Abstract  

Eight analogs of cholesterol (1) containing a benzophenone group have been synthesized as prospective photoaffinity labels for studies in cellular sterol efflux and HDL formation. Six of these compounds (4–9) have the photophore replacing different portions of the cholesterol alkyl side chain, and two (10 and 11) have it attached via nitrogen at carbon 3. The suitability of these analogs as cholesterol surrogates was determined by examining their ability to replace \(^{3}H\) cholesterol (FC) in fibroblasts preequilibrated with \(^{3}H\) cholesterol. All eight analogs were effective in replacing natural 1 \(^{3}H\) cholesterol in competition with \(^{3}H\) cholesterol for apolipoprotein A-I-induced efflux. These are the first compounds shown to replace cholesterol successfully in a complex pathway of multiple intracellular steps. The results suggest an unexpected tolerance of biological membranes regarding the incorporation of sterols of differing chemical structure.—Spencer, T. A., P. Wang, D. Li, J. S. Russel, D. H. Blank, J. Huuskonen, P. E. Fielding, and C. J. Fielding. Benzophenone-containing cholesterol surrogates: synthesis and biological evaluation. J. Lipid Res. 2004. 45: 1510–1518.

Supplementary key words  

cholesterol • cellular sterol efflux • apolipoprotein A-I • photoactivable sterols • photoaffinity labeling

As part of a study of cellular cholesterol efflux and HDL formation, we are investigating how cholesterol (free cholesterol (FC)), interacts with the proteins caveolin and apolipoprotein A-I (apoA-I) in these processes. Photoactivable analogs of FC could provide information about transient complexes between FC and these proteins by forming permanent covalent links that can serve to identify their binding sites (1–3). Recently, two analogs of FC (2 and 3, Fig. 1) containing diazirine groups have been prepared (4, 5) and used in photolabeling experiments (4–8). Analog 2 ("photocholesterol") has been used in a variety of biochemical media to photolabel synaptophysin (4), proteolipid protein (6), and proteins in Caenorhabditis elegans (7). Evidence has been presented that 2 can serve as a substitute for FC in model membranes (9). Analog 3 and its linoleate ester were shown in Chinese hamster ovary cells to effect labeling of several proteins, including caveolin (5).

In the present work, the benzophenone group was chosen as photophore because it offers several distinct advantages (1, 10–12). It is readily activated, like the diazirine group, by light at a wavelength (ca. 350 nm) that is relatively nondestructive to proteins. It is hydrophobic and, therefore, well suited for incorporation into most portions of the cholesterol structure. It is very stable and will survive intact and be traceable through complex cellular processes. And, finally, unlike diazirines, benzophenones are efficient and selective for labeling amino acid \(\alpha\) carbon atoms.

This paper describes the synthesis of four pairs of analogs of FC containing benzophenone groups (compounds 4–11, Fig. 2A). In three of these pairs (4–9) the benzophenone moiety extends, or replaces most of, the sterol alkyl side chain; in the fourth pair (10, 11), the photophore is attached at carbon 3 (C3) via an amide linkage. The schematic of Fig. 2B shows the approximate area that the incorporated photophore occupies in each of the eight analogs, giving an idea of the extension at either end of the sterol tetracycle that exists in the cases of 4–9 or 10 and 11.

One of these analogs, compound 4, designated FC benzophenone (FCBP), has already been used to substitute successfully for 47% of cellular FC without perturbing smooth muscle cell function, to photolabel caveolin effectively, and to obtain evidence that FC transferred to apoA-I was mainly derived from caveolin-rich domains.

Abbreviations: apoA-I, apolipoprotein A-I; C3, carbon 3; DEAD, diethyl azodicarboxylate; DMF, dimethylformamide; EI-HRMS, electron-impact high resolution mass spectrometry; FC, free cholesterol; FCBP, FC benzophenone; THF, Tetrahydrofuran.

