Structural specificity in the suppression of HMG-CoA reductase in human fibroblasts by intermediates in bile acid biosynthesis

Magnus Axelson,[1,*] Olle Larsson,[†] Jie Zhang,[**] Junichi Shoda,[**] and Jan Sjövall[**]

Departments of Clinical Chemistry* and Tumor Pathology,† Karolinska Hospital, and Department of Medical Biochemistry and Biophysics,** Karolinska Institutet, Stockholm, Sweden

Abstract The effect of bile acid precursors on the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase was investigated. Cholesterol and 34 of its derivatives, including 23 potential intermediates in bile acid biosynthesis, were incubated with cultures of human fibroblasts for 24 h in the absence or presence of lipoproteins, and the activity of HMG-CoA reductase was then determined. In the absence of lipoproteins, many of the bile acid intermediates were inhibitory at a high concentration (2.5 μM), while only three, 27-hydroxycholesterol, 7α,27-dihydroxy-4-cholesten-3-one, and 7α,12α,27-trihydroxy-4-cholesten-3-one, caused a significant suppression at lower concentrations (often >80% suppression at 0.25 μM). Even at 0.06 μM these sterols caused >50% suppression of the enzyme activity. In addition, 27-hydroxy-4-cholesten-3-one, not usually considered to be an intermediate in bile acid biosynthesis, was a very potent inhibitor. Comparative studies showed that the effect of the three bile acid precursors was similar to that of 25-hydroxy-, 24-hydroxy-, and 7-oxo-cholesterol and 3β-hydroxy-5α-cholest-8(14)-en-15-one. The presence of lipoproteins decreased or eliminated the inhibitory effect of most intermediates. Studies of the metabolism of the three most potent inhibitors in the fibroblasts indicated that the suppression was due to the compounds per se and not to products of their metabolism. The results show that a few specific intermediates in the formation of bile acids are potent suppressors of HMG-CoA reductase. Alternative biosynthetic pathways to bile acids may then have different regulatory roles in cholesterol biosynthesis, depending on the biological activities of the intermediates involved.—**Axelson, M., O. Larsson, J. Zhang, J. Shoda, and J. Sjövall.** Structural specificity in the suppression of HMG-CoA reductase in human fibroblasts by intermediates in bile acid biosynthesis. *J. Lipid Res.* 1995. **36:** 290–298.

Supplementary key words oxysterols • bile acid precursors • cholesterol synthesis • cholesterol metabolism

Bile acids are the major metabolites of cholesterol in humans (1, 2). Cholic and chenodeoxycholic acids can be formed via alternative pathways involving a large number of oxygenated steroid intermediates (1–4). Oxygenated sterols with structures resembling those of such intermediates are potent suppressors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (5, 6), the rate-limiting enzyme of cholesterol biosynthesis. In contrast to many of these oxysterols, intermediates in bile acid synthesis are formed in the liver (1–4, 7) and are also present in the blood (8–10). As a possible regulatory function of these intermediates has not previously been investigated, we have made a comprehensive screening of their effects on the activity of HMG-CoA reductase. Human diploid fibroblasts were used as test system (11) in order to limit enzymatic conversions of the intermediates that would be extensively metabolized in liver cells. It was also considered important to use human cells due to possible species differences in cholesterol regulation. The results show that intermediates containing a 27-hydroxy group and a 3-oxo-4-ene structure are potent suppressors whereas other compounds, with the exception of 27-hydroxycholesterol, have less or no inhibitory effect at physiological levels.

MATERIALS AND METHODS

Steroids

Common C_{24} bile acids, 3β-hydroxy-5-cholenoic acid and 25-hydroxy-, 24-hydroxy-, and 7-oxo-cholesterol were from Steraloids Inc. (Wilton, NH). The following compounds were kind gifts from colleagues: 3β-hydroxy-5α-cholest-8(14)-en-15-one from Prof. G. Schroepfer, Houston, Texas; 7α-hydroxy-4-cholesten-3-one and 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) from Prof. I. Björkhem, Huddinge Sweden; 5β-cholestane-3α,7α-diol, 7α,12α-dihydroxy-4-cholesten-3-one and 5β-cholestane-3α,7α,12α-triol from Dr. K. Wikvall, Uppsala, Sweden; 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) from Prof. T. Hoshita, Hiroshima, Japan, and

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; DHCA, 3α,7α-dihydroxy-5β-cholestanoic acid; THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; ODS, octadecylsilane; GC/MS, gas–liquid chromatography–mass spectrometry.

[1]To whom correspondence should be addressed.

kryptogenin (3β,27-dihydroxy-5-cholestene-16,22-dione) from Dr. L. Tökés, Syntex Research, Palo Alto, CA. Kryptogenin was used as the starting material for the synthesis of cholest-5-ene-3β,27-diol (27-hydroxycholesterol), cholest-5-ene-3β,7α,27-triol (7α,27-dihydroxycholesterol), 7α,27-dihydroxy-4-cholesten-3-one, 3β-hydroxy-5-cholestenoic acid, 3β,7α-dihydroxy-5-cholestenoic acid, and 7α-hydroxy-3-oxo-4-cholestenoic acid as described previously (12). 3β,7α-Dihydroxy-5-cholenoic acid and 7α-hydroxy-3-oxo-4-cholenoic acid were prepared by oxidation of 3β-hydroxy-5-cholenoic acid (12). 27-Hydroxy-4-cholesten-3-one, 3-oxo-4-cholestenoic acid, and 3-oxo-4-cholenoic acid were prepared by oxidation of the corresponding 3β-hydroxy-Δ^5 steroids with cholesterol oxidase (12). 5β-Cholestane-3α,7α,27-triol and 7α,27-dihydroxy-5β-cholestan-3-one were prepared from DHCA essentially as described previously (7). 5β-Cholestane-3α,7α,12α,27-tetrol, 7α,12α,27-trihydroxy-4-cholesten-3-one, and 7α,12α-dihydroxy-3-oxo-4-cholestenoic acid were all synthesized from THCA and 7α,12α-dihydroxy-3-oxo-4-cholenoic acid from cholic acid (7).

