOTs (1.30 g, 1.0 mmol) were added. After the mixture was stirred at 55°C for 2 weeks, the

product was collected by filtration, washed with $CHCl_3$–MeOH-H_2O 65:35:8, and purified by column chromatography on silica gel, eluting with solvent system A. Suspended silica gel was removed by dissolving the product in a minimum amount of H_2O and precipitating the product with 2-PrOH, giving 240 mg (40% yield) of silica gel free product as a white solid; R_f 0.10 (solvent system A); (for comparison purposes in this solvent system: CDOTs, R_f 0.59 and CD, R_f 0.41); ^{1}H NMR (D_2O) δ 7.24–6.91 (m, 4H), 4.85–5.05 (m), 4.40–4.65 (m, overlap with HDO), 3.5–3.85 (m), 2.90–3.00 (m, 4H), 2.78–2.82 (m, 4H); ^{13}C NMR (D2O) δ 102.5–104.5 (m), 81.5–83.5 (m), 74.37–74.57 (m), 72.5–74.5 (m), 60.6–62.6 (m).

1,6-Di-(6^A-thio-β-cyclodextrinyl)-hexane, hexyl-*S*-CD dimer. The compound was prepared in 20% yield from 1,6-dibromohexane by the procedure described above; R_f 0.10 (solvent system A); ^{1}H NMR (D_2O) δ 4.95-5.01 (m), 4.54–4.86 (m, overlap with HDO), 3.72–4.12 (m), 3.43–3.60 (m), 2.84–2.95 (m, 4H), 2.58–2.68 (m, 4H), 1.36–1.94 (m, 8H).

6-Deoxy-6-*N*-cyclodextrin-*N,N*-6′-(dimethylammoniomannityl)-1-*N,N*-dimethylamine, CD-mannitol-(NMe_2)$_2$. This compound was prepared from 3,4-*O*-isopropylidene-d-mannitol, which was converted to the 1,2,5,6-bis-epoxide. The epoxide was opened with dimethylamine hydrochloride in the presence of potassium carbonate. The resulting 1,6-bis-*N,N*-dimethylaminomannitol was allowed to react with CDOTs in pyridine. ^{1}H NMR (D_2O) δ 4.95–5.30 (m), 4.65–4.76 (m, overlap with HDO), 3.30–4.20 (m), 2.83–2.88 (m, 4H, CH2N), 2.44–2.70 (m, 12H, NMe); ^{13}C NMR (D_2O) δ 105.10–105.68 (m), 84.49–86.67 (m), 74.17–76.71 (m), 62.32–65.67 (m), 58.72, 46.29.

Synthesis of double-decker cyclophanes

The cyclophanes used in this study were previously shown to form strong complexes with cholesterol as well as with other steroids in vitro in polar protic solvents (21, 22). Their unique binding properties are based on their large hydrophobic cavities that are formed by eight bridged aromatic moieties. Solubility of the racemic hosts is achieved by the positively charged ammonium groups located at the periphery of the molecules, thereby not affecting the hydrophobic character of the cavities. The dimensions of the cavities are tailor-made for the incorporation of the hydrophobic tetracyclic scaffold of the steroids. They measure 11 × 11 × 8 Å (depth × length × width, cyclophane 1) and 11 × 10 to 11 × 8 Å (cyclophane 2), respectively, allowing the axial inclusion of the steroidal guests. Cyclophane 1 was found to form complexes with cholesterol with free binding energies of −8.2 kcal mol^{-1} (association constant K_a = 1.1 × 1061 mol^{-1} T = 295 K) in water and −4.3 kcal mol^{-1} (K_a = 1500 L mol^{-1}, T = 298 K) in methanol as well as with other steroids in water and methanol (21, 22). Cyclophane 2 bound cholesterol in methanolic solution with a free binding energy of −3.8 kcal mol^{-1} (K_a = 600 L mol^{-1}, T = 298 K). The structures of both double-decker cyclophanes are illustrated in **Fig. 2**.

