Effects of a novel 2,3-oxidosqualene cyclase inhibitor on the regulation of cholesterol biosynthesis in HepG2 cells

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Abstract Within the cholesterol biosynthesis cascade, the enzyme 2,3-oxidosqualene cyclase [EC 5.4.99.7] is of special interest due to its dual function: cyclization of 2,3-monoepoxysqualene to lanosterol and 2,3;22,23-diepoxysqualene to oxylanosterol. Further determination of the significance of this enzyme for the intracellular cholesterol homeostasis was done with BIBX 79, a new potent, specific inhibitor of this enzyme. In HepG2 cells the effects of BIBX 79 on cholesterol biosynthesis, 2,3-oxidosqualene cyclase as well as HMG-CoA reductase activities were studied. BIBX 79 is a potent inhibitor of sterol biosynthesis in HepG2 cells (IC_{50} 4×10^{-9} M). No other enzyme within the cholesterol biosynthesis cascade was significantly inhibited as was evidenced by a radio HPLC detection system. In contrast to simvastatin, no direct interaction with HMG-CoA reductase was observed. When incubating HepG2 cells for 16 h with the HMG-CoA reductase inhibitor simvastatin (10^{-6}–10^{-10} M) HMG-CoA reductase activity was increased up to 180%. BIBX 79 did also affect HMG-CoA reductase activity under these conditions: in concentrations of BIBX 79 $\geq 10^{-9} \leq 10^{-7}$ M, where a partial inhibition of 2,3-oxidosqualene cyclase is observed, HMG-CoA reductase activity was decreased. However, higher concentrations of BIBX 79 that totally blocked 2,3-oxidosqualene cyclase led to an increase in HMG-CoA reductase activity. This effect of BIBX 79 on HMG-CoA reductase is thought to be mainly mediated by oxysterols that are formed by the cyclization of 2,3;22,23-diepoxysqualene. 2,3;22,23-Diepoxysqualene is preferentially cyclized by the 2,3-oxidosqualene cyclase and, consequently, only high inhibitor concentrations will also block 2,3;22,23-diepoxysqualene cyclization. Thus, by partial blockade of this enzyme, both an inhibition of lanosterol and subsequently cholesterol formation as well as a concomitant effect on HMG-CoA reductase can be achieved. Both effects complement each other and lead to an effective control of cholesterol biosynthesis. It is therefore concluded that 2,3-oxidosqualene cyclase plays a crucial role in the regulation of intracellular cholesterol homeostasis. 2,3-Oxidosqualene cyclase inhibitors offer an attractive approach for novel lipid-lowering agents.—**Mark, M., P. Müller, R. Maier, and B. Eisele.** Effects of a novel 2,3-oxidosqualene cyclase inhibitor on the regulation of cholesterol biosynthesis in HepG2 cells. *J. Lipid Res.* 1996. **37:** 148–158.

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Hypercholesterolemia is a major risk factor for the development of atherosclerotic vascular diseases. A clear correlation between plasma cholesterol levels and CHD morbidity and mortality has been demonstrated (1). With the recent results of the Scandinavian Simvastatin Survival Study (4S) it has been convincingly shown that cholesterol lowering will lead to a decrease in cardiovascular events as well as in cardiac and overall mortality (2).

Reducing the rate of cholesterol biosynthesis has been shown to be an effective therapeutic approach to lowering elevated plasma cholesterol levels. The only class of compounds with that mechanism of action available for therapy so far are the HMG-CoA reductase inhibitors. These compounds (e.g., lovastatin, simvastatin, pravastatin, and fluvastatin) inhibit the rate-limiting enzyme of the cholesterol synthesis cascade, HMG-CoA reductase (3, 4).

Apart from that step other enzymes within the cholesterol biosynthesis pathway could serve as possible intervention points for drug therapy. Amongst these the enzyme 2,3-oxidosqualene cyclase (OSC) [EC 5.4.99.7] seems to be an attractive target. Due to the position in

Abbreviations: OSC, 2,3-oxidosqualene cyclase; MES, 2,3-monoepoxysqualene; DES, 2,3;22,23-diepoxysqualene; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; LPDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline.

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the biosynthesis cascade, inhibition of this enzyme avoids problems caused either by reduced synthesis of other products, e.g., t-RNA, dolichol, ubiquinone, and isoprene units or by accumulation of sterol intermediates.

Furthermore inhibition of OSC might have other advantages due to the special function of this enzyme. In addition to directly cyclizing 2,3-monoepoxysqualene (MES) to lanosterol, this enzyme also catalyzes the conversion of 2,3;22,23-diepoxysqualene (DES) into 24,25-epoxylanosterol. The latter is eventually transformed to 24,25-epoxycholesterol which is a known repressor of HMG-CoA reductase (5–7). Therefore, an up-regulation of HMG-CoA reductase activity as seen with HMG-CoA reductase inhibitors (8–11), which might limit the efficacy of that class of compounds, should be avoided.

Inhibition of OSC should then combine two synergistically acting mechanisms: *1)* decrease of the amount of lanosterol formed (i.e., direct inhibition of cholesterol biosynthesis), and *2)* repression of HMG-CoA reductase through the formation of oxysterols such as 24,25-epoxycholesterol (i.e., regulation of cholesterol biosynthesis).

Up to now either only weak inhibitors (12, 13) of the enzyme OSC have been described or compounds that also interfere with other steps of the cholesterol biosynthesis cascade (14, 15).

In this report we describe the mechanism of action of a potent and selective inhibitor of OSC. Derived from that mode of action of BIBX 79, the role and attractiveness of the enzyme OSC as a target enzyme for the design of hypolipidemic drugs becomes clear. A model will be discussed on the regulation of cholesterol biosynthesis.

EXPERIMENTAL PROCEDURES

Chemicals

Cholesterol was obtained from Serva Heidelberg Germany and digitonin was from Merck, Darmstadt Germany. AG 1-X8 the anion exchange resin came from Bio-Rad. Ketoconazole was purchased from Sigma. U18666A was synthesized in the labs of Dr. Karl Thomae GmbH. [2-^{14}C]acetate and DL-3-[^{14}C]hydroxy-3-methylglutaryl coenzyme A were obtained from New England Nuclear.