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(13). In the present work, isotope dilution was used to test the ability of each of the analogs 4–11 to substitute for FC in cells labeled with $^{3}H$FC, which is known to equilibrate completely with cellular FC pools (14). Cellular phospholipids and FC are transferred from $^{3}H$FC-labeled cultured fibroblast monolayers or other peripheral cells to the extracellular medium in a process facilitated by the presence of apoA-I. If an unlabeled analog of FC is introduced into such cells, successful substitution for FC would lead to proportional dilution of the label and reduction of the radioactivity returned to the medium by efflux. Those photoactivatable analogs of FC that meet this demanding standard could provide a novel approach to determining the organization of lipids and proteins in the membranes of living cells.

**MATERIALS AND METHODS**

**Chemical synthesis**

*General methods.* $^1H$ and $^{13}C$ NMR spectra, unless otherwise noted, were taken in CDCl$_3$ at 300 MHz and 75 MHz, respectively. The chemical shifts are reported in units of $^1$H and $^{13}$C NMR spectra, unless otherwise noted. Tetrahydrofuran (THF) and ether were distilled from sodium/benzophenone. Acetone was distilled onto 4 Å molecular sieves. Benzene was dried over 4 Å molecular sieves for 6 h, then distilled from calcium hydride. Dimethylformamide (DMF) and pyridine were distilled from calcium hydride onto 3 Å molecular sieves. Methanol was distilled from calcium hydride. Acetic acid was distilled from calcium hydride onto 3 Å molecular sieves. CH$_2Cl_2$ was distilled from acetic anhydride. All reactions were magnetically stirred. All reagents, unless otherwise noted, were obtained from Aldrich Chemical Co.

3a,5-Cyclo-22-(p-benzophenony)-5a-23,24-bisnorcholan-6β-ol 6-methyl ether(16). In Procedure A, according to modification of a procedure by Marecak et al. (15), a solution of 221 mg (0.64 mmol) of 12 in 1 ml of THF was added dropwise to a solution of 190 mg (0.96 mmol) of 14 and 201 mg (0.77 mmol) of triphenylphosphine in 1 ml of THF with stirring at room temperature (rt) under nitrogen. This solution was cooled to 0°C, treated with 134 mg (0.77 mmol) of diethyl azodicarboxylate (DEAD), warmed to rt over 2 h, and stirred for an additional 22 h. The THF was evaporated and, according to modification of a procedure by Mitsunobu (16), the residue was treated with 3 × 10 ml of ether and filtered. The ether was evaporated and the residue (232 mg) was chromatographed on silica gel (1:19; EtOAc:hexane) to afford 145 mg (68%) of colorless

![Fig. 1. The structures of cholesterol and two recently reported photoactivatable analogs are shown.](image)

and stirred for an additional 22 h. The THF was evaporated and, according to modification of a procedure by Mitsunobu (16), the residue was treated with 3 × 10 ml of ether and filtered. The ether was evaporated and the residue (232 mg) was chromatographed on silica gel (1:19; EtOAc:hexane) to afford 145 mg (68%) of colorless 4-

![Fig. 2. A: The structures of the eight benzophenone-containing cholesterol analogs described in this paper. B: Schematic depiction of the elongating effect on the cholesterol structure when converted to compounds 4–9 or 10 and 11.](image)
1H NMR (500 MHz) 7.82–7.70 (m, 4H), 7.47–7.40 (m, 2H), 7.34–7.26 (m, 3H), 7.12–7.07 (m, 1H), 3.97–3.92 (dd, J = 9.0, 3.5 Hz, 1H), 1.74–1.67 (dd, J = 9.0, 7.0 Hz, 1H), 3.54–3.46 (4H), 2.30 (m, 2H), 2.06–0.38 (m, 20H), 1.12 (dd, J = 6.6 Hz, 3H), 1.00 (s, 3H), 0.72 (s, 3H); 31C NMR 196.7, 159.6, 139.0, 137.8, 132.5, 132.0, 129.3, 128.4, 122.7, 119.5, 115.2, 82.3, 75.4, 56.7, 54.3, 53.0, 48.2, 43.5, 41.3, 40.3, 36.6, 35.4, 35.2, 33.5, 30.7, 28.1, 25.1, 24.4, 22.9, 21.6, 19.5, 17.6, 13.3, 12.5. EI-HRMS (M+1) calcd for C36H52O3: 526.3474. Found: 526.3443.