$^2H_7$-labeled versions of the side-chain oxygenated bile acid precursors (without a 12α-hydroxy group) were also prepared (12) and used as internal standards in the metabolism studies.

Cell culture conditions

Low-passage (passage 2–6) human diploid fibroblasts, line GM08333, obtained from NIGMS, Coriell Institute for Medical Research, Camden, NJ, were grown in monolayers in tissue culture flasks maintained in a 95% air/5% CO_2 atmosphere at 37°C in a humidified incubator. Cells were cultured in Modified Eagle's Medium supplemented with nonessential amino acids and 10% (v/v) fetal calf serum.

For experimental purposes, cells were cultured in dishes. Cells were seeded at a density of 5,000 cells per cm². The experiments were started 48–72 h later, at which time a cell density of approximately 20,000 per cm² had been reached.

Steroids were tested at concentrations 0.06–2.5 μM, and were added in an ethanol solution to the cell media containing 10% fetal calf serum, with or without lipoproteins. The ethanol concentration was 22 mM in all incubations. In the latter case, lipoproteins had been removed from the serum by adsorption to Cab-O-Sil (Eastman Kodak Co., Rochester, NY) as described by Weinstein (13). The concentrations of total cholesterol in the serum before and after treatment with Cab-O-Sil were 1.13 and 0.1 mM, respectively. Cell growth was not affected by the treatment with Cab-O-Sil (cf. ref. 13). Incubations were carried out in duplicate for 1–24 h. Control cells were incubated in the same way but without steroids. Each steroid was tested in at least two separate experiments at 0.25 μM, and the most potent steroids were tested more than five times. For metabolism studies, 25–63 nmol of the steroid substrates was incubated with the fibroblasts (cell number 4–6 × 10^6) in 10–25 ml medium (concentration of steroid was 2.5 μM) without lipoproteins for 72 h.

Analytical procedures

Determination of HMG-CoA reductase activity. Cells in 50-mm dishes were, after the indicated treatments, rinsed twice in phosphate-buffered saline and harvested for assay of cellular HMG-CoA reductase activity as described elsewhere (14). In brief, cell lysates were incubated in 200 mM potassium phosphate, 20 mM dithiothreitol, 40 mM glucose 6-phosphate, 5 mM NADPH, and 5 units/ml of glucose-6-phosphate dehydrogenase. After a 15-min preincubation at 37°C, 0.9 nmol [^{14}C]HMG-CoA (57 mCi/mmol) and unlabeled HMG-CoA (the final concentration of HMG-CoA was 100 μM) were added for a 60-min incubation at 37°C. The final reaction volume for each sample was 60 μl. The reaction was stopped by addition of 5 μl 5 M HCl which also allowed lactonization of the produced [^{14}C]mevalonate. After addition of known amounts of [^{3}H]mevalonolactone (internal standard) one aliquot was used to separate [^{14}C]mevalonolactone from [^{14}C]HMG-CoA by ion-exchange chromatography according to the description of Edwards, Lemongello, and Fogelman (15), and one aliquot was used for spectrophotometric determination of protein content. The ^{3}H and ^{14}C radioactivity was analyzed by a scintillation counter that was equipped with a program for automatic correction for quenching and spill-over (Beckman LS 5000 TA). The lower limit of determination was usually about 1 pmol/min per mg protein.

Analysis of steroid metabolites. To the incubation medium (10 ml) was added a mixture of $^2H_7$-labeled internal standards (about 0.3–1.0 nmol of each) dissolved in 0.5 ml of methanol, and the medium was then diluted with 10 ml of 0.5 M aqueous triethylamine sulphate. Steroids were then extracted on a column (1.5 × 0.8 cm) of octadecylsilane (ODS)-bonded silica (Preparative C_{18}; particle size 55–105 μM, Waters Associates Inc., Milford, MA) in water at 64°C (16). The column was then washed with 10 ml of water and 5 ml of 10% aqueous methanol prior to elution of steroids with two 10-ml portions (A and B) of 85% aqueous methanol (7). Group separation of neutral steroids and bile acids was achieved by chromatography on a column (6 × 0.4 cm) of the lipophilic anion exchanger, triethyl-aminohydroxypropyl-Sephadex LH-20 in HCO_3^--form packed in 85% aqueous methanol (7). The two fractions collected from the ODS-bonded silica column were passed through the anion exchanger in the order of B and A, followed by a rinse with 5 ml of methanol. The combined eluate contained the neutral steroids. Unconjugated bile acids were then eluted with 4 ml of 0.15 M acetic acid in 95% aqueous methanol and this fraction was taken to dryness prior to derivatization.

The neutral steroid fraction was taken to dryness and redissolved in 2 ml of hexane–dichloromethane 1:4 (v/v) and was further purified by chromatography on a column (3 × 0.4 cm) of Unisil (Clarkson Chemical Company, Williamsport, PA) (17) packed in hexane and washed with 5 ml of hexane–dichloromethane 1:4 (v/v) prior to use. After application of the sample, the column was washed with 10 ml of the same solvent and the steroids were then eluted with 10 ml of ethyl acetate. This fraction was taken to dryness and derivatized.

The flasks with the cell layers were treated with 3 × 12 ml of 40% aqueous ethanol and finally 5 ml of ethanol in order to release the cells and extract steroids from the culture flask. The cells and the ethanolic solutions were then transferred to tubes and held in an ultrasonic bath for 15 min. After centrifugation, the supernatants were combined. The cells were then treated a second time with ethanol (5 ml) in the ultrasonic bath and after centrifugation all supernatants were combined. To this solution was added $^2H_7$-labeled steroid internal standards (usually between 0.5–1.0 nmol of each) in methanol and the mixture was taken to dryness in vacuo. The residue was redissolved in 3 ml of methanol and 2 ml of water was added. The sample was passed through a column (1.5 × 0.8 cm) of ODS-bonded silica in water. After a rinse with 5 ml of water, the alcohol in the combined eluates was removed in vacuo and the remaining aqueous solution was reextracted on the same column. The column was then washed with 10 ml of water and 5 ml of 10% aqueous methanol. Neutral steroids and bile acids were then eluted with two 10-ml portions of 85% aqueous methanol (fractions A and B). These two fractions were then treated as described for those obtained from the incubation medium.