BIBX 79

Based on expanded molecular modeling investigations of the cyclization cycle of MES to lanosterol (16–18), BIBX 79 (*trans*-N-(4-chlorobenzoyl)-N-methyl-(4-dimethylaminomethylphenyl)-cyclohexylamine) (**Fig. 1**) was designed as an analogue of the tetracyclic C-20 carboniumion "protolanosterol" formed during MES-

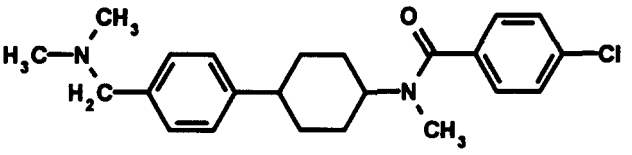

Fig. 1. Chemical structure of BIBX 79.

cyclization. Thereby the compound should mimic the steric and electronic properties of protolanosterol. In addition, the compound should be able to suppress cyclization by interfering with the proton-initiated oxirane ring-opening of 2,3-oxidosqualene. The synthesis of BIBX 79 was performed by the following steps.

(I) 4-(4-Dimethylaminomethylphenyl) cyclohexanone. To a solution of n-butyl lithium (1.12 l, 1.6 mol in hexane) in THF (1.5 l) a solution of 4-bromo-N,N-dimethylbenzylamine (364 g, 1.7 mol, from 4-bromobenzylbromide and dimethylamine in toluene, b.p.$_{11}$ 105°C) in THF (500 ml) was added (-65°C, N_2). After 30 min at -65°C, 1.4-cyclohexandionmonoethylenketal (276.4 g, 1.72 mol) in THF (1.1 l) was added, the mixture was stirred for 30 min at -65°C, then stirred overnight at room temperature (N_2) and poured into a mixture of ice water (6 l) and ethyl acetate (1.8 l). After stirring for 10 min, the aqueous phase was extracted with ethyl acetate (4 × 800 ml) and the combined extracts were worked up (brine, Na_2SO_4) to give a residue that was crystallized from diisopropyl ether (1.2 l) to give 1-(4-dimethylaminomethylphenyl)-4-ethylendioxycyclohexanol (419 g, 84.6%): mp 84–86°C.

This product (224.4 g, 0.77 mol) was dehydrated by refluxing in toluene (2.4 l, 3.5 h) in the presence of *p*-toluenesulfonic acid monohydrate (150.3 g, 0.79 mol) and ethylene glycol (390 ml) with continuous removal of the formed water. The mixture was poured into ice water (1 l), the pH was raised to 12–13 (2 N NaOH) and the aqueous phase was extracted with toluene (2 × 500 ml). Workup (brine, Na_2SO_4) of the combined extracts gave 1-(4-dimethylaminomethylphenyl)-4-ethylendioxycyclohex-1-ene (211 g, 100%) as a yellow oil.

The crude material (325 g, 1.19 mol) dissolved in a mixture of ethyl acetate (3240 ml) and methanol (1380 ml) was hydrogenated over Pd/$BaSO_4$ (78 g) at room temperature (1.5 h, 5 bar). Removal of catalyst and solvents gave a yellow brownish oil (305 g) consisting of a mixture of 1-(4-dimethylaminomethylphenyl)-4-ethylendioxycyclohexane as the main product and 1-(4-methylphenyl)-4-ethylendioxycyclohexane as the by-product. This oil (132 g) was stirred in 2 N HCl (730 ml) for 3.5 h at room temperature. To remove the by-product, the acid solution was extracted with ethyl acetate (3 × 300 ml), then the pH was raised to 13–14 under cooling

(50% NaOH, N_2) and the solution was extracted with ethyl acetate (3 × 400 ml). Workup (brine, Na_2SO_4) of the combined extracts gave 4-(4-dimethylamino-methylphenyl)cyclohexanone (87.3 g): mp 64–67°C.

(II) trans *4-(4-Dimethylyaminomethylphenyl)-N-methylcyclohexylamine.* 4-(4-Dimethylaminomethylphenyl)-cyclohexanone (118, 0.51 mol), molecular sieve Å 3 (190 g, Fluka) and a solution of methylamine in toluene (640 ml, 2.04 mol) were vigorously stirred for 15 h at room temperature and then molecular sieve and solvent were removed. The residue was dissolved in methanol (650 ml); sodium borohydride (14 g, 0.37 mol) was added during 1 h with cooling (-8 to -4°C); the mixture was stirred for 3 h at room temperature, and then the solvent was removed. The residue was suspended in water (600 ml) and concentrated hydrochloric acid was added to pH 1–2 with cooling. After 1 h at room temperature the pH was brought to 12–13 (50% NaOH) with cooling; the solution was extracted with ethyl acetate under pH control and the extract was concentrated (Na_2SO_4). To separate the isomers, the residue was dissolved in ethyl acetate (50 ml). A solution of benzoic acid (62 g, 0.51 mol) in hot ethyl acetate (220 ml) was added, followed by hot diisopropyl ether (200 ml). After 2 h at -15°C, the solid was filtered off, dissolved in a mixture of ice water (500 ml) and methylene chloride (200 ml), the pH was adjusted to 14 (50% NaOH), and the aqueous phase was extracted with methylene chloride (3 × 150 ml). Workup (Na_2SO_4) of the combined extracts gave *trans* 4-(4-dimethylaminomethylphenyl-N-methylcyclohexylamine) (55.3 g, 44%): mp 56–58°C.

(III) trans-*N-(4-Chlorobenzoyl)-N-methyl-4-(4-dimethylaminomethylphenyl) cyclohexylamine.* A solution of 4-chlorobenzoyl chloride (7.1 g, 40.6 mmol) in methylene chloride (20 ml) was added to a solution of *trans* 4-(4-dimethylaminomethylphenyl)-N-methylcyclohexylamine (10 g, 40.5 mmol) in methylene chloride (100 ml) at room temperature. After 2 h the mixture was poured into water, the aqueous phase was made alkaline (NaOH) and extracted three times with methylene chloride. Workup (Na_2SO_4) of the combined extracts and purification by chromatography (alumina N, act. III, ICN, ethyl acetate) gave *trans*-N-(4-chlorobenzoyl)-N-methyl-4-(4-dimethylaminomethylphenyl) cyclohexylamine (12.5 g, 80%): mp 154–156°C.

HepG2 cells

The human hepatoma cell line HepG2 was obtained from B. B. Knowles (Wistar Institute/Philadelphia). Cells were routinely grown at 37°C in a humidified 5% CO_2 atmosphere in Eagles Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum and 8 mM glutamate without addition of antibiotics.