22-(b-Methylphenoxy)-23,24-bisnorcholestan-6β-ol-6-methyl ether (17). In Procedure A for 17, as in Procedure A for the preparation of 16, 400 mg (1.16 mmol) of 12 and 345 mg (1.74 mmol) of 15 gave 265 mg (43%) of yellow oily 17 containing trace amounts of reduced DEAD, as judged by 1H NMR.

In Procedure B for 17, similar to Procedure B for the preparation of 16, a solution of 456 mg (2.30 mmol) of 15, 74 mg (0.23 mmol) of tetra-n-butylammonium bromide, and 92 mg (2.30 mmol) of NaN3 in 5 ml of CH2Cl2, and the resulting mixture was heated at 40°C for 5 days. The aqueous layer was extracted with CH2Cl2, and the combined extracts were washed with brine, dried, filtered, and evaporated to afford 380 mg of residue that was chromatographed (1:30: EtOAc:n-hexane) to afford 287 mg (83%) of colorless oil 17. 1H NMR 7.81–7.76 (m, 2H), 7.59–7.53 (m, 1H), 7.49–7.42 (m, 2H), 7.36–7.26 (m, 3H), 7.12–7.06 (m, 1H), 3.96–3.90 (dd, J = 9.0, 5.0 Hz, 1H), 3.74–3.66 (dd, J = 9.0, 7.0 Hz, 1H), 3.58–3.46 (m, 3H), 2.30 (m, 2H), 2.06–0.88 (m, 20H), 1.12 (dd, J = 6.6 Hz, 3H), 1.00 (s, 3H), 0.72 (s, 3H); 31C NMR 196.7, 159.6, 141.0, 139.0, 137.9, 132.6, 132.0, 129.3, 128.5, 128.1, 118.0, 116.9, 115.2, 82.3, 75.4, 56.7, 54.3, 53.0, 48.2, 43.5, 41.3, 40.3, 36.6, 35.4, 35.2, 33.5, 30.7, 28.1, 25.1, 24.4, 22.9, 21.6, 19.5, 17.6, 13.3, 12.5. EI-HRMS (M+1) calcd for C36H52O3: 526.3474. Found: 526.3443.

22-(b-Methylphenoxy)-23,24-bisnorcholestan-6-one-3β-ol (5). As in the preparation of 4, 165 mg (0.31 mmol) of 17 gave 138 mg (86%) of colorless 5: mp 87–90°C. Recrystallization from 2:1 pentane:acetone gave 60 mg of 5: mp 104.2–106.6°C; 1H NMR 7.81–7.76 (m, 2H), 7.60–7.55 (m, 1H), 7.48–7.42 (m, 2H), 7.35–7.26 (m, 3H), 7.12–7.07 (m, 1H), 5.35 (br. 1H), 3.97–3.92 (dd, J = 9.0, 3.5 Hz, 1H), 3.73–3.68 (dd, J = 9.0, 7.0 Hz, 1H), 3.53 (br. 4H), 2.30 (m, 2H), 2.06–0.88 (m, 20H), 1.12 (dd, J = 6.6 Hz, 3H), 1.00 (s, 3H), 0.72 (s, 3H); 31C NMR 196.9, 159.6, 141.0, 139.0, 137.9, 132.6, 132.0, 129.3, 128.5, 128.1, 118.0, 116.9, 115.2, 82.3, 75.4, 56.7, 54.3, 53.0, 48.2, 43.5, 41.3, 40.3, 36.6, 35.4, 35.2, 33.5, 30.7, 28.1, 25.1, 24.4, 22.9, 21.6, 19.5, 17.6, 13.3, 12.5. EI-HRMS (M+1) calcd for C36H52O3: 526.3474. Found: 526.3443.