Prior to derivatization, triacontane (about 0.7 nmol) was added to the fraction. Bile acids were methylated with freshly prepared diazomethane at 0°C in 2 ml of methanol–diethyl ether 1:9 (v/v). Trimethylsilyl ethers were prepared by addition of about 0.1 ml of pyridine–hexamethyldisilazane–trimethylchlorosilane 3:2:1 (by vol.), and heating at 60°C for 30 min. The reagents were removed under a stream of nitrogen and the derivatives were redissolved in hexane (9).

Gas–liquid chromatography–mass spectrometry (GC/MS) was carried out using a Finnigan 1020 instrument housing a fused-silica column (30 m × 0.32 mm) coated with a 0.25 μm layer of SE-30 (DB-1) (J & W Scientific Inc., Rancho, Cordova, CA) ending in the ion source. An on-column injection device was used. The oven temperature was about 50°C during the injection and, after 6 min, was rapidly increased to 190°C and then programmed from 190 to 285°C and the electron energy was 40 eV. Repetitive scanning (20 scans × min^{-1}) over the *m/z* range 50–800 was started after a suitable delay (7).

The identification of a steroid was based on the retention time and the complete mass spectrum which were compared with those of the authentic steroid. The amounts of identified steroids were calculated from fragment ion current chromatograms by comparing the peak areas given by the steroid metabolites with those given by the corresponding $^2H_7$-labeled internal standards (18).

RESULTS

Twenty-three potential intermediates in bile acid biosynthesis were tested for their effects on the activity of HMG-CoA reductase in human fibroblast cultures. For comparison, cholesterol, cholic, chenodeoxycholic, deoxycholic, and lithocholic acids as well as known inhibitory oxysterols, i.e., 24-hydroxy-, 25-hydroxy-, and 7-oxo-cholesterol (5) and 3β-hydroxy-5α-cholest-8(14)-en-15-one (6), were also tested. In the first part of the study, different incubation conditions were examined. For this purpose, precursors of chenodeoxycholic acid were used, as they include 27-hydroxycholesterol, which has previously been shown to be a potent inhibitor of HMG-CoA reductase (19). The effects of these compounds at a concentration of 2.5 μM in the absence and presence of lipoproteins in the incubation media are summarized in **Table 1.** As expected, the activity of HMG-CoA reductase in the control cells was much higher (about fivefold) in the absence than in the presence of lipoproteins (20).

In the absence of lipoproteins, essentially all of the intermediates suppressed the enzyme activity significantly (activity ≤29% of the control cells). The most potent compounds seemed to be 27-hydroxycholesterol, 7α,27-dihydroxycholesterol, and 7α,27-dihydroxy-4-cholesten-3-one (enzyme activity ≤7% of control cells). The presence of lipoproteins decreased or eliminated the inhibitory effect of most intermediates. As 2.5 μM was considered to be high and not physiological and as lipoproteins reduced the response to the steroids, further tests were carried out at lower concentrations and in the absence of lipoproteins.

At 0.25 μM, larger differences in the inhibitory properties were revealed. 27-Hydroxycholesterol and 7α,27-dihydroxy-4-cholesten-3-one were most inhibitory, causing about 90% suppression of the enzyme activity (Table 1). Even at 0.06 μM the suppression was greater than 50% (**Table 2**). The two bile acid precursors were at least as potent as the established oxysterol inhibitors 25-hydroxy-, 24-hydroxy-, and 7-oxocholesterol and 3β-hydroxy-5α-cholest-8(14)-en-15-one (Tables 1 and 2). Side-chain oxygenated 3-oxo-Δ^4 steroids without a 7α-hydroxy group were also tested. As shown in Table 2, 27-hydroxy-4-cholesten-3-one suppressed HMG-CoA reductase to the same extent as its 7α-hydroxylated analog.

Precursors of cholic acid, i.e., 12α-hydroxylated steroids, were tested at 0.25 μM concentration (**Table 3**). Only 7α,12α,27-trihydroxy-4-cholesten-3-one was a highly

TABLE 1. Activity of HMG-CoA reductase in human fibroblasts

Steroid Structure[a]	Activity of HMG-CoA Reductase[b]		
	2.5 μM + Lipoproteins[c]	2.5 μM	0.25 μM
	% of control		
3β-Hydroxy-Δ steroids			
C^{5}-3β-ol	NT[d]	69	94
C^{5}-3β,27-ol	31	7	12
CA^{5}-3β-ol	65	9	36
B^{5}-3β-ol	72	8	34
C^{5}-3β,25-ol	25	-[e]	-[e]
C^{5}-3β,24 (RS)-ol	32	9	24
5α-$C^{8(14)}$-3β-ol-15-one	21	NT	17
3β,7α-Dihydroxy-Δ^{5} steroids			
C^{5}-3β,7α-ol	80	17	46
C^{5}-3β,7α,27-ol	95	6	25
CA^{5}-3β,7α-ol	55	22	117
B^{5}-3β,7α-ol	92	29	119
7α-Hydroxy-3-oxo steroids			
C^{4}-7α-ol-3-one	76	12	20
C^{4}-7α,27-ol-3-one	83	5	10
CA^{4}-7α-ol-3-one	78	17	30
B^{4}-7α-ol-3-one	52	13	43
5β-C-7α,27-ol-3-one	NT	NT	81
3α,7α-Dihydroxy-5β steroids			
5β-C-3α,7α-ol	NT	NT	110
5β-C-3α,7α,27-ol	NT	NT	116
5β-CA-3α,7α-ol	NT	NT	119
5β-B-3α,7α-ol	89	29	116

Fibroblasts were incubated for 24 h with chenodeoxycholic acid precursors and some oxysteroids at concentrations of 2.5 μM in the presence of lipoproteins and at 2.5 μM or 0.25 μM in the absence of lipoproteins in the incubation medium. Similar results were obtained in repeated experiments (see Materials and Methods).