For the experiments, if not otherwise stated, cells were grown in 6-well plates (Greiner, Germany). Cells were near confluence at the start of the experiment. Prior to any addition of test substances, the medium was changed to MEM supplemented with 5% fetal calf lipoprotein-deficient serum (LPDS) and 8 mM glutamate. LPDS was prepared by ultracentrifugation (20 h, 8°C and 100,000 *g*) of fetal calf serum with ρ adjusted to 1.25 g/l by addition of KBr. LPDS was then dialyzed extensively against phosphate-buffered saline (PBS) and resterilized prior to use by filtration (0.2 µm).

Sterol synthesis in HepG2 cells

Cells were incubated for 16 h with BIBX 79 dissolved in dimethyl sulfoxide (final test concentration 0.1%). Then [^{14}C]acetate (0.2 mM, 53 MBq/l) was added for another 2 h. Media were removed, cell layers were washed with PBS, and then 0.1 M NaOH was added. After taking aliquots for protein determination, the remainder was saponified with 10% ethanolic KOH for 30 min at 60°C. The nonsaponifiable lipids were extracted with petroleum ether, evaporated and dissolved again in acetone–ethanol. After precipitation with digitonin, samples were centrifuged, and the precipitate was washed with ethanol and dissolved in methanol. Radioactivity was measured by liquid scintillation counting. Cell proteins were determined according to the method described by Lowry et al. (19) with human serum albumin as standard. Results were expressed as nmol [^{14}C]acetate incorporated into sterols per mg cell protein.

2,3-Oxidosqualene cyclase activity

HepG2 cells grown in 25-cm^2 culture flasks were washed with PBS and after freezing and thawing the cells were scraped off and sonicated. Aliquots of 1 ml cell homogenate were assayed for enzyme activity in 0.1 M potassium phosphate buffer (pH 7.4). The reaction was started by addition of BIBX 79 (dissolved in dimethyl sulfoxide, final test concentration 0.1%) and [^{14}C]MES (14 µM, 74 KBq/mg). Final incubation volume was 1.0 ml. In the displacement experiments, [^{14}C]DES (14 µM, 49 KBq/mg) was used. After 1 h incubation at 37°C, the reaction was stopped by addition of 2.5 ml 10% ethanolic KOH. Lipids were extracted with petroleum ether and after evaporation samples were dissolved in chloroform containing 1 mg/ml lanosterol. Lipids were separated by TLC (petroleum ether–diethylether 5:1 (v/v)) and [^{14}C]lanosterol or [^{14}C]epoxylanosterol was counted. Inhibition of OSC was calculated as % [^{14}C]lanosterol or [^{14}C]epoxylanosterol versus control after subtraction of blanks.

[^{14}C]MES as well as [^{14}C]DES used in these experiments were prepared as follows. To confluent HepG2

cells grown in 25-cm^2 plastic culture flasks, [^{14}C]acetate (37 MBq/flask) together with BIBX 79 (10^{-5} M) dissolved in DMSO (final test concentration 0.1%) was added. After 18 h incubation, medium was removed and the cells were washed with PBS. Further procedure is described in the next paragraph. HPLC fractions were collected and identification as well as quantification of the peaks was done by comparison with MES and DES. Specific activities of [^{14}C]MES and [^{14}C]DES were calculated accordingly.

Specificity of BIBX 79 versus 2,3-oxidosqualene cyclase

To subconfluent HepG2 cells grown in 25-cm^2 plastic culture flasks, [^{14}C]acetate (925 KBq per flask) together with BIBX 79, ketoconazole or U18666A (dissolved in dimethyl sulfoxide, final test concentration 0.1%) were added. After 2.25 h of incubation media were removed and the cells were washed with PBS. Cells were then treated with trypsin, suspended, and lipids were extracted according to the method described by Bligh and Dyer (20). The extract was dissolved in 0.5 ml $CHCl_3$ and purified over Amchro Bond-Elut columns. Therefore, the total extract was transferred to the columns, eluated by 1 min centrifugation at 200 *g*, and the columns were washed twice with $CHCl_3$. The eluate was evaporated to dryness and then the lipids were extracted into methanol for separation by HPLC on a 4.6 × 120 mm ODS-Hypersil 5 µm with a guard column 4.6 × 40 mm ODS-Hypersil 5 µm using methanol–water 95:5 (v/v) as the mobile phase at 1 ml/min. The column effluent was monitored by an on-line radioactivity detector (Raytest, Ramona 5). For peak identification by UV monitoring, a nonradioactive standard mixture was added to deliver between 2.5 and 10 µg/ml of the individual compounds to the samples. The following precursors could be separated (retention time in min.): 25-hydroxycholesterol (4.37), 15-ketocholesterol (5.60), 24,25-epoxycholesterol (5.65) DES (7.95), desmosterol (14.65), 7-dehydrocholesterol (15.95), MES (17.10), cholesterol (19.00), lanosterol (19.65), dihydrolanosterol (25.25), and squalene (43.33). ^{14}C activity-containing peaks isolated at designated retention times were positively identified by mass spectroscopy. Signals were calculated as percentage of the control cholesterol peak. The detection limit of the system was at about 1% of the control cholesterol peak which corresponds to about twice the basal noise level.

HMG-CoA reductase activity

Direct inhibition. Cells were grown to subconfluency and cultivated for 24 h in MEM medium containing 5% LPDS. The cells were washed with PBS and after freezing and thawing the cells were scraped off into buffer A (10 mM EDTA, 50 mM potassium phosphate, Brij 96 0.25%, pH 7.4). After sonication, aliquots of 40 µl of the homogenate were assayed for enzyme activity in a 0.1 M potassium phosphate buffer containing 20 mM glucose-6-phosphate, 5 mM $NADP^+$ and 0.5 U glucose-6-phosphate dehydrogenase. Substances to be tested were dissolved in dimethyl sulfoxide with the final test concentration of DMSO being 0.1%. The reaction was started by addition of [^{14}C]HMG-CoA (15 MBq/l, 25 µM). After incubation for 2 h at 37°C, the reaction was stopped by the addition of 20 µl of 12 mM mevalonolactone dissolved in 5 M HCl. Aliquots of the incubation media were chromatographed over an AG 1-X8 column and [^{14}C]mevalonolactone was eluated with H_2O from the column. After addition of scintillation cocktail, the radioactivity was counted.