(25R)-26-(b-Benzoylphenoxy)-cholest-5-ene-3β,26-diol (18), mp 110°C (lit. 112°C). To a solution of 101 mg (0.31 mmol) of 17 containing 1% of potassium carbonate in water, was added 3 ml of 60% EtOAc, and 4 Å molecular sieves were added, and the resulting mixture was heated at 60°C for a further 1 h. The solvent was removed by distillation, and the residue was chromatographed (1:1:200; EtOAc:n-hexane:petroleum ether) to give 613 mg (86%) of colorless 18. EI-HRMS (M+1) calcd for C39H56O4: 568.3965. Found: 568.3965.
3β-Butyldimethylsilyloxy-17β-(3-benzylbenzoyloxy)androst-5-ene (23). As in the preparation of 22, 995 mg (2.46 mmol) of 19 and 1.35 g (4.92 mmol) of 21, mp 66–68°C, prepared according to the procedure of Zhao et al. (21), gave 4.57 g of residue, which was chromatographed (1:30–1:20; EtOAc:hexane) to give 1.93 g of 23 as a white solid that was used directly in the next step. Puriﬁcation by similar chromatography gave 23 mp 73.2–74.8°C; 1H NMR (500 MHz) 7.85 (m, 3H), 7.71 (m, 1H), 7.62 (m, 2H), 7.52 (m, 3H), 5.33 (m, 1H), 4.62 (s, 2H), 3.47 (m, 2H), 2.29 (m, 1H), 2.20 (m, 1H), 2.0–1.72 (m, 4H), 1.62–1.27 (m, 8H), 1.18–0.87 (m, 5H), 1.03 (s, 3H), 0.85 (s, 3H), 0.08 (s, 6H); 13C NMR 197.0, 141.1, 140.0, 137.9, 137.8, 132.6, 131.5, 130.3, 129.4, 129.0, 128.5, 128.5, 128.5, 128.1, 127.2, 122.5, 56.4, 50.7, 50.3, 42.5, 40.2, 39.8, 39.5, 39.1, 36.8, 36.4, 36.1, 32.1, 29.4, 28.5, 28.3, 24.5, 24.1, 23.1, 22.8, 21.2, 19.6, 19.0, 12.1. Anal. calcd for C39H54SiO3·H2O: C, 82.72; H, 9.33; N, 2.36. Found: C, 82.71; H, 9.35; N, 2.35.

3β-Hydroxy-17β-(3-benzylbenzoyloxy)-androst-5-ene (9). As in the preparation of 8, 1.93 g of 23 afforded 6.1 g of residue, which was chromatographed (1:4–1:1; EtOAc:hexane) to give 782 mg (66%) of colorless azide: mp 85–86.5°C.

3a,5-Cyclo-6β-methoxy-3α-cholestan-10-ol 6-methyl ether (28). A solution of 680 mg (1.9 mmol) of 3,5-dibromo-4-hydroxybenzophenone (27), mp 155–156°C, prepared according to the method of Blakey, Jones, and Scarborough (30), and 61 mg (0.19 mmol) of tetrabutylammonium bromide in 2 ml of 2 M NaOH was treated with 365 mg (0.80 mmol) of 13 in 5 ml of EtOAc and heated at reﬂux for 4 days. The aqueous layer was removed and the EtOAc layer was washed with 2 × 10 ml of water, dried, ﬁltered, and evaporated to afford 1.1 g of residue, which was evaporated to dryness and precipitated with 3 ml of 1:2 EtOAc:hexane to precipitate essentially identical with those for 27 that was collected by ﬁltration. The ﬁltrate was evaporated and the residue (350 mg) was chromatographed (1:4; EtOAc:hexane) to afford 442 mg (80%) of colorless, waxy mass: mp: soften 72–76°C; 1H NMR (500 MHz) 7.79 (s, 2H), 7.70 (m, 2H), 7.67 (m, 1H), 7.54 (m, 2H), 4.03 (dd, J = 9.0, 3.5 Hz), 1H), 3.88 (m, 1H), 3.73 (s, 3H), 2.88 (br, 1H), 2.11–1.12 (m, 16H), 1.35 (J = 6.6 Hz, 3H), 1.08 (s, 3H), 0.95–0.89 (m, 4H), 0.85 (s, 3H), 0.70 (m, 1H), 0.48 (m, 1H); 13C NMR 193.4, 157.3, 136.8, 135.4, 134.7, 133.1, 130.1, 128.8, 118.7, 82.6, 78.8, 56.8, 56.5, 52.8, 48.2, 43.3, 43.3, 40.3, 38.1, 35.4, 33.3, 33.6, 30.8, 28.3, 25.2, 24.6, 23.0, 21.6, 19.5, 17.6, 13.3, 12.5. EI-HRMS (M+·) calcd for C22H45BrO2·Br: 682.1657. Found: 681.1650. Anal. calcd for C22H45Br2O2·H2O: C, 61.55; H, 6.60. Found: C, 61.27; H, 6.74.