Statistical analyses

Values are the mean (in triplicate) ± SD unless otherwise indicated. The criterion for significance was $P \leq 0.05$. The EC_{50} of MβCD and double-decker cyclophane 2 was determined through a sigmoidal variable slope analysis using GraphPad Prism (San Diego, CA). To determine the relationship between the shuttling capacity and the cholesterol-solubilizing ability of each of the β-cyclodextrin derivative and cyclophanes, a linear regression analysis was performed using GraphPad Prism.

RESULTS

Determination of the effective concentration at which MβCD acts as a shuttle

Because of the limited quantities of cyclodextrins and cyclophanes available for these studies, complete dose–response curves could not be used to determine the shuttling capacity of each synthesized cyclodextrin derivative and double-decker cyclophane. Previously, it was demonstrated that MβCDs exhibit greater shuttling capacity when compared to other modified β-cyclodextrins such as 2OHpβCD, carboxymethyl β-CD, and tetradecasulfated βCD (7). Therefore, when analyzing the synthetic βCDs for their shuttling capacity, our "benchmark" for comparison was MβCDs. For our studies, we needed to determine the EC_{50} for stimulation of efflux in CHOK1 cells. A dose response of cholesterol efflux from CHOK1 cells was obtained using several concentrations of MβCD alone or MβCD with 5% human serum as described. **Figure 3** demonstrates the efflux capacity of the MβCD alone and of MβCD with 5% human serum. The calculated shuttle effect of MβCD was established by subtracting the sum of the efflux values obtained from

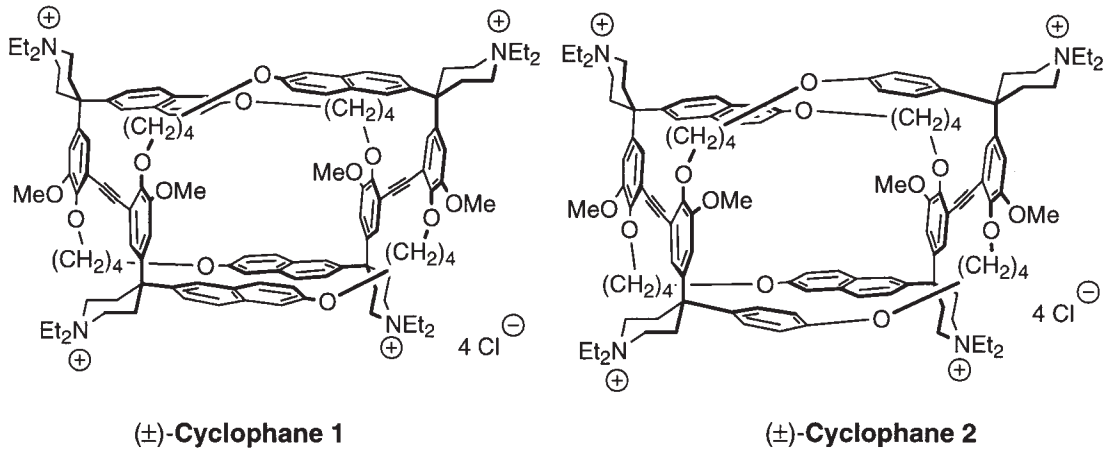

Fig. 2. Representation of the structures of cyclophanes 1 and 2.