Regulation. Subconfluent HepG2 cells were cultivated for 16 h in MEM medium containing 5% LPDS. Substances to be tested were added to this medium directly (dissolved in DMSO, final test concentration 0.1%). Cells were thereafter thoroughly washed with MEM and PBS to remove all the remainder of the substances that could interfere directly with HMG-CoA reductase. Determination of enzyme activity was performed as described above.

Calculation of results

Calculation of IC_{50} values was performed by fitting the function $y = b+[a*k/(k + x^n)]$ to the data using the program Sigma Plot (Jandel Scientific). Y is the measured enzyme activity, x is the concentration of the inhibitor, n is the Hill coefficient, a is the difference between the upper and lower asymptotes of the dose response curve, and b is the baseline level. IC_{50} values were defined as half maximal effect concentrations.

RESULTS

Inhibition of sterol biosynthesis by BIBX 79

To determine the inhibitory effect of BIBX 79 on sterol biosynthesis, HepG2 cells were incubated for 16 h with the substance. This long incubation period was chosen in order to obtain as an overall result of the OSC inhibition the sum of the direct inhibition as well as the regulatory effect on cholesterol biosynthesis. BIBX 79 led to a concentration-dependent decrease of sterol biosynthesis in concentrations > 10^{-10} M; the plateau was reached with a concentration of 10^{-7} M. The IC_{50} value was calculated as 3.8×10^{-9} M (**Fig. 2**).

Inhibition of 2,3-oxidosqualene cyclase

HepG2 cell homogenate was used to further identify BIBX 79 as an inhibitor of OSC. Therefore [^{14}C]mono-

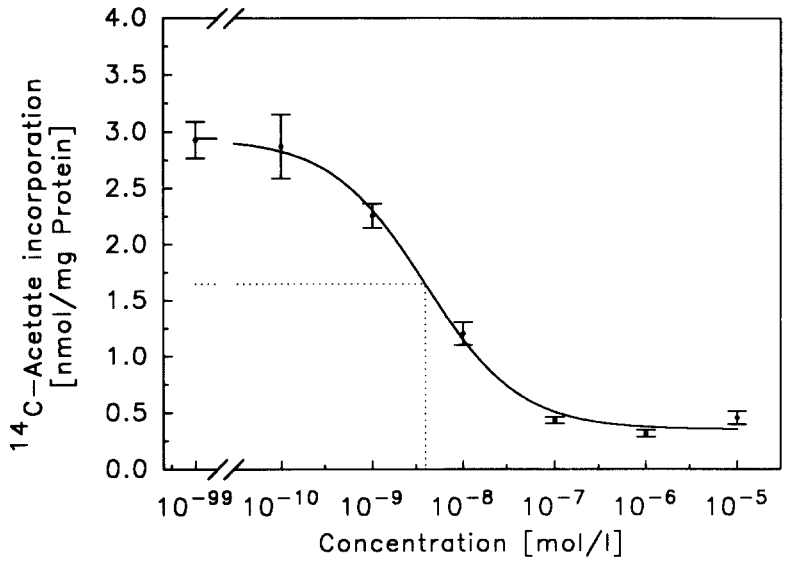

Fig. 2. Effect of BIBX 79 on sterol biosynthesis. HepG2 cells were preincubated with BIBX 79 dissolved in dimethyl sulfoxide (final test concentration 0.1%) for 16 h and further incubated with [^{14}C]acetate for another 2 h. Nonsaponifiable lipids were extracted and precipitated with digitonin. Radioactivity was measured by liquid scintillation counting. Results are expressed as nmol [^{14}C]acetate incorporated in sterols per mg cell protein. Experiments were run in quadruplicate (mean ± SD).

epoxysqualene was used as a substrate. BIBX 79 inhibited the OSC reaction in concentrations ≥ 10^{-10} M. The IC_{50} value was calculated as 6×10^{-9} M (**Fig. 3**).

Specificity of BIBX 79 versus 2,3-oxidosqualene cyclase

Because it has been shown that accumulation of sterols other than cholesterol (e.g., desmosterol) can eventually lead to massive side effects (21, 22), it was mandatory to exclude any significant interference of BIBX 79 with the so-called late steps in the cholesterol biosynthesis pathway. For that purpose, a radio HPLC system was set up which allowed the detection of precursors in the cholesterol biosynthesis cascade. As can be seen in **Table 1**, BIBX 79 in concentrations > 10^{-9} M led to a decrease in cholesterol synthesis. Concomitantly, the accumulation of the two precursors of the OSC step (MES as well as DES) was seen (**Fig. 4**).

24,25-Epoxycholesterol as one prominent (and the only detectable) representative of the regulatory oxysterol class was detected at low levels, but in significant amounts only in concentrations of BIBX 79 of 10^{-8} and 10^{-7} M. No epoxycholesterol was seen at higher concentrations of BIBX 79. This finding confirms the dual role of OSC and also gives an idea on the mode of action of a competitive OSC inhibitor like BIBX 79. With 10^{-8} M BIBX 79, only small amounts of DES were detected. With the occurrence of epoxycholesterol with the same concentration of inhibitor it can be concluded that further cyclization of DES to epoxycholesterol is possi-

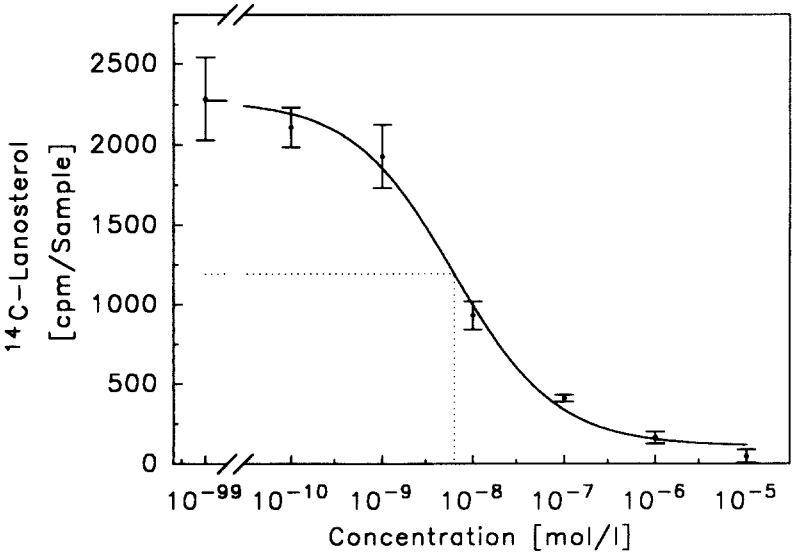

Fig. 3. Inhibition of 2,3-oxidosqualene cyclase by BIBX 79 in cell-free extract of HepG2 cells. HepG2 cell homogenate was incubated with BIBX 79 dissolved in dimethyl sulfoxide (final test concentration 0.1%) together with [^{14}C]MES. After 1 h incubation was stopped by addition of 10% ethanolic KOH. Lipids were extracted with petroleum ether and separated by TLC. Lanosterol bound was scraped off and radioactivity was measured by liquid scintillation counting. Inhibition of OSC was calculated as % [^{14}C]lanosterol versus control after subtraction of blanks. Results are the mean of four experiments (mean ± SD).