Reduction of 28 over 20% Pd(OH)2 on carbon. According to a modiﬁcation of a procedure by Dorman et al. (31), a solution of 38 mg (0.056 mmol) of 28 and 50 μl (0.36 mmol) of triethylamine in 5 ml of EtOAc was added to 38 mg of 20% Pd(OH)2 on carbon (Degussa type). The reaction mixture was ﬂushed 3X with H2 and stirred under a balloon of H2 at rt. Hydrodebromination went to completion within 5 min as indicated by tlc analysis (1:4; EtOAc:hexane), with only trace amounts of ketone reduction. Reduction of the ketone went nearly to completion within 25 min as indicated by tlc. The product was obtained from the 5 min reduction by ﬁltration through a pad of Celite, washing the ﬁltrate with saturated NH4Cl solution, water, and brine, drying, ﬁltering, and evaporating to afford 16 with 1H NMR and IR spectra essentially identical with those for 16 prepared using 14. The product similar obtained from the 25 min reduction was the alcohol formed by reduction of the carbonyl group: 1H NMR 7.38–7.21 (m, 7H), 6.84–6.76 (m, 2H), 5.79 and 5.75 (2s, 1H), 3.96–3.85 (s, 1H), 3.72–3.59 (m, 1H), 3.51 (s, 3H), 2.75 (br, 1H), 3.25.
Biochemical experiments

Human apoA-I was obtained from centrifugally isolated human HDL delipidated with ethanol and diethyl ether, and dissolved in 6 M urea, 0.01 M tri(hydroxymethyl)amino-methane–HCl buffer, pH 8.1. apoA-I, which was about 60% of the total protein in HDL, was purified by molecular sieved column chromatography over Sephadex G-150 in the same buffer, based on the procedure of Brewer, Ronan, and Bishop (32). Fractions containing apoA-I (>99%) were dialyzed against 1 mM Na-phosphate buffer, pH 8.1 (0.8–1 mg protein/ml), flash frozen, stored at −70°C, and thawed immediately before use.

[3H][1,2]FC (50–55 mCi/mmole) was from Perkin Elmer/NEN. FBS from the cell culture facility contained 65 μg ml−1 of FC, determined with cholesterol oxidase (33). Human skin fibroblasts were grown to near confluence in 10% v/v of FBS in DMEM (2.25 μg ml−1 FC) in 12-well plastic dishes (1 ml medium per well). Under these conditions, the cell FC content was 2.2 ± 0.2 μg FC per dish. For the final 48 h, 5 μCi of [3H]FC was included for each milliliter of medium for control incubations. In the other incubations, 5 μCi of [3H]FC per milliliter of medium was included, together with an amount of unlabeled FC or photoactivatable analog (one of 4–11) representing the molar equivalent of the sum of cellular and medium FC. These mixtures of labeled and unlabeled sterol were dissolved in DMSO and added with stirring to a final solvent concentration of 1–3% v/v. DMSO alone was added to control incubations.

To assay apoA-I-dependent efflux, the cells were washed (3×) with phosphate-buffered saline, then incubated overnight in DMEM containing 1 mg ml−1 of high molecular weight dextran (Pharmacia, T-500) as oncotic agent. The cells were then washed with the same medium and incubated (3 h) in the presence or absence of 10 μg ml−1 apoA-I. At the end of incubation, the medium was collected, centrifuged (5000 g, 10 min, 4°C) to pellet any unattached cells, and assayed for [3H]label. Efflux of FC was linear over this incubation period. apoA-I-dependent FC efflux was expressed as [efflux in the presence of apoA-I] − [efflux in the absence of apoA-I] under the same experimental conditions. Both total and apoA-I-dependent efflux were linear with time over the period of the assay. apoA-I-independent efflux was <20% of total efflux under the conditions of these experiments.