[a] C, cholestane; CA, cholestanoic acid; B, cholanoic acid. Superscript indicates position of double bond; Greek letters denote configuration of 5-hydrogen or hydroxyl groups.

[b] The activity of HMG-CoA reductase in control cells with and without lipoproteins was 11 and 58 pmol/min per mg protein, respectively.

[c] Incubation medium contained lipoproteins (see Materials and Methods).

[d] NT, not tested.

[e] See Table 2.

TABLE 2. Activity of HMG-CoA reductase in human fibroblasts

Steroid Structure[a]	Activity of HMG-CoA Reductase[b]		
	0.06 μM	0.25 μM	2.5 μM
	% of control cells		
3β-Hydroxy-Δ^{5} steroids			
C^{5}-3β,27-ol	36	32	-[c]
C^{5}-3β,25-ol	69	49	15
7α-Hydroxy-3-oxo-Δ^{4} steroid			
C^{4}-7α,27-ol-3-one	43	30	-[c]
3-Oxo-Δ^{4} steroids			
C^{4}-27-ol-3-one	49	20	13
CA^{4}-3-one	68	33	33
B^{4}-3-one	121	71	37

Fibroblasts were incubated for 24 h with some bile acid precursors and oxysteroids at concentrations of 0.06, 0.25, and 2.5 μM in lipoprotein-free medium. Similar results were obtained in repeated experiments (see Materials and Methods).

[a] For abbreviations, see Table 1.

[b] The activity of HMG-CoA reductase in control cells was 32 pmol/min per mg protein.

[c] See Table 1.

TABLE 3. Activity of HMG-CoA reductase in human fibroblasts

Steroid Structure[a]	Activity of HMG-CoA Reductase[b]
	% of control cells
27-Hydroxycholesterol	36
7α,12α-Dihydroxy-3-oxo steroids	
C^4-7α,12α-ol-3-one	121
C^4-7α,12α,27-ol-3-one	39
CA^4-7α,12α-ol-3-one	90
B^4-7α,12α-ol-3-one	69
5β-C-7α,12α,27-ol-3-one	92
3α,7α,12α-Trihydroxy-5β steroids	
5β-C-3α,7α,12α-ol	125
5β-C-3α,7α,12α,27-ol	122
5β-CA-3α,7α,12α-ol	87
5β-B-3α,7α,12α-ol	106

Fibroblasts were incubated for 24 h with cholic acid precursors at a concentration of 0.25 μM in lipoprotein-free medium. Similar results were obtained in repeated experiments (see Materials and Methods).

[a] For abbreviations, see Table 1.

[b] The activity of HMG-CoA reductase in control cells was 44 pmol/min per mg protein.

potent suppressor of HMG-CoA reductase, whereas the other 12α-hydroxylated compounds had much less or no effect on the enzyme activity.

The four most potent inhibitors were tested with regard to the time course of the cellular response. Incubation of 0.25 μM inhibitor with fibroblasts for different lengths of time showed that the enzyme activity decreased relatively rapidly (**Fig. 1**). The maximum effect was reached within 7–24 h with 27-hydroxycholesterol and 7α,12α,27-trihydroxy-4-cholesten-3-one and after 24 h with 7α,27-dihydroxy-4-cholesten-3-one and 27-hydroxy-4-cholesten-3-one. These results show that a 24-h incubation period was suitable for determination of the potency of the steroids.

Because metabolism may modify the effects, the extent of metabolism of selected 27-hydroxylated intermediates was studied in separate experiments using a 72 h incubation time. The steroids were isolated by a method permitting collection of metabolites with a wide range of polarities. Screening for metabolites was done by GC/MS, and searches were made for potential metabolites formed by hydroxylations, oxidations and reductions, and combinations of these reactions. The results are summarized in **Table 4.** The three most potent inhibitors of HMG-CoA reductase, 27-hydroxycholesterol, 7α,27-dihydroxy-4-cholesten-3-one and 27-hydroxy-4-cholesten-3-one, were oxidized to a limited extent to the corresponding C_{27} acids. There was no detectable conversion of 27-hydroxycholesterol into the potent 27-hydroxy-4-cholesten-3-one. In contrast, substantial amounts of 5-cholestene-3β,7α,27-triol were converted into 7α,27-dihydroxy-4-cholesten-3-one, and both compounds were also oxidized to the corresponding acids (Table 4).

A search was made for 7α-hydroxylation of 27-hydroxycholesterol in the fibroblasts. This reaction has recently been shown to be catalyzed by enzyme(s) different from cholesterol 7α-hydroxylase (18, 21–23). Although this reaction was seen in one experiment and artifacts or contamination could be excluded (results not shown), this result could not be reproduced in a number of subsequent experiments using the same and different conditions. The results therefore suggest that this reaction may not generally occur in fibroblasts.