TABLE 1. [^{14}C]cholesterol and [^{14}C]cholesterol biosynthesis intermediates in HepG2 cells

	Cholesterol	Monoepoxysqualene	Diepoxysqualene	Epoxycholesterol	Desmosterol	Lanosterol	Dihydrolanosterol
Control	100	bql	bql	bql	bql	bql	bql
BIBX 79							
1E-9M	113.8 ± 16.0	bql	bql	1.6 ± 0.3	bql	bql	bql
1E-8M	76.8 ± 2.8	22.9 ± 5.2	6.2 ± 2.8	8.0 ± 0.4	2.0 ± 0.0	bql	bql
1E-7M	19.1 ± 3.1	71.8 ± 4.0	33.3 ± 16.1	6.1 ± 2.4	2.9 ± 1.9	bql	bql
1E-6M	2.5 ± 1.4	96.8 ± 3.3	43.8 ± 20.2	bql	bql	bql	bql
Ketoconazole							
1E-9M	134.4 ± 22.4	bql	bql	bql	bql	bql	bql
1E-8M	122.8 ± 35.5	bql	bql	bql	bql	bql	bql
1E-7M	31.2 ± 12.6	bql	bql	bql	bql	56.2 ± 22.7	26.3 ± 2.6
1E-6M	bql[a]	bql	bql	bql	bql	101.0 ± 25.7	47.5 ± 20.6
U18666A							
1E-9M	105.9 ± 12.5	bql	bql	bql	4.73	bql	bql
1E-8M	41.7 ± 13.5	2.5 ± 0.0	bql	4.4 ± 3.1	37.9 ± 5.7	bql	bql
1E-7M	bql	46.3 ± 10.5	23.7 ± 8.2	7.6 ± 3.7	10.3 ± 3.5	bql	bql
1E-6M	bql	112.2 ± 0.1	46.3 ± 24.1	bql	bql	bql	bql

For experimental conditions, see legend to Fig. 4. Values were calculated as percent of the control cholesterol peak. Control cholesterol values were set as 100%; bql, below quality limit (< 1% of control peak). Experiments were done in duplicate (BIBX 79, ketoconazole) or in triplicate (U18666A). Values are given as means ± SD. In experiments with ketoconazole and U18666A, in concentrations of > 10^{-7} M, unidentified sterol related peaks were detected at a total level of ca. 15% of the control cholesterol peak.

[a]Due to overlapping between cholesterol and lanosterol peaks, the bql level for cholesterol and lanosterol in this set of experiments is at ~10% of control.

ble. With 10^{-7} M BIBX 79, a further increase in MES and DES was seen but without a parallel increase, even a slight decrease, in epoxycholesterol values. This indicates that at this concentration BIBX 79 also competed with the cyclization of DES that was completely blocked at 10^{-6} M. With 10^{-8} and 10^{-9} M BIBX 79, small amounts of desmosterol occurred. However, the values were only about double the detection limit in our system and are outweighed by far by the precursors MES and DES (Fig. 4). It was therefore concluded that BIBX 79 is a selective OSC inhibitor and no accumulation of sterol products was expected.

The significance of the findings with BIBX 79 becomes even clearer when comparing the HPLC pattern of BIBX 79 with those of ketoconazole and U18666A. Ketoconazole is an inhibitor of cholesterol biosynthesis and blocks mainly the P450-mediated C14-demethylation of lanosterol (23). This finding could be confirmed under our experimental conditions where, beginning with concentrations of 10^{-7} M, a decrease in cholesterol formation was seen with a parallel increase in lanosterol and dihydrolanosterol formation.

U18666A is described as an inhibitor of OSC (24) but also as an inhibitor of the reduction of desmosterol to cholesterol (25). In our experiments U18666A was confirmed as a potent inhibitor of cholesterol biosynthesis that blocks both enzymatic steps, OSC as well as desmosterol reductase. Our data indicate that U18666A preferentially inhibits the late step enzyme of cholesterol biosynthesis. At concentrations of ≥ 10^{-7} M, U18666A blocked OSC to a comparable extent as BIBX 79, but with lower concentrations (≥ 10^{-9} ≤ 10^{-7} M) of U18666A significant amounts of desmosterol were detected. Epoxycholesterol occurred at concentrations of 10^{-7} and 10^{-8} M U18666A, whereas at 10^{-6} M both compounds, U18666A and BIBX 79, totally blocked OSC to an extent where cyclization of DES is also prohibited. These data are in fair consistence with the effects described by Boogaard, Griffioen, and Cohen (24).