ABC1 mRNA levels in control fibroblasts, or fibroblasts equilibrated with FC or one of 4–11, were determined by RT-PCR as previously described (34). In brief, RNA was reverse transcribed with Superscript II enzyme. PCR was carried out with Taq polymerase (Qiagen) using SYBR Green as fluorophor. Glyceraldehyde 3-phosphate dehydrogenase mRNA was the control.

RESULTS

Synthesis

Compound 4, in which the benzophenone group is attached to C22 of a truncated cholesterol framework via an ether linkage, was prepared either by Mitsunobu coupling of the C22 alcohol 12 (17, 35, 36) with 4-hydroxybenzophenone (14) to give 43% yield of 16, or, preferably, by phase transfer catalyzed alkylation of C22 iodide 13 (17, 35) with 14 to afford 16 in 81% yield (Fig. 3). Acidic hydrolysis of steroid 16 gave 68% of 4. Isomeric analog 5 was prepared by the same two methods via coupling with 3-hydroxybenzophenone (15) to give 17 in 43% yield from 12 and in 83% yield from 13, followed by acidic hydrolysis to afford 86% of 5.

Analogs 6 and 7, having the benzophenone group at the end of the cholesterol side chain, were synthesized in 74% and 52% yield, respectively, by selective Mitsunobu reaction at the primary hydroxyl group of 27-hydroxysterol, which had been prepared by the method of Kim et al. (18), with 14 or 15, respectively. Analog 8 was prepared in 70% overall yield from the known (20) 3β-butyldimethylsilyl derivative 19 of androst-5-en-3β,17-diol (18) by Ag2O promoted alkylation (19) with 4-bromomethyl benzophenone (20), prepared by the method of Zhao et al. (21), to produce 22, followed by deprotection of the 3β-hydroxyl group (Fig. 4A). Analog 9 was comparably prepared in 66% overall yield from 19 and 3-bromomethylbenzophenone (21) via 23.

Fig. 3. The synthetic pathway to cholesterol analogs 4 and 5 via 16 and 17 and the pathway for synthesis of [3H]4 via 28 are shown.

Fig. 4. A: The synthetic pathway to cholesterol analogs 8 and 9. B: The synthetic pathway to cholesterol analogs 10 and 11.
We desired also to prepare cholesterol analogs having the benzophenone moiety at the opposite end of the structure from its location in 4–9. Because it was considered potentially important to retain the hydrogen bonding capability of the C5 hydroxyl group of cholesterol itself, amides 10 and 11 were selected as candidates of this type. For synthesis of 10 and 11, cholesterol was converted to 3-amino-cholest-5-ene (24) by modifications of procedures for reactions developed by Barton and Morgan (22) and Jarreau, Khung-Huu, and Goutarel (23), and this amine was acylated with the acid chlorides prepared in situ from benzophenone carboxylic acids 25, to give 59% of 10, and 26, to give 77% of 11 (Fig. 4B).

As described in the next section, all eight analogs show promise as biochemical surrogates for cholesterol. For the initial photoaffinity labeling experiments, it was decided to use the first analog prepared, FCBP (4) (13). In order to facilitate analysis of the photoaffinity labeling products and for use in additional types of experiments, isotopically-labeled FCBP was needed. A procedure for preparing tritiated 4 was developed via the dibromo analog 28 of intermediate 16. Compound 28 was prepared in 80% yield by use of the known (30) dibromohydroxybenzophenone 27 for alkylation by 13 (Fig. 3). Careful hydrogenation using Degussa Pd(OH)$_2$/C catalyst (31) effected debrumination to 16 without carbonyl group reduction, and this procedure, followed by acid hydrolysis of the s-teroid moiety, has been used with commercial catalytic tritiation to afford [3H]4. As noted above, FCBP (4) and [3H]FCBP have already proved to be excellent biochemical research tools (13).