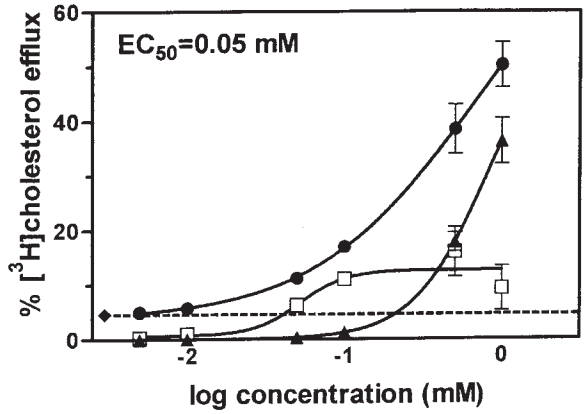

Fig. 3. Effect of MβCD concentration on the efflux of cholesterol from CHOK1 cells. Monolayers of CHOK1 cells were grown and labeled with [^{3}H]cholesterol as described in Materials and Methods. The fractional efflux of labeled cholesterol was determined after a 2-h incubation of the monolayers with a combination of 5% human serum and the indicated amount of MβCD (●) or MβCD alone (▲). The calculated shuttle effect (□) of MβCD was established by subtracting the sum of the efflux values obtained from MβCD alone and 5% human serum alone (dotted line) from the efflux value of the combination of 5% HS and MβCD. The EC_{50} of MβCD was determined using a variable slope sigmoidal dose response analysis as described in Materials and Methods.

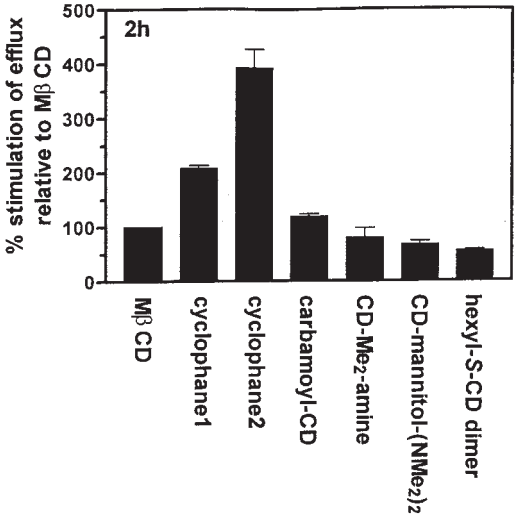

Fig. 4. Effect of stimulation of efflux of a sample of cyclodextrin derivatives or cyclophanes relative to MβCD. Monolayers of CHOK1 cells were grown and labeled with [^{3}H]cholesterol as described in Materials and Methods. The fractional efflux of labeled cholesterol was determined after a 2-h incubation of the monolayers with 0.05 mm of each βCD alone or combined with 5% human serum. The stimulation of efflux of the compounds relative to MβCD (100%) is depicted in the graph.

MβCD alone and 5% human serum alone from the efflux value of the combination of 5% HS and MβCD. The EC_{50} of MβCD for CHOK1 cells was calculated to be 0.05 mm using a variable slope sigmoidal dose response analysis as described in Materials and Methods. In all subsequent studies, the shuttling capacity of each cyclodextrin derivative or cyclophane was determined at 0.05 mm and compared to the stimulation of efflux observed for MβCD at the same concentration.

Comparison of the shuttle capacity of different cyclodextrin and cyclophane derivatives incubated with 5% human serum

To determine the ability of each macrocyclic receptor to shuttle cholesterol to lipoprotein "sinks," efflux of cholesterol to the βCDs ± 5% human serum was performed in CHOK1 cells as previously described. The calculated shuttle effect of the each βCD and double-decker cyclophane was established by subtracting the sum of the efflux values obtained from βCDs alone and 5% human serum alone from the efflux value of the combination of 5% HS and the βCDs. **Figure 4** shows the shuttling ability of the two double-decker cyclophane compounds and four of the βCDs relative to MβCD (100%). As can be seen from the graph, the shuttling ability of the β-cyclodextrin derivatives was similar to that of MβCD. However, both double-decker cyclophanes greatly surpassed the shuttling ability of MβCD. These results led to experiments to determine the effective concentration of the cyclophane compounds. **Figure 5** shows the dose response of cyclophane **2**. The calculated shuttle effect of the double-decker cyclophane **2** was established by subtracting the sum of the efflux values obtained from cyclophane **2** alone and the 5% human serum alone from the efflux value of the combination of 5% HS and cyclophane **2**. As can be seen, the effective concentration of this compound is calculated to be 0.01 mM, which suggests that it is approximately 5-fold more efficient than MβCD.