DISCUSSION

Effects of bile acid precursors on HMG-CoA reductase

In the course of studies on the biological significance of different biosynthetic pathways to bile acids in humans, we have screened the effect of essentially all bile acid intermediates on HMG-CoA reductase. An overview of the apparent relative potencies of the intermediates is shown

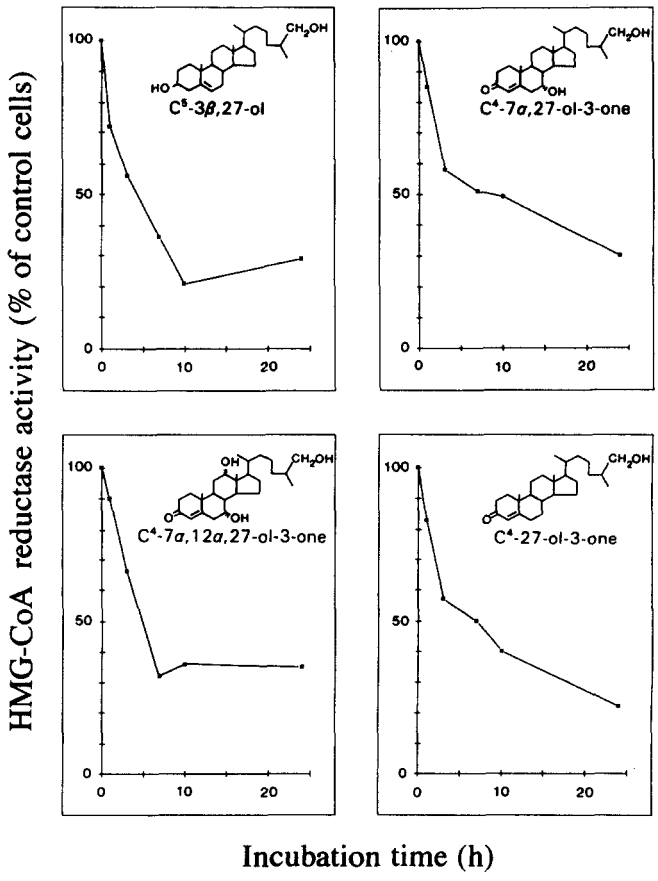

Fig. 1. Time-response curves for four of the most potent HMG-CoA reductase inhibitors found. The steroids were incubated at a concentration of 0.25 μM in lipoprotein-free medium with human fibroblasts, and the cells were harvested at the indicated times.

TABLE 4. Formation of metabolites of inhibitory bile acid precursors in human fibroblasts during a 72-h incubation in lipoprotein-free medium

Substrate[a]	Amount	Metabolites Identified[a]	Amount of Steroid Found[b] In Medium	In Cells[c]
	nmol		*nmol*	
C^5-3β,27-ol	63[d]		25.7	37.5
		CA^5-3β-ol	3.1	0.1
C^5-3β,7α,27-ol	25		0.1	1.2
		C^4-7α,27-ol-3-one	1.6	3.2
		CA^5-3β,7α-ol	0.1	<0.1
		CA^4-7α-ol-3-one	0.8	0.2
C^4-7α,27-ol-3-one	25		2.9	9.9
		CA^4-7α-ol-3-one	1.1	0.3
C^4-27-ol-3-one	25		2.2	9.9
		CA^4-3-one	0.5	0.2

[a] For abbreviations, see Table 1.
[b] Losses probably due to degradation or incomplete extraction of substrate or its metabolites from cells and dish wall.
[c] May include steroids attached to the wall of the incubation dish.
[d] In 25 ml incubation medium; the other incubations in 10 ml medium.

in **Fig. 2.** While 27-hydroxylation of the side-chain enhanced the inhibitory effect, further oxidation to a C_{27} acid and side-chain shortening seemed to decrease the potency of the steroid. The steroid nucleus was also of importance and the most potent inhibitors contained either a 3β-hydroxy-Δ^5 or a 3-oxo-Δ^4 structure. The double bond was essential (see also Tables 1 and 3), whereas hydroxyl groups in the 7α- and 12α-positions of the 3-oxo-Δ^4 steroids seemed to have neither positive nor negative effects.

Several factors may affect the apparent potency of the inhibitors in the test system used. The cellular uptake and the intracellular distribution of the added steroid are ob-

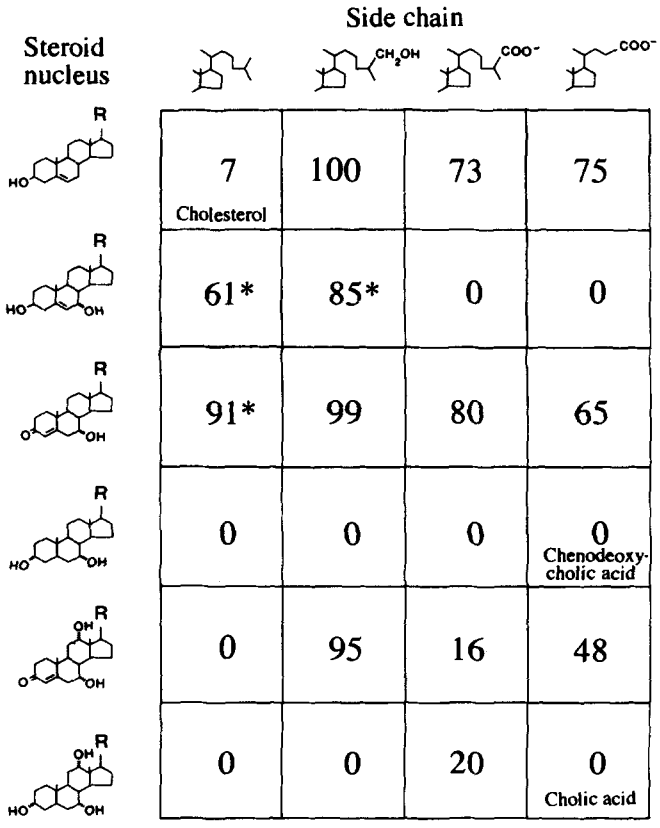

Fig. 2. Apparent inhibitory potencies of intermediates in bile acid biosynthesis on HMG-CoA reductase activity in human fibroblasts determined after incubations for 24 h and at a steroid concentration of 0.25 μM in lipoprotein-free medium. Potencies (in %) are expressed relative to that of 27-hydroxycholesterol (= 100%), as the suppression of HMG-CoA reductase activity varied in different experiments. Median suppression induced by 27-hydroxycholesterol was 71% of control cells (range 52–88%). An asterisk indicates that the apparent inhibitory potency may be due to metabolism, as the compound can be converted to 7α,27-dihydroxy-4-cholesten-3-one in human fibroblasts. Corresponding values for related oxysteroids and bile acids were: 27-hydroxy-4-cholesten-3-one = 118; 3-oxo-4-cholestenoic acid = 99; 3-oxo-4-cholenoic acid = 43; 7α,27-dihydroxy-5β-cholestan-3-one = 22; 7α,12α,27-trihydroxy-5β-cholestane-3-one = 12; lithocholic acid = 16; deoxycholic acid = 7; 24-hydroxycholesterol = 86; 25-hydroxycholesterol = 75; 7-oxocholesterol = 100; and 3β-hydroxy-5α-cholest-8(14)-en-15-one = 94.