**Cellular equilibration of sterols**

As described in Materials and Methods, the extracellular medium of cultured fibroblasts was enriched with [3H]FC alone, with the same level of [3H]FC + unlabeled FC in a mass equivalent to total FC in cells plus medium, or with [3H]FC + unlabeled photoactivable analog to the same molar concentration. The cells and medium were incubated at 37°C for 48 h to equilibrate sterol across the cell membrane. The labeled medium was removed and replaced by DME medium containing 1 mg ml$^{-1}$ of high molecular weight dextran as an oncotic agent, in the presence or absence of apoA-I (10 µg ml$^{-1}$). As shown in Fig. 5, there was approximately a 50% decrease in apoA-I-dependent medium radioactivity if the specific activity of [3H]FC was reduced by ca. 50% by inclusion of unlabeled FC in the culture medium. As also shown in Fig. 5, there was a similar decrease when each of the benzophenone-modified FC analogs replaced unlabeled FC.

Cells incubated with [3H]FC only had a sterol content of 2.2 ± 0.1 µg, while cells incubated with medium supplemented with unlabeled FC or analog had a sterol content of 2.1 ± 0.2 µg. These data indicate that equilibration with either FC or one of 4–11, under the conditions described in Materials and Methods, reflected sterol exchange and not simply sterol loading of the cells. To obtain further evidence that the results in Fig. 5 reflect sterol exchange rather than loading, the mRNA levels for the ABCA1 gene were measured for the cells incubated with sterol-supplemented medium. The ABCA1 gene, whose product mediates HDL genesis, is highly sensitive to sterol loading, and it has been reported (37, 38) that mRNA levels were upregulated 4- to 10-fold by a FC-albumin suspension that promotes FC uptake, but that FC efflux was not upregulated. In contrast, the mRNA levels in the cells incubated with FC or 4–11 supplemented medium in 10% FBS in the current investigation were essentially unchanged from control (Fig. 6). These data confirm the
conclusion that sterol analogs 4–11 were each effective in substituting for FC in the cellular pools contributing to apoA-I-dependent sterol efflux.

DISCUSSION

Photoactivable FC analogs capable of substituting successfully for FC in the membranes of living cells could provide novel data on membrane lipid-protein and lipid-lipid associations at the molecular level, including those complexes too transient or unstable to be isolated by classical fractionation techniques. FC plays several different roles in mammalian cells (39). It promotes the condensation of phospholipid acyl chains in synthetic or natural membrane bilayers and facilitates the separation of FC/sphingolipid-rich, liquid-ordered microdomains (lipid rafts) (40–42). In addition, single molecules of FC form stoichiometric complexes with recognition sites on sterol-binding proteins, including caveolin, Niemann-Pick type C2, steroidogenic acute regulatory, and the benzodiazepine receptor protein (13, 43–45). These recognition sites include both hydrophobic and positively charged amino acid residues and often conform to the motif V/L_{1–4}Y_{x1–4}R/K, where x is any amino acid (46). It seems likely that most sterol-dependent biological pathways include both bulk phase and monomolecular effects, so a successful FC analog must substitute for FC in both types of interaction. For this reason, we chose as the test for acceptability as an FC surrogate a complex pathway, apoA-I-dependent sterol efflux from recycling low density lipoprotein FC (47), the major source of free sterol in FBS (48). The initial uptake of FC depends mainly on the membrane properties of coated pits (49, 50). Intracellular recycling is vesicular, possibly dependent on both membrane properties and specific FC-protein interactions (51). The binding of intracellular FC to cell-surface caveolae depends on FC-caveolin binding (13). Successful replacement of FC in apoA-I-dependent sterol efflux thus represents a challenging standard for a prospective cholesterol surrogate.

In the present investigation, seven analogs of FC (compounds 5–11) have been prepared in addition to FCBP (4). Five of these, like FCBP, have the benzophenone moiety replacing or extending the ring D side chain, occupying together the approximate area relative to the sterol tetracyclic core shown schematically in Fig. 2B. The other two (10 and 11) have the photophore appended via an amide linkage to C3, extending the opposite end of the structure roughly by the area also shown in Fig. 2B, while maintaining the possibility of hydrogen bonding by the 3β-substituent. Each of these eight analogs was tested to see if it could compete with FC in apoA-I-induced cellular sterol efflux. The dilution of [3H]FC by analogs 4–11 was essentially the same in all cases as that by unlabeled FC (Fig. 5). These eight benzophenone-containing FC analogs are the first shown to replace FC successfully in the major pools involved in a complex pathway of multiple intracellular steps.