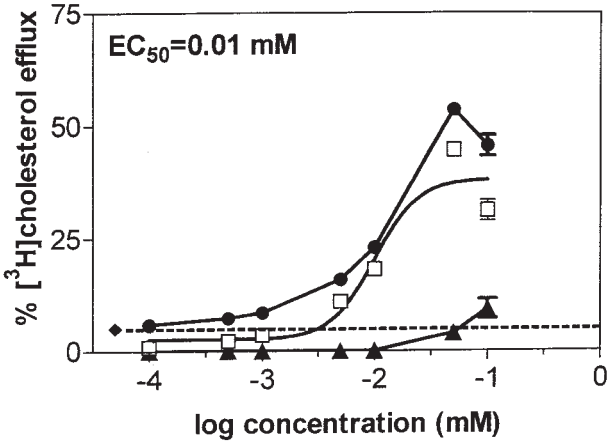

Fig. 5. Effect of double-decker cyclophane 2 concentration on the efflux of cholesterol from CHOK1 cells. Monolayers of CHOK1 cells were grown and labeled with [^{3}H]cholesterol as described in Materials and Methods. The fractional efflux of labeled cholesterol was determined after a 2-h incubation of the monolayers with a combination of 5% human serum and the indicated amount of cyclophane **2** (●) or cyclophane **2** alone (▲). The calculated shuttle effect (□) of cyclophane **2** was established by subtracting the sum of the efflux values obtained from cyclophane **2** alone and 5% human serum alone (dotted line) from the efflux value of the combination of 5% HS and cyclophane **2.** The EC_{50} of cyclophane **2** was determined using a variable slope sigmoidal dose response analysis as described in Materials and Methods.

TABLE 1. Relationship between cholesterol-solubilizing ability and stimulation of cholesterol efflux of β-cyclodextrin derivatives and double-decker cyclophanes

Treatment	Molar Ratio (host:1 cholesterol)	% Stimulation Relative to MβCD
Cyclophane 2[a] (n = 2)	4	392.18 ± 34.33
Cyclophane 1[a] (n = 2)	199	209.10 ± 4.96
Carbamoyl-CD (n = 3)	3656	119.27 ± 4.62
MβCD (n = 6)	7	100
CD-Me_2-amine	564	80.28 ± 17.87
CD-mannitol-$(Nme_2)_2$	501	67.52 ± 6.53
Hexyl-*S*-CD dimer	39	55.01 ± 3.01
CD-Et_2-amine	482	50.97 ± 6.65
2OHpβCD	930	46.02 ± 5.46
CD-amine	985	38.52 ± 16.51
Carbamoyl-CD dimer	443	33.92 ± 0.45
CD-*N*-Xyl-amine	340	32.29 ± 7.46
m-Xyl-*S*-CD dimer	67	29.30 ± 5.98

The cholesterol-solubilizing capacity of each βCD and double-decker cyclophane compounds at 5 mm in MEM-HEPES (pH 7.2) was determined as described in Materials and Methods (n = 1 unless otherwise indicated). The stimulation of efflux relative to MβCD (100%) was determined as described. These values are mean ± SD (in triplicate).

[a] At receptor concentration of 5 mm, the solubility limits of the double-decker cyclophanes may have been exceeded, causing an apparent low solubilizing efficiency.

