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THE EFFECTS OF BM 15.766, AN INHIBITOR OF 7-DEHYDROCHOLESTEROL Δ^7-REDUCTASE, ON CHOLESTEROL BIOSYNTHESIS IN PRIMARY RAT HEPATOCYTES

JOHANNES AUFENANGER, JOHANNES PILL,* FELIX H. SCHMIDT and KARLHEINZ STEGMEIER
Medical Research Department, Boehringer Mannheim GmbH, D-6800 Mannheim, Federal Republic of Germany

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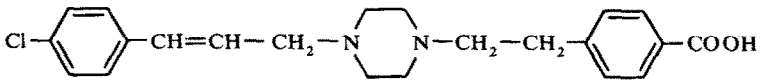
Abstract—The effect of the piperazine derivative BM 15.766 (4-(2-[1-(4-chlorocinnamyl)piperazin-4-yl] ethyl]-benzoic acid) on the biosynthesis of sterols was investigated in adult rat hepatocytes in primary monolayer culture. The substance led to a dose-dependent reduction of cholesterol in the serum of various species of animals such as rat, dog and marmoset.

BM 15.766 showed a dose-dependent action on the incorporation of ^{14}C-acetate in neutral, non-saponifiable lipids. The inhibition of the overall incorporation was 10–12% (10^{-5} M). No inhibition was observed in the hepatocytes over the entire dose range of 10^{-8} M to 2×10^{-5} M, while the release of the neutral lipids from the hepatocytes into the culture medium was reduced by up to 40%.

The biosynthesis of cholesterol could be reduced by more than 90%. Simultaneously, 7-dehydrocholesterol levels rose in the cells and, to a less marked extent, in the medium. This can be interpreted as an indication that 7-dehydrocholesterol is incorporated into the cell membrane, which results in a lower release of 7-dehydrocholesterol into the medium in comparison with controls.

The site of attack is the inhibition of the $\Delta^{5,7}$-sterol Δ^7-reductase. The formation of desmosterol and cholestatrienol as well as other 7-dehydrocholesterol precursors could also be demonstrated.

After longer incubation, there was an additional accumulation of squalene and lanosterol with simultaneous reduction of 7-dehydrocholesterol by BM 15.766, whereas the total ^{14}C-acetate incorporation in neutral lipids was increased.



Scheme 1. Structural formula of BM 15.766.

Epidemiological studies have repeatedly shown that the incidence of atherosclerosis and coronary heart disease are closely related to hyperlipidaemia especially hypercholesterolaemia [1, 2]. Therefore an intense search is being conducted for therapeutically active substance that either reduce plasma levels or prevent a rise therein. In the early 1960s, experiments were carried out with triparanol [3] and AY 9944 [4, 5], which inhibit cholesterol biosynthesis immediately before cholesterol formation. These substances, however, did not prove useful clinically because of adverse reactions and an accumulation of desmosterol and 7-dehydrocholesterol. The latter accumulates especially in certain tissue on treatment with AY 9944 and shows a particular affinity to lung tissue [5]. A further attempt at selective inhibition in an early step namely 3-hydroxy-3-methyl-glutaryl(HMG)-CoA-reductase was carried out with compactin and mevinolin [6]. Other substances, such as clofibrate and bezafibrate, have multilocular sites of attack in the lipid metabolism but they do not inhibit cholesterol biosynthesis selectively.

Former experiments in our laboratory showed a lipid lowering effect of certain piperazine derivatives in different species of animals [7]. This is a report on a new substance, BM 15.766, 4-(2-[1-(4-chlorocinnamyl)piperazin-4-yl]ethyl)benzoic acid, from a series of piperazine derivatives that has been synthesized in the laboratories of Boehringer Mannheim.

The liver represents the central site of lipid metabolism. Rat hepatocytes in primary culture have proved to be particularly suitable for such metabolic investigations [8–11].