viously of importance. The amounts of steroids in the cell fraction were not determined because of the difficulty in distinguishing between intracellular steroid and steroid adsorbed to the cell surface. Regardless of possible differences in cellular uptake, the finding that 27-hydroxycholesterol and intermediates with a 27-hydroxy-3-oxo-Δ^4 structure are potent suppressors of HMG-CoA reductase is valid.

Metabolism of a steroid can also affect its activity. Although metabolic conversions of sterols are more limited in fibroblasts than in liver cells, two reactions found in bile acid biosynthesis have previously been demonstrated in fibroblasts: 27-hydroxylation of sterols, including 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one (24), and the conversion of 7α-hydroxycholesterol into 7α-hydroxy-4-cholesten-3-one (25). Such reactions may explain the (unexpectedly) high inhibitory activities of the two intermediates lacking a 27-hydroxy group by converting them into 7α,27-dihydroxy-4-cholesten-3-one (Fig. 2). The relatively strong suppression of HMG-CoA reductase by 5-cholesten-3β,7α,27-triol may also be due, at least partly, to the conversion into the 3-oxo-Δ^4 derivative as shown in Table 4. In a previous study by Esterman et al. (19), 5-cholestene-3β,7α,27-triol had very little effect on the activity of HMG-CoA reductase. This difference could be due to a lower activity of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase in their cell lines or to a species difference.

However, it is reasonable to assume that the observed effects of 27-hydroxycholesterol and the 27-hydroxylated 3-oxo-Δ^4 steroids were due to the steroids per se and not to a metabolite. Their oxidation to acids yielded less potent inhibitors and oxidation of 27-hydroxycholesterol to the 3-oxo-Δ^4 derivative was not observed. The finding that 7α,27-dihydroxy-4-cholesten-3-one, 7α,12α,27-trihydroxy-4-cholesten-3-one, and 27-hydroxy-4-cholesten-3-one

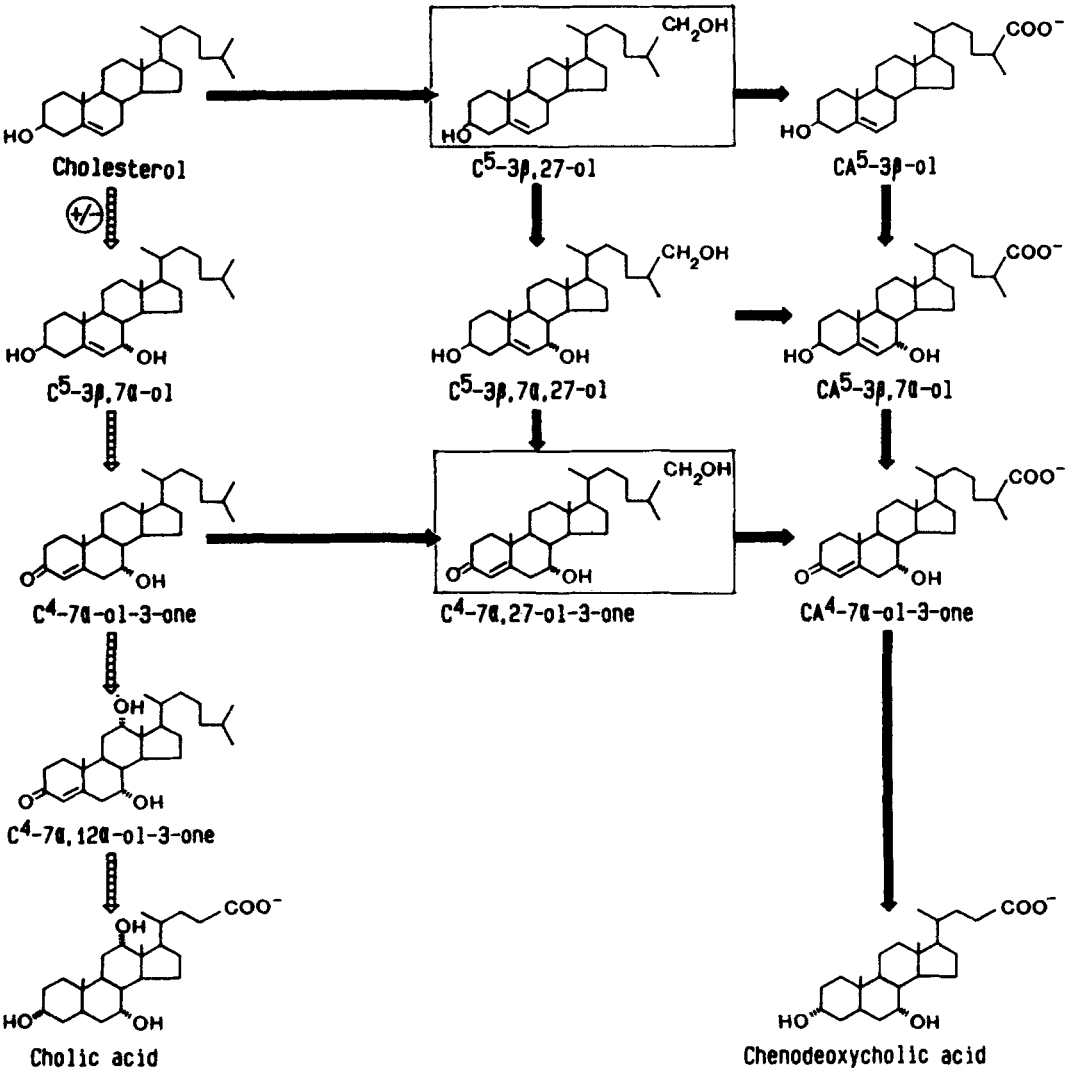

Fig. 3. Model for the biosynthesis of bile acids in the human liver. Potent HMG-CoA reductase inhibitors are indicated in frame.