Determination of the cholesterol solubilizing ability of different cyclodextrin derivatives

To determine whether there is a relationship between the ability of the βCDs or cyclophanes to effectively shuttle cholesterol from cells to lipoproteins in serum and their ability to complex with cholesterol, cyclodextrin:[^{3}H]cholesterol complexes were made as described in Materials and Methods and the cyclodextrin:cholesterol molar ratio was determined. The molar ratio of MβCD:cholesterol, which has been previously determined (26), was measured in each experiment to test the reproducibility of the assay. **Table 1** shows the cholesterol solubilizing ability and the calculated stimulation of efflux (shuttling capacity) relative to MβCD of each of the βCDs and the two double-decker cyclophanes. There appears to be no relationship between the ability of the compounds to solubilize cholesterol and their ability to stimulate cholesterol efflux to serum lipoproteins. The compounds that exhibit high molar ratios may either indicate that the structure of the cyclodextrin may not be conducive for complexation with cholesterol or that the cyclodextrin derivatives themselves have very low water solubility.

DISCUSSION

Cyclodextrins have been extensively used as pharmacological agents for the delivery of water-insoluble drugs (18, 28–30). It has been proposed that cyclodextrin compounds might be useful as drugs that could modify cholesterol metabolism (31, 32). However, the renal toxicity observed (33) and the need for intravenous injection and rapid clearance (34) of known cyclodextrins would limit the use of these compounds as pharmacological agents to enhance cholesterol removal from cells in vivo. More recently, cyclodextrins have been used as experimental tools to manipulate the cholesterol content of cultured cells in order to study a variety of aspects of cellular cholesterol metabolism (12, 13, 17, 26). Two general protocols have been used in these cell studies. *1*) When incubated with cells at high concentrations (10–100 mm), cyclodextrins caused a net efflux of cholesterol from the cells. In addition, studies from this laboratory have demonstrated that cyclodextrins can be complexed with various concentrations of cholesterol and, when incubated with cells, these complexes can deliver sterols to cells and, thus, increase net cholesterol content (26). *2*) At lower concentrations (<1 mm), cyclodextrins can be used as cholesterol shuttles that have the ability to catalyze the exchange of cholesterol from the cell membrane to serum lipoproteins (7). It is this ability to catalyze free cholesterol exchange between cells and lipoproteins that provides the greatest potential for the use of cyclodextrins as agents to modify reverse cholesterol transport. Used in vivo as extracellular cholesterol shuttles, cyclodextrins would be administered at low concentrations to speed the exchange of cholesterol between cellular and lipoprotein compartments without changing the concentration gradients driving net cholesterol flux. The ideal in vivo cholesterol shuttle would have a high efficiency for acquiring cell membrane cholesterol and have the ability to readily transfer that cholesterol to lipoproteins. As pharmacological agents, the ideal compounds would be absorbed via the intestine, have a reasonable half-life in the plasma and interstitial compartments, and be non-toxic.

The structural characteristics of cyclodextrins that would determine their cholesterol-shuttling efficiency are not known. The number of compounds that have been examined is very limited. Of the compounds that have been studied, MβCD was shown to be more effective than 2OHpβCD, and sulfated-cyclodextrins were found to be completely inactive (7). In the present investigation, we screened a number of new cyclodextrins for their cholesterol-shuttling capacity. In addition, we examined two double-decker cyclophanes, compounds that have been specifically designed to have internal dimensions to accommodate a cholesterol molecule and to serve as a "cage for cholesterol" (21).

A variety of different properties can determine the efficiency of potential cholesterol shuttles. For example, the ability of the cyclodextrin derivative to serve as an appropriate host molecule to encapsulate cholesterol is an important determinant for its shuttle capacity. We have demonstrated that the ability of MβCD and of 2OHpβCD to solubilize cholesterol was variable. We found that the maximum cholesterol solubilizing value for each was related to the concentration of the cyclodextrin in the medium (26). For example, a 25 mm solution of MβCD yielded a saturated MβCD:cholesterol molar ratio of 5:1. If this solution was diluted to 5 mm, the complex shed cholesterol and a new ratio was established at 8:1. In order to determine whether any relationship existed between the shuttling capacity of the cyclodextrin derivatives or cyclophanes and their ability to solubilize cholesterol, we determined