To investigate the action of BM 15.766 on sterol biosynthesis, the alteration of the [2-^{14}C]acetate incorporation in neutral lipids and of the conversion of 7-dehydrocholesterol to cholesterol was measured in comparison with controls. The first mentioned technique is mostly used for screening purposes in our laboratory.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Chemicals. BM 15.766 was synthesized in the Chemical Research Department of Boehringer Mannheim, F.R.G. [2-^{14}C]acetate, sodium salt, was obtained from Amersham Buchler, F.R.G.; cholesterol from Boehringer Mannheim; 7-dehydrocholesterol and lanosterol from Sigma Chemie GmbH, Munich, F.R.G.; squalene from Roth, Karlsruhe, F.R.G., and desmosterol from Serva, Heidelberg, F.R.G. The chemicals and media required for cell culture were obtained from Boehringer Mannheim, unless otherwise stated. All other chemicals were obtained in the necessary degrees of purity from the usual chemical suppliers.

Primary hepatocytes cell culture. Primary hepatocytes were obtained under sterile conditions from male and female rats (oestrus phase), weighing between 190 and 240 g, using the collagenase recirculation technique modified from Berry and Friend [9–12] (cellular yield: 2–3 × 10^8 hepatocytes per liver, trypan blue exclusion: ≥ 95%) and cultivated as a monolayer in Dulbecco's modified Eagle's medium [13] with the following additives: 16.5% fetal calf serum, L-glutamine (4 mM), ornithine (0.4 mM), insulin CS (0.25 IU/ml, Hoechst, Frankfurt, F.R.G.), streptomycin (100 μg/ml) and penicillin (100 IU/ml). 3 ml of the hepatocyte suspension (10^6 cells/ml) were plated on a petri dish (6 cm in diameter) with a semipermeable membrane (Petriperm®, Heraeus, Hanau, F.R.G.). The cells were incubated at 37° in 10% CO_2 and 90% air and the medium was exchanged after 16 hr for a lipid-free medium with the addition of [2-^{14}C]acetate (37 kBq/ml) in 100 μM acetate and BM 15.766 dissolved in DMSO (final concentration 0.2%).

For investigating the conversion of 7-dehydrocholesterol, hepatocytes were incubated for 48 hr with [U-^{14}C]7-dehydrocholesterol (5 μg/ml) with a specific activity of 4.44 kBq/ml at a final concentration of about 1.3 × 10^{-5} M in the presence of the inhibitory substance.

Protein determination. The protein determinations in the cell lysate were carried out with the Coomassie blue method [14].

Incorporation and determination of the radioactive precursor into nonsaponifiable neutral lipids. Special experimental conditions are shown under the respective graphs. After incubation of the cells with radioactive substrate, the monolayer was removed from the membrane surface with a rubber spatula, washed twice in isotonic sodium chloride solution

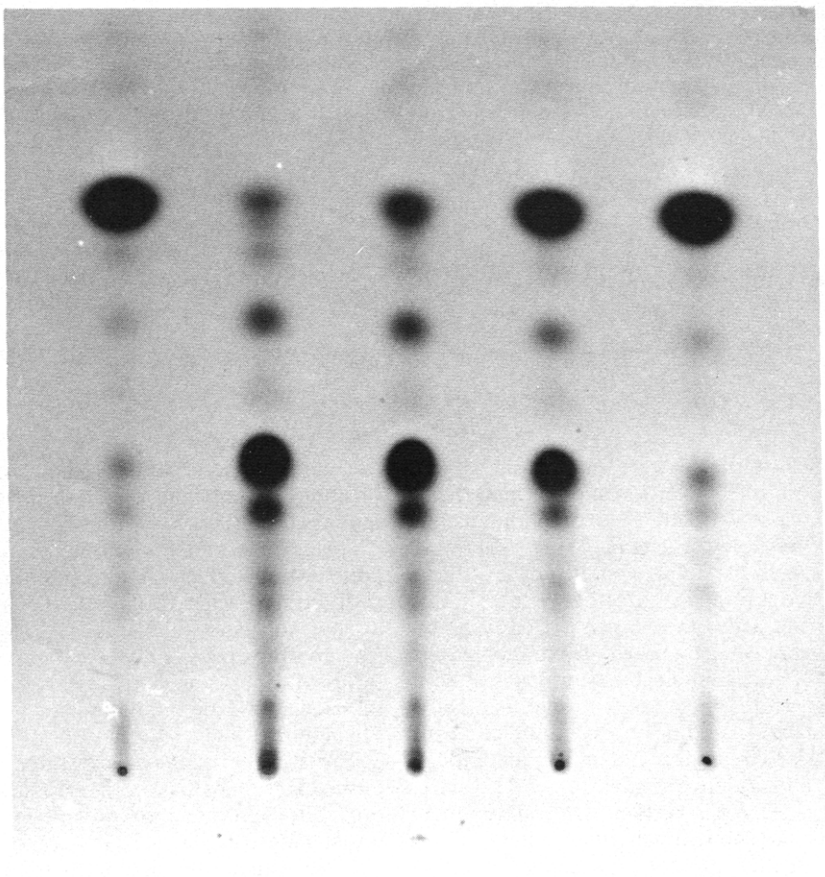


Fig. 1. Autoradiography of the neutral nonsaponifiable lipids after silver ion thin-layer chromatography; dose–response relationship of BM 15.766 (2 × 10^{-5}, 10^{-5}, 10^{-6}, 10^{-7} M and control) to ^{14}C-acetate incorporation in cholesterol and precursors (corresponding to decrease of the R_f-values: lanosterol, squalene, cholesterol, desmosterol, sterol I, sterol II, 7-dehydrocholesterol, sterol III and cholestatrienol) in male rat hepatocytes monolayer culture. The incubation time in serum-free MEM-Dulbecco was 48 hr.

and centrifuged at 4000 *g*. The cell pellet was taken up in 3 ml deionized water and homogenized with ultrasound (Sonifier B 12, Branson). An aliquot of 1.4 ml cell homogenate or culture medium was saponified at 75° in twice its volume of 0.5 M KOH in absolute ethanol. The neutral, nonsaponifiable lipid fractions were extracted using a column with a coarse-pored kieselguhr (Extrelut®, Merck, Darmstadt, F.R.G.; [15]) with *n*-heptane directly into the scintillation vessels. The radioactivity was measured in Aqualuma® (Baker) in a fluid scintillation spectrometer (Packard Tricarb 460 C).

Thin-layer chromatography of the radioactive lipids. The eluate containing the neutral, nonsaponifiable lipids obtained with the Extrelut® was evaporated to dryness in a vacuum concentrator (Vacuum Concentrater, Savant), taken up in 50 µl mobile phase (*n*-heptane/ethyl acetate 2:1, v/v) and applied with an Autospotter (Desaga, Heidelberg, F.R.G.) to the TLC-plates. The pre-coated silica gel plates (silica gel 60 F 254, 20 × 20 cm, Merck, Darmstadt, F.R.G.) had been impregnated for about 4 min with a 3% silver nitrate solution in 97% methanol, allowed to dry briefly in the air and activated at 120° for 15 min. The plates were developed twice, reproducing the path length exactly. The solvent was allowed to evaporate on each occasion in the air. Standards were run on separate paths and were visualized either colorimetrically using Liebermann-Burchard reagent [16] or in u.v.-light.

Qualitative and quantitative evaluation of the thin-layer chromatograms. An X-ray film (Kodak SB), coated on one side, was placed on the TLC-plate and exposed at −20°. The radioactivity, localized by developing the X-ray film, was then scratched out and extracted directly into Aqualuma® [17] and determined in the scintillation spectrometer.

Gas liquid chromatography and mass spectroscopy. The spots on the TLC-plates were extracted after scratching off the silica gel into methanol/ethyl acetate 3:2 (v/v) and, following precipitation of the silver ions with HCl, evaporated to dryness in a vacuum. The crudely purified cholesterol intermediates were converted to the trimethyl-silylether derivatives, further separated by gas chromatography and examined by mass spectroscopy. Mass spectrometer: Finnigan MAT 312, energy: 70 eV, column: 50 m, temperature: 10–200°: 20°/min, 200–280°: 5°/min, carrier gas: helium.

Isolation of [U-^{14}C]*7-dehydrocholesterol*. ^{14}C-7-dehydrocholesterol was synthesized biochemically in the hepatocytes from [2-^{14}C]acetate in the presence of the inhibitor BM 15.766 and separated via a silica gel column (100 × 2.6 cm) impregnated with 3% silver nitrate solution in 97% methanol. It was eluted with n-heptane/ethyl acetate 4:1 (v/v) under nitrogen at a pressure of 50 kPa at a flow rate of 1 ml/min. The [U-^{14}C]7-dehydrocholesterol obtained was rechromatographied by TLC with *n*-heptane, di-isopropylether and glacial acetic acid 60:40:4 (v/v), was subsequently extracted and, after precipitation of the silver ions, was recrystallized twice from ethanol.