MES as well as DES are both cyclized by the enzyme OSC. DES is the product of further enzymatic oxidation of MES by squalene epoxidase. Only DES cyclization ultimately leads to regulatory active oxysterol molecules whereas cyclization of MES will result in the formation of lanosterol and finally cholesterol. When inhibition of OSC results in the above-mentioned dual effect on cholesterol biosynthesis, it is a prerequisite that formation of lanosterol is inhibited and simultaneous cyclization of DES to epoxylanosterol is possible. A higher affinity of DES to the enzyme, or in other words a preferential cyclization of DES over MES, could be one alternative to achieve this goal. Preferential cyclization of DES has been recently reported by Boutaud, Dollis, and Schuber (26) and was also confirmed in the course of these experiments. When equimolar amounts of MES and DES were added to an HepG2 homogenate, which was used as the source of the enzyme, DES was preferentially cyclized. Whereas DES inhibited the cyclization of MES, when added in equimolar amounts, by 80% (**Fig. 5A**), under identical conditions MES did not affect the formation of epoxylanosterol from DES (Fig. 5B). While a 10-fold excess of DES almost abolished the

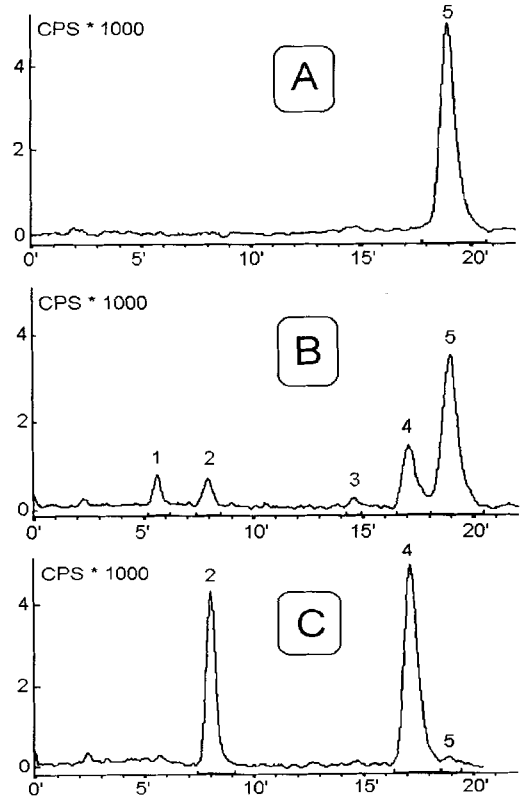

Fig. 4. Formation of different cholesterol precursors in the presence of BIBX 79. HepG2 cells were incubated with BIBX 79 dissolved in dimethylsulfoxide (final test concentration 0.1%). After 15 min [^{14}C]acetate was added and incubation was followed for 2 h. Cells were then treated with trypsin and lipids were extracted by the method of Bligh and Dyer. After purification over Amchro Bond-Elut columns, lipids were separated by HPLC. The column effluent was monitored by an on-line radioactivity detector. CPS, counts per second. Panel A: control; panel B: BIBX 79 10^{-8} M; panel C: BIBX 79 10^{-6} M; 1, epoxycholesterol, 2, diepoxysqualene, 3, desmosterol, 4, monoepoxysqualene, 5, cholesterol.

formation of lanosterol (Fig. 5A), more than a 50-fold excess of MES was necessary to reduce formation of epoxylanosterol to 50% of basal values (Fig. 5B).

Inhibition and regulation of HMG-CoA reductase

To discriminate between a direct interaction and a regulatory effect of BIBX 79 on HMG-CoA reductase, different sets of experiments were performed. To demonstrate that BIBX 79 does not directly inhibit HMG-CoA reductase, HepG2 homogenate was used as a source for this enzyme. Even in high concentrations (10^{-5} and 10^{-6} M) BIBX 79 did not exhibit any inhibitory effect on the enzyme (98 and 97% of control dpm, respectively) whereas with simvastatin, as expected, a concentration-dependent inhibition was seen with an IC_{50} value of 3.5×10^{-11} M (**Fig. 6**). In contrast to that approach, an indirect effect of OSC inhibition on HMG-CoA reductase was observed when HepG2 cells were incubated for 16 h. This experimental approach parallels the experiment on sterol biosynthesis (Fig. 2).

As already described by many groups, inhibition of HMG-CoA reductase with statins leads to a tremendous up-regulation of enzyme activity under in vitro as well as under in vivo conditions (8–11, 27). This was confirmed here when HepG2 cells were incubated with simvastatin for 16 h (**Fig. 7**).

A quite different result was obtained when instead of simvastatin the OSC inhibitor BIBX 79 was used. After 16 h incubation, high concentrations of BIBX 79 led to an increase in HMG-CoA reductase activity whereas with concentrations of BIBX 79 $\geq 10^{-9}$ M and $\leq 10^{-7}$ M a decreased enzyme activity was observed (Fig. 7). Such a "U shaped" concentration-effect relationship was also reported for experiments with U18666A (24).

This finding fits quite nicely with the observations made with the radio HPLC experiment (Table 1 and Fig. 4) and supports the general idea of the reaction

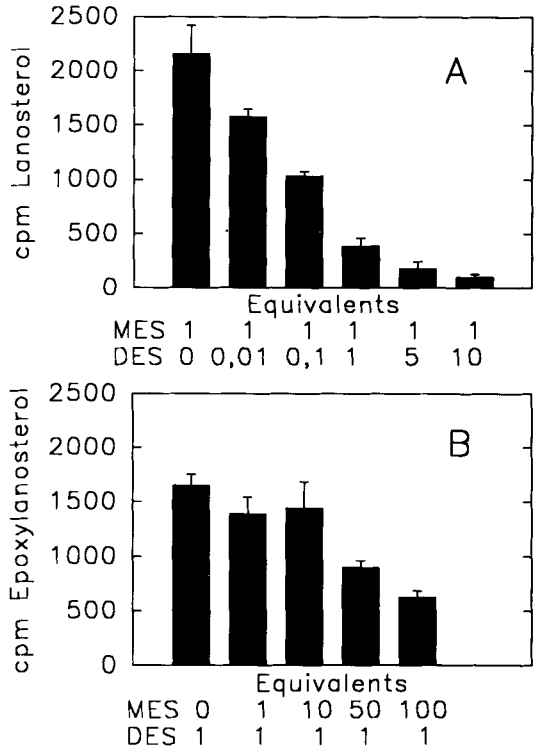

Fig. 5. Competition of 2,3-monoepoxysqualene (MES) with 2,3;22,23-diepoxysqualene (DES) for cyclization by 2,3-oxidosqualene cyclase. Panel A: HepG2 cell homogenate was incubated with a fixed concentration of [^{14}C]MES and increasing concentrations of DES. Incubation conditions as given in Fig. 3. [^{14}C]lanosterol was counted. Given is the mean ± SD of four experiments. Panel B: HepG2 cell homogenate was incubated with a fixed concentration of [^{14}C]DES and increasing concentrations of MES. Incubation conditions as given in Fig. 3. [^{14}C]epoxylanosterol was counted. Given is the mean ± SD of four experiments.