the saturated molar ratio of a 5 mm solution of each cyclodextrin derivative and cyclophane to [^{3}H]cholesterol. It should be noted that an assumption of this assay was that all of the cyclodextrins were completely soluble at this 5 mm concentration. Therefore, the observation that a particular cyclodextrin yielded high molar ratios could be due to either an inability to incorporate the sterol or the inability of the cyclodextrin derivatives themselves to be solubilized under our experimental conditions. In either case, such cyclodextrins would not be useful for the shuttling of cholesterol between cells and lipoproteins. We found that the cholesterol-solubilizing ability of the test compounds differed considerably. The two cyclophanes were the most efficient shuttles and also exhibited low molar ratios; however, there was no direct correlation between the shuttling capacity and the cholesterol-solubilizing ability of the other cyclodextrin derivatives. Thus, a simple determination of the capacity of a cyclodextrin derivative to solubilize cholesterol is not sufficient to allow a prediction of the efficiency of the compound to act as a cholesterol shuttle.

As discussed above, two properties of a cholesterol acceptor that would influence shuttling efficiency are solubility and sterol complexing ability. Other properties that would be important are size, charge, and specificity for the cholesterol molecule. Size and charge could affect the interaction of the acceptor with the cell membrane by influencing the access of the acceptor to specific membrane domains such as caveolae (35, 36). In addition, a charge on the acceptor could result in high affinity binding of the acceptor to the membrane, and such binding would limit its capacity to shuttle membrane cholesterol. In the present study, a number of charged β-cyclodextrin derivatives were examined for their shuttling capacity with none of them exhibiting any significant effect (data not shown). It is important to note that these charged compounds would be expected to have high water solubility because they contain multiple quaternary groups. Therefore, their low cholesterol-shuttling ability is most likely due to an inability to effectively complex with cholesterol. Finally, an effective shuttle must be able not only to complex with cholesterol, but also easily release that cholesterol to the "sink," in this case, serum lipoproteins. Acceptors with an optimal size for accommodating cholesterol in a tight host/guest complex could serve as effective vehicles for the stimulation of cholesterol efflux but may not be effective in catalyzing the exchange of cholesterol between cell and serum compartments.

The ability of β-cyclodextrins to incorporate lipids other than sterols is limited (37), and the modifications of the cyclodextrin molecule that were performed in this study were directed to the external face of the molecule, and would not be expected to change the dimensions of the hydrophobic core which is the primary determinant for lipid solubilization. Thus, it is unlikely that the differences between cyclodextrins can be attributed to selective competition by other lipids present in serum. At this time there is no information to establish whether there is some selectivity in the cyclodextrin-mediated exchange of cholesterol between cells and serum lipoproteins. Preliminary experiments have established that some of the cell cholesterol is recovered in the medium as cholesteryl ester, a product of the lecithin:cholesterol acyltransferase reaction. However, the kinetics of this esterification have not been determined. In addition, we have observed cyclodextrin-mediated exchange of cholesterol upon incubation of cells with either HDL or LDL (G. H. Rothblat, unpublished data), but we do not know if cell cholesterol is initially preferentially transferred to one type of lipoprotein upon incubation with serum.

In summary, the present study describes methods that were used to determine the ability of a number of β-cyclodextrin derivatives and two double-decker cyclophane compounds to catalyze the exchange of cholesterol between cells and lipoproteins. Our results clearly demonstrate that the double-decker cyclophanes were the most effective cholesterol shuttles. Under a standardized set of experimental conditions the cyclophanes were at least 3-fold more efficient than MβCD (Figs. 4 and 5). None of the synthetic β-cyclodextrin derivatives proved to be more effective than the MβCD. However, as demonstrated, the increased ability of the double-decker cyclophanes over MβCD to effectively catalyze the exchange of labeled cholesterol from cells to serum lipoproteins suggests that it will be possible to develop artificial cholesterol shuttles that may be pharmacologically relevant agents for the stimulation of reverse cholesterol transport.

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