At first sight, these results appear to conflict with the high degree of specificity reported for sterol incorporation into biological membranes (52). Such physical specificity had been thought responsible for the normally low levels in mammalian plasma and tissues of plant sterols, which differ in structure from FC primarily in the alkyl side chain (53). However, the mechanism of this effect was recently shown to be mediated retroactively via the ABCG5/ABCG8 transporter complex (54, 55), which promotes the selective efflux of plant sterols from intestinal cell membranes. A number of recent observations support the idea that for incorporation into membranes, a sterol must have a sufficiently large and quite hydrophobic side chain, but that structural specificity is not required. Cholestaatrienol is an example of a sterol with the side chain of FC, but with an otherwise slightly modified structure, which can replace FC in membranes (56). It has been reported that tracer levels of 7,7-azocholestanol (3) mimic FC in its distribution between intracellular membrane compartments (5), and that 6,6-azocholestanol (2) is incorporated at high levels into model membranes (9). Sterols with modified side chains that act like FC in association with phospholipids include dehydroergosterol (57).

Stigmasterol, sitosterol, and a synthetic C10 side-chain analog, each also containing a somewhat larger side chain than FC, were all found to mimic FC in lipid raft formation (58). FCBP (4) is incorporated at high levels in human aortic smooth muscle cells, despite replacement of a major portion of the side chain by the likewise hydrophobic benzophenone moiety (13). In contrast, 22 and 25(7-nitrobenz-2-oxa-1,3-diazol-4-yl)cholesterol, with even larger and distinctly more polar side chains, are packed in membranes quite differently from FC (59). A synthetic analog lacking six carbons of the side chain (58) and several similarly truncated, variously substituted steroids (60) did not promote lipid raft formation. The success in the current work of analogs 4–9 as replacements for FC (1) is consistent with the requirement for a large hydrophobic side chain, which can be somewhat bigger than that in FC. However, even in the most extended side-chain analogs (6 and 7), the overall length, as judged from examination of Dreiding molecular models, is only ca. 2 Å longer than the fully extended C18 saturated fatty acid component of the phospholipid, with which it might compete for packing.

All natural sterols and all the synthetic analogs discussed above, with the exception of 10 and 11, retain the 3β-hydroxy group of FC. There has been general agreement that this functional group is important in the orientation of FC in biological membranes (59, 61). It was, therefore, of interest and somewhat unexpected to find that replacement of up to 50% of cellular FC with either 10 or 11, in which the 3β-hydroxyl group had been replaced with a large amidobenzophenone group, was without effect on the ability of human skin cells to efflux sterol to apoA-I. Although the amide linker had been selected specifically because it retains the hydrogen bond donor and receptor capabilities of the hydroxyl group, the data in Fig. 5 suggest that the 3β-hydroxyl group per se is not
essential for biological activity, at least as assayed by promotion of apoA-I-dependent sterol efflux. The polarity of the amide link is probably sufficient to maintain an “upright” orientation of the molecule in biological membranes (59), despite the attached benzophenone group. The precise positioning of this hydrophobic appendage is not revealed by the current investigation.

There is, in principle, no reason why a sterol analog that can replace FC in binding to proteins should also mimic FC in its condensing function in membranes. For example, 3β-doxylecholesterol displaces FC from intestinal proteins (62), but does not replace FC in synthetic membrane bilayers (59). The progesterin promegestone displaces FC from several membrane proteins, including the benzodiazepine receptor (45) and the HIV-1 nef protein (63), but does not condense membrane phospholipids or promote raft formation (60). In contrast, FCBP (4) mimics FC (1) in both protein binding and in the delivery of cellular sterol to apoA-I (13), and the present research shows that each of seven additional benzophenone derivatives can also substitute successfully in the major roles of sterol in the cell.

In summary, the present data suggest that a variety of cholesterol analogs containing benzophenone groups at either end of the sterol structure can serve as satisfactory substitutes for FC in studying lipid-protein and lipid-lipid complexes in living cells. Further studies are in progress to determine whether replacement of part of the sterol tetracyclic nucleus with a benzophenone moiety can also afford potentially useful cholesterol surrogates.

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