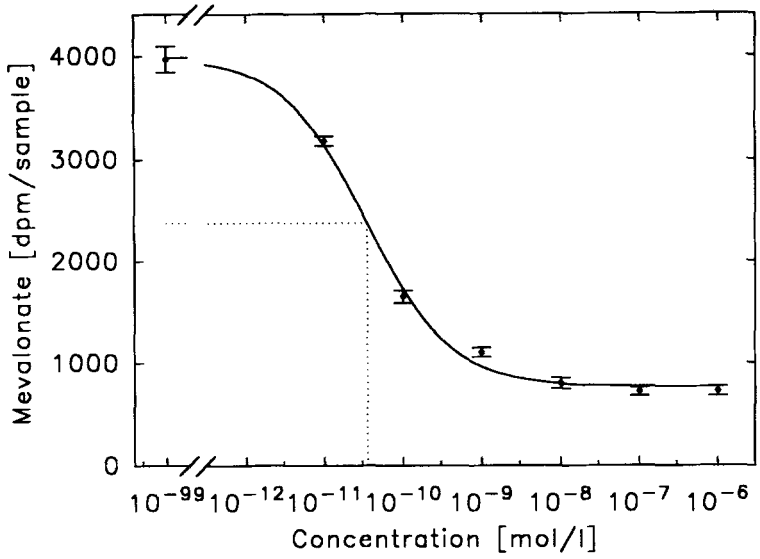

Fig. 6. Inhibition of HMG-CoA reductase activity in the presence of simvastatin. HepG2 cell homogenate was incubated with simvastatin (dissolved in dimethyl sulfoxide, final test concentration 0.1%) together with [^{14}C]HMG-CoA. After 2 h the reaction was stopped by addition of mevalonolactone in HCl. Aliquots of the incubate were chromatographed over AG 1-X8 columns. [^{14}C]mevalonolactone was eluted with H_2O and, after addition of scintillation cocktail, radioactivity was counted. Experiments were run in quadruplicate. Given is the mean ± SD.

mechanism of the squalene oxidase/OSC system. Inhibition of OSC with BIBX 79 will first lead to an increase of the precursor molecule MES which is, at least partially, further oxidized by squalene oxidase to DES. Only DES can lead, via the cyclization by OSC, to the formation of epoxylanosterol and finally to epoxycholesterol. This compound and similar molecules are able to interfere with HMG-CoA reductase by down-regulation of transcription. Because, as shown above, DES is preferentially cyclized, low to moderate concentrations of the inhibitor will lead to an inhibition of the cyclization of MES without interfering with the cyclization of DES to epoxylanosterol. Only when the inhibitor concentration is further increased, is the cyclization of DES decreased and ultimately blocked. In consequence, no oxysterols as negative control elements on HMG-CoA reductase are formed, which results then in an up-regulation of HMG-CoA reductase activity triggered by a decrease in cytoplasmic cholesterol concentration.

DISCUSSION

2,3-Oxidosqualene cyclase plays an important role in the regulation of intracellular cholesterol homeostasis and is therefore an attractive target for drug intervention. This enzyme catalyzes the step from the last non-steroidic to the first steroidic intermediate in the sterol biosynthesis pathway and, more important, it is involved in the formation of regulatory active intermediates. The special function and the significance of this enzyme are a consequence of its ability to cyclize both MES as well as DES, the further oxidized form of MES; furthermore, both substrates compete with each other for the cyclization process. With the help of BIBX 79, a potent and selective inhibitor, we were able to clearly outline the significance of OSC for the intracellular cholesterol homeostasis. Partial inhibition of the OSC will lead to the blockade of the cyclization process of MES to lanos-

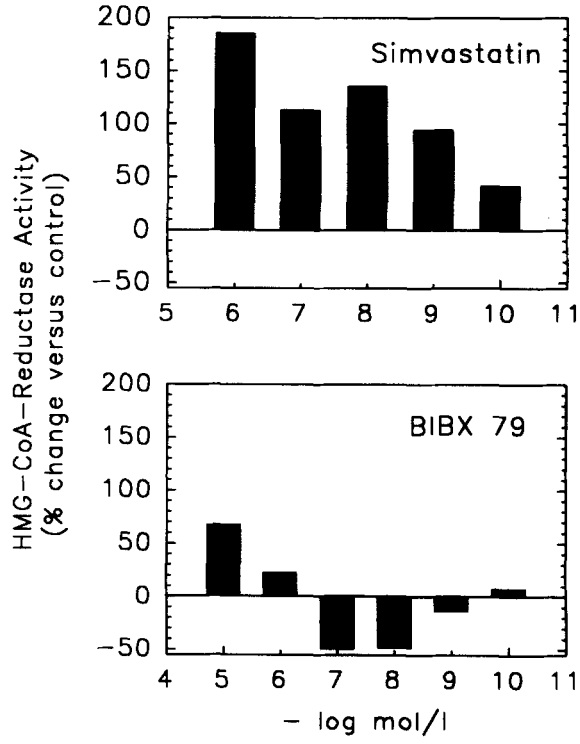

Fig. 7. Effect of simvastatin and BIBX 79 on HMG-CoA reductase activity in HepG2 cells after 2 and 16 h incubation. HepG2 cells were either incubated for 16 h with simvastatin or BIBX 79 (dissolved in dimethyl sulfoxide, final test concentration 0.1%). Cells were then thoroughly washed with MEM and PBS to remove all remainder of the substances that could interfere with HMG-CoA reductase. Determination of enzyme activity was performed as described in Fig. 6. Results are expressed as % change versus control. Experiments were run in quadruplicate.

terol. As a result, MES will accumulate and be subject to further oxidation by squalene oxidase [E.C. 1.14.99.7] (28). DES will be formed and will compete for the cyclization process with MES as well as with a competitive inhibitor like BIBX 79. As shown here, DES is preferentially cyclized by the enzyme. This is most probably due to the fact that the DES molecule, based on its C_{2v}-symmetry, can be equally and effectively cyclized from both ends of the molecule whereas the unsymmetric molecule MES has to adopt the right conformation and orientation of the oxirane ring to be accepted by the active site of OSC. Boutaud et al. (26) report on a 5-fold specificity ratio in favor of a cyclization of DES versus MES. Furthermore, about a 7-fold higher concentration of an inhibitor (U18666A) is needed for a 50% reduction of epoxylanosterol formation when compared with the reduction of lanosterol formation. Thus DES will be able to compete with the inhibitor more effectively and will be cyclized to epoxylanosterol under conditions where cyclization of MES is almost totally blocked. As a result, epoxycholesterol, as well as probably other regulatory active oxysterols, can be formed that interfere with the cholesterol biosynthesis by regulation of enzyme activity such as acetoacetyl coenzyme A thiolase (29), HMG-CoA synthase (30), farnesyl pyrophosphate synthetase (31) and, most importantly, HMG-CoA reductase (6). Oxylanosterol itself has been found to regulate HMG-CoA reductase activity on a posttranscriptional level (15). Furthermore, the degradation of the enzyme is also enhanced in the presence of oxidolanosterol. This is in contrast to the effects described with epoxycholesterol where an interference with the HMG-CoA reductase on the transcriptional level seems to be the most prominent effect. Therefore, different oxysterols complement one another in the regulation of the cholesterol biosynthesis.