are potent inhibitors of HMG-CoA reductase is new. The 3-oxo-Δ^4 structure is also present in many steroid hormones. This could indicate that the C_{27} steroids act via a protein of the steroid receptor family (26). Such a receptor would be different from the cytosolic oxysterol binding protein previously described, which seems to have a low affinity for 3-oxo-Δ^4 steroids (27–29). However, further studies are required to determine the mechanisms of action of this new group of inhibitors.

Possible biological role of inhibitory intermediates

The results indicate that a few specific bile acid precursors could be regulators of cholesterol biosynthesis provided that their activity is not limited to fibroblasts and that their intracellular levels are sufficiently high. The concentrations of 27-hydroxycholesterol, 7α,27-dihydroxy-4-cholesten-3-one, and 7α,12α,27-trihydroxy-4-cholesten-3-one in liver cells are not known but in plasma they are normally about 0.1–0.3, 0.01–0.02, and 0.002 μM, respectively (8, 10, 30). In patients with stimulated bile acid synthesis, plasma levels of the two 3-oxo-Δ^4 steroids can be raised more than 10-fold (10, 30, 31). If levels in plasma in any way reflect those in liver cells, one would expect 27-hydroxycholesterol and 7α,27-dihydroxy-4-cholesten-3-one to be the most, and 7α,12α,27-trihydroxy-4-cholesten-3-one the least important of the inhibitors, especially under normal conditions. An intracellular formation of 27-hydroxy-4-cholesten-3-one has not been demonstrated.

Interestingly, the two inhibitors, 27-hydroxycholesterol and 7α,27-dihydroxy-4-cholesten-3-one, represent the first committed intermediates in what is considered to be the two major biosynthetic pathways to chenodeoxycholic acid in humans (7, 10, 32, 33) (**Fig. 3**). Thus, it may be hypothesized that the synthesis of chenodeoxycholic acid may have an inhibitory role in cholesterol biosynthesis through the formation of 27-hydroxycholesterol and/or 7α,27-dihydroxy-4-cholesten-3-one (Fig. 3). The formation of cholic acid seems less likely to result in levels of 7α,12α,27-trihydroxy-4-cholesten-3-one in the liver that can significantly affect HMG-CoA reductase.

In extrahepatic tissues, only 27-hydroxycholesterol is expected to be formed and have a potential regulatory role. However, if the release of 3-oxo-Δ^4 intermediates from the liver is increased, e.g., when bile acid biosynthesis is stimulated (30, 31) a suppressive effect of these compounds on extrahepatic HMG-CoA reductase cannot be excluded.

Ms. Birgitta Mörk provided excellent technical assistance. This work was supported by the Swedish Medical Research Council (grants no. 03X-219 and 03X-7890) and the Swedish Cancer Society (grant no. 03XCC), Karolinska Institutet and the Scandinavia-Japan Sasakawa Foundation.

Manuscript received 7 January 1994 and in revised form 2 September 1994.

REFERENCES

1. Björkhem, I. 1985. Mechanism of bile acid biosynthesis in mammalian liver. *In* Sterols and Bile Acids. H. Danielsson, and J. Sjövall, editors. Elsevier, Amsterdam. 231–278.
2. Vlahcevic, Z. R., D. M. Heuman, and P. B. Hylemon. 1991. Regulation of bile acid synthesis. *Hepatology.* **13:** 590–600.
3. Björkhem, I. 1992. Mechanism of degradation of the steroid side chain in the formation of bile acids. *J. Lipid Res.* **33:** 455–471.
4. Russel, D. W., and K. D. R. Setchell. 1992. Bile acid biosynthesis. *Biochemistry.* **31:** 4737–4749.
5. Kandutsch, A. A., H. W. Chen, and H-J. Heiniger. 1978. Biological activity of some oxygenated sterols. *Science.* **201:** 498–501.
6. Kim, H-S., W. K. Wilson, D. H. Needleman, F. D. Pinkerton, D. K. Wilson, F. A. Quiocho, and G. J. Schroepfer, Jr. 1989. Inhibitors of sterol synthesis. Chemical synthesis, structure, and biological activities of (25R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one, a metabolite of 3β-hydroxy-5α-cholest-8(14)-en-15-one. *J. Lipid Res.* **30:** 247–261.
7. Axelson, M., B. Mörk, and G. T. Everson. 1991. Bile acid synthesis in cultured human hepatoblastoma cells. *J. Biol. Chem.* **266:** 17770–17777.
8. Javitt, N. B., E. Kok, S. Burstein, B. Cohen, and J. Kutscher. 1981. 26-Hydroxycholesterol. Identification and quantitation in human serum. *J. Biol. Chem.* **256:** 12644–12646.
9. Axelson, M., B. Mörk, and J. Sjövall. 1988. Occurrence of 3β-hydroxy-5-cholestenoic acid, 3β,7α-dihydroxy-5-cholestenoic acid, and 7α-hydroxy-3-oxo-4-cholestenoic acid as normal constituents in human blood. *J. Lipid Res.* **29:** 629–641.
10. Axelson, M., and J. Sjövall. 1990. Potential bile acid precursors in plasma—possible indicators of biosynthetic pathways to cholic and chenodeoxycholic acids in man. *J. Steroid Biochem.* **36:** 631–640.
11. Brown, M. S., and J. L. Goldstein. 1974. Suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and inhibition of growth of human fibroblasts by 7-ketocholesterol. *J. Biol. Chem.* **249:** 7306–7314.
12. Shoda, J., M. Axelson, and J. Sjövall. 1993. Synthesis of potential C_{27}-intermediates in bile acid biosynthesis and their deuterium-labeled analogs. *Steroids.* **58:** 119–125.
13. Weinstein, D. B. 1979. A single-step adsorption method for removal of lipoproteins and preparation of cholesterol-free serum. *Circulation.* **59-60, suppl. II.** 54.
14. Cavenee, W. K., H. W. Chen, and A. A. Kandutsch. 1981. Regulation of cholesterol biosynthesis in enucleated cells. *J. Biol. Chem.* **256:** 2675–2681.
15. Edwards, P. A., D. Lemongello, and A. M. Fogelman. 1979. Improved methods for the solubilization and assay of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Lipid Res.* **20:** 40–46.
16. Axelson, M., and B-L. Sahlberg. 1981. Solid extraction of steroid conjugates from plasma and milk. *Anal. Lett.* **14:** 771–782.
17. Jänne, O., R. Vihko, J. Sjövall, and K. Sjövall. 1969. Determination of steroid mono- and disulfates in human plasma. *Clin. Chim. Acta.* **23:** 405–412.
18. Axelson, M., J. Shoda, J. Sjövall, A. Toll, and K. Wikvall. 1992. Cholesterol is converted to 7α-hydroxy-3-oxo-4-cholestenoic acid in liver mitochondria. Evidence for a mitochondrial sterol 7α-hydroxylase. *J. Biol. Chem.* **267:** 1701–1704.
19. Esterman, A. L., H. Baum, N. B. Javitt, and G. J. Darlington. 1983. 26-Hydroxycholesterol: regulation of hydroxy-