Presentation of the results. All the results presented in this paper were obtained from two to three independent studies with different hepatocyte preparations. The incorporation rate is either in terms of absolute cellular protein or in direct comparison with the controls as relative rates. Controls were assigned a value of 1.00 both for hepatocytes and for culture medium. The latter mode of presentation is necessary because of the absolute content of radioactivity in some experiments is 10% lower in the medium than that in the hepatocytes. The standard deviations are <10% and were omitted.

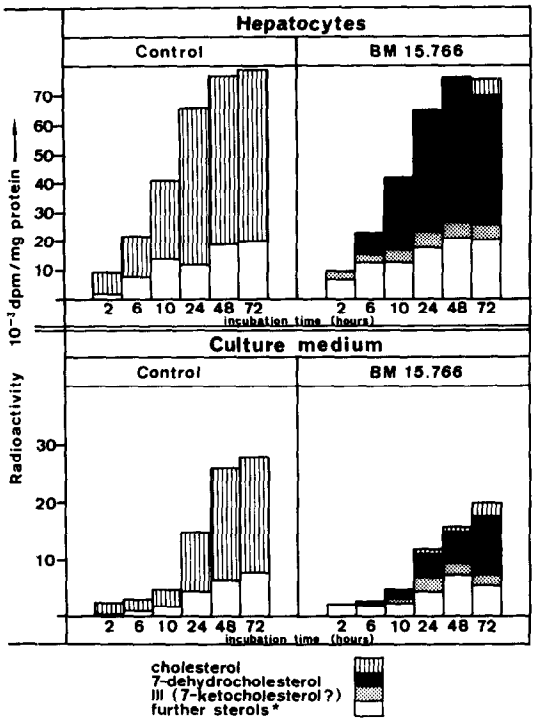


Fig. 2. Biosynthesis of sterols from ^{14}C-acetate in male rat hepatocytes monolayer cultures in the absence and presence of BM 15.766 (10^{-5} M) related to the incubation time (2–72 hr). One single column represents the mean of six cultures from three different male rats. Standard deviation <10%, not shown here. The cells were incubated as described after 16 h preliminary culture in MEM-Dulbecco and 16% fetal calf serum in serum-free MEM-Dulbecco medium with the addition of [2-^{14}C]acetate (37 kBq/ml). After each incubation period the cells and medium were saponified. The neutral lipids were isolated and separated with silver ion thin-layer chromatography. The individual spots were scratched off and their radioactivity content determined. * Lanosterol, squalene, desmosterol, sterol I and sterol II.

RESULTS

Identification of the cholesterol and precursors

Lanosterol, squalene, cholesterol, desmosterol and 7-dehydrocholesterol were reliably identified according to concurrent R_f-values in thin-layer chromatography (Fig. 1) and with the GLC–MS coupling. Cholestatrienol could only be determined as such; no statement could be made about the position of the double bonds. From experiments with the inhibitors AY 9944 and triparanol, there are indications for cholesta-5,7,24-trien-3β-ol [18]. The substances designated I and II show an indication

of cholesta-5,8(14)-dien-3β-ol and cholesta-5,8(9)-dien-3β-ol according to mass spectrometry. The substance III is probably 7-ketocholesterol.

Kinetics of ^{14}C-acetate incorporation

The hepatocytes of the female rats had an incorporation rate of ^{14}C into neutral lipids that was 2–4 times higher than that of the males. The male and female hepatocytes showed essentially the same incorporation behaviour, however. Only under the influence of BM 15.766 the ^{14}C incorporation was in the female hepatocytes relatively lower than that in the males.

The histograms (Fig. 2) show the quantitative distribution of the cholesterol and its precursors in the male rat hepatocytes and in their medium. In control hepatocytes, cholesterol dominates throughout the entire incubation period with about 80%. There were scarcely any cholesterol precursors. Of the other sterols, desmosterol showed up particularly in the initial phase (6–10 hr) and contributed about 12% of the total activity. In the hepatocytes treated with BM 15.766, the synthesis of cholesterol was almost completely inhibited at the beginning (2 hr). 7-Dehydrocholesterol and a small amount of substance III, which probably represents 7-ketocholesterol, appeared in its place. 7-dehydrocholesterol rose steeply and constituted the dominant fraction of the cholesterol precursors. Present in much smaller quantities but characteristic of inhibition with BM 15.766 were the intermediate stages I