The effects of this regulation on the key enzyme of the cholesterol biosynthesis, HMG-CoA reductase, have been studied in the present report. The only regulatory oxysterol found in our experimental setting was epoxycholesterol whereas other molecules such as oxidolanosterol were not detected. This is supported by data of Dollis and Schuber (12) where epoxycholesterol was also found as the only measurable oxysterol after OSC inhibition. Oxysterols at either the transcriptional level or at posttranscriptional sites will influence the substrate flux through the biosynthesis pathway from acetate to cholesterol. With a partial inhibition of OSC, the amount of MES and DES formed as well as the concentration of the resulting oxysterols will depend on the activity of the HMG-CoA reductase. The lower the activity of this enzyme the lower is the substrate flux, eventually leading to lower amounts of MES, DES, and oxysterols which, in turn, will lead to a decrease of the down-regulatory effects on HMG-CoA reductase. That means that the circuit from HMG-CoA reductase via the OSC to MES, DES, and oxysterols and back to HMG-CoA reductase is a feedback loop with a self-regulating and self-limiting

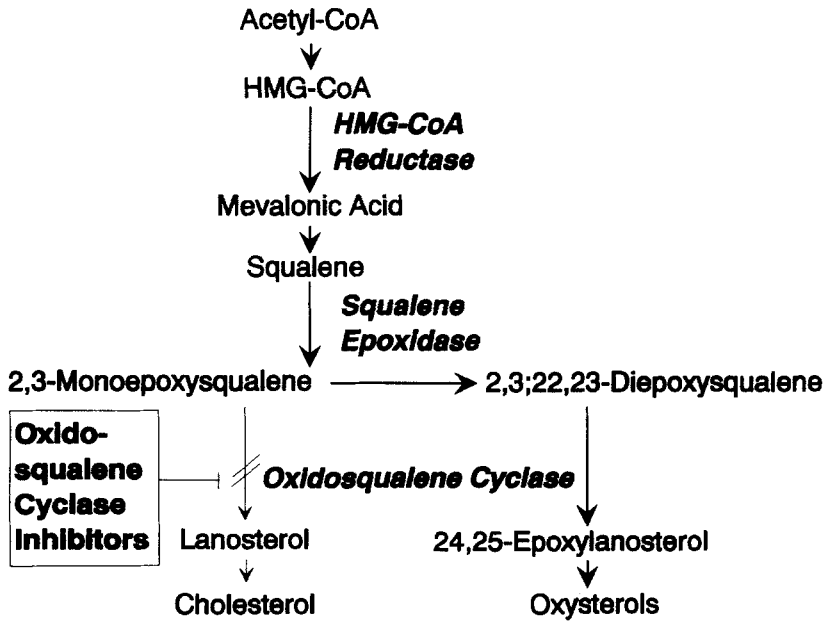

Fig. 8. Schematic model on the role of 2,3-oxidosqualene cyclase in the regulation of intracellular cholesterol homoeostasis. Partial inhibition of OSC will lead to an inhibition of lanosterol and cholesterol formation. Concomitantly, oxysterols can accumulate by cyclization of DES to epoxylanosterol. Oxysterol will control HMG-CoA reductase activity and so control substrate flux through the biosynthesis cascade.

mechanism: the concentration of oxysterols is low enough to avoid a total down-regulation of HMG-CoA reductase but sufficiently high enough to exclude an up-regulation of the HMG-CoA reductase activity as seen e.g., with simvastatin. The explanation for that up-regulation seen with simvastatin is, of course, due to an interference with the early step in the cholesterol biosynthesis leading to a decrease of substrate flux and a lower rate of oxysterol formation. This highlights the outstanding position and also the physiological role of the OSC enzyme. By interfering with this enzyme, a physiologically occurring regulatory mechanism is utilized and so an overshooting reaction is avoided.

Total inhibition of OSC with either high concentrations of a potent inhibitor like BIBX 79 or a noncompetitive or even suicide inhibition will ultimately also block the cyclization of DES with the consequence of a total inhibition of oxysterol formation. The resulting effect then will be identical to that seen with an HMG-CoA reductase inhibitor, i.e., an up-regulation of the HMG-CoA reductase activity (**Fig. 8**).

As there is a reasonable therapeutic window where (as seen with BIBX 79) a partial inhibition of the OSC enzyme can be obtained, the synergistic potential of the inhibition of cholesterol biosynthesis and the regulation via oxysterols can be utilized. Both facets obtained with an inhibition of OSC will then complement each another and their single effects will be potentiated. Ever when the concentration of MES is decreased due to the down-regulation of HMG-CoA reductase, the efficacy of a given concentration of a competitive OSC inhibitor will be increased. Overall, this phenomenon, described here, makes the OSC an attractive target for the development of hypolipidemic agents. In contrast to other inhibitors of enzymes within the cholesterol biosynthesis pathway, like squalene synthase (32) or squalene oxidase (33), there will be no increase and accumulation of precursors as the pathway and substrate flux through the biosynthesis cascade is self-limited, provided a partial inhibition of OSC is obtained. It is further hypothesized that a net reduction of cholesterol biosynthesis will be the result and one might anticipate that this is translated into a net inhibition of VLDL and LDL production. This mechanism of action would then lead to a quite distinct mode of action and possible therapeutic advantage over the statins that do not act via a net inhibition of cholesterol biosynthesis but act preferentially via an increase of LDL receptor number thereby leading to an enhanced LDL catabolism. BIBX 79 and similar compounds offer the possibility for a novel approach for lipid-lowering therapy. However, further studies are needed to substantiate this quality of OSC inhibitors.

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