methylglutaryl-CoA reductase activity in Chinese hamster ovary cell culture. *J. Lipid Res.* **24:** 1304–1309.
20. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1973. Regulation of 3-hydroxy-3-methylgutaryl coenzyme A reductase activity in human fibroblasts by lipoproteins. *Proc. Natl. Acad. Sci. USA.* **70:** 2162–2166.
21. Toll, A., J. Shoda, M. Axelson, J. Sjövall, and K. Wikvall. 1992. 7α-Hydroxylation of 26-hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid by cytochrome P-450 in pig liver microsomes. *FEBS Lett.* **296:** 73–76.
22. Björkhem, I., B. Nyberg, and K. Einarsson. 1992. 7α-Hydroxylation of 27-hydroxycholesterol in human liver microsomes. *Biochim. Biophys. Acta.* **1128:** 73–76.
23. Shoda, J., A. Toll, M. Axelson, F. Pieper, K. Wikvall, and J. Sjövall. 1993. Formation of 7α- and 7β-hydroxylated bile acid precursors from 27-hydroxycholesterol in human liver microsomes and mitochondria. *Hepatology.* **17:** 395–403.
24. Skrede, S., I. Björkhem, E. A. Kvittingen, M. S. Buchmann, S. O. Lie, C. East, and S. Grundy. 1986. Demonstration of 26-hydroxylation of C_{27}-steroids in human skin fibroblasts, and a deficiency of this activity in cerebrotendinous xanthomatosis. *J. Clin. Invest.* **78:** 729–735.
25. Buchmann, M. S., E. A. Kvittingen, H. Nazer, T. Gunasekaran, P. T. Clayton, J. Sjövall, and I. Björkhem. 1990. Lack of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase in fibroblasts from a child with urinary excretion of 3β-hydroxy-Δ^5-bile acids. A new inborn error of metabolism. *J. Clin. Invest.* **86:** 2034–2037.
26. Evans, R. M. 1988. The steroid and thyroid hormone receptor superfamily. *Science.* **240:** 889–895.
27. Kandutsch, A. A., and E. B. Thompson. 1980. Cytosolic proteins that bind oxygenated sterols. Cellular distribution, specificity and some properties. *J. Biol. Chem.* **255:** 10813–10826.
28. Taylor, F. R., S. E. Saucier, E. P. Shown, E. J. Parish, and A. A. Kandutsch. 1984. Correlation between oxysterol binding to a cytosolic binding protein and potency in the repression of hydroxymethylglutaryl coenzyme A reductase. *J. Biol. Chem.* **259:** 12382–12387.
29. Dawson, P. A., N. D. Ridgway, C. A. Slaughter, M. S. Brown, and J. L. Goldstein. 1989. cDNA cloning and expression of oxysterol-binding protein, an oligomer with a potential leucine zipper. *J. Biol. Chem.* **264:** 16798–16803.
30. Axelson, M., B. Mörk, and J. Sjövall. 1991. Studies on biosynthetic pathways to cholic and chenodeoxycholic acids in humans. *In* Bile Acids as Therapeutic Agents. From Basic Science to Clinical Practice. G. Paumgartner, A. Stiehl, and W. Gerok, editors. Kluwer, Dordrecht. 53–62.
31. Axelson, M., M. Mörk, and J. Sjövall. 1991. Ethanol has an acute effect on bile acid biosynthesis in man. *FEBS Lett.* **281:** 155–159.
32. Andersson, K. E., E. Kok, and N. B. Javitt. 1972. Bile acid synthesis in man: metabolism of 7α-hydroxycholesterol-^{14}C and 26-hydroxycholesterol-^{3}H. *J. Clin. Invest.* **51:** 112–117.
33. Swell, L., J. Gustafsson, C. C. Schwartz, L. G. Halloran, H. Danielsson, and Z. R. Vlahcevic. 1980. An in vivo evaluation of the quantitative significance of several potential pathways to cholic and chenodeoxycholic acids from cholesterol in man. *J. Lipid Res.* **21:** 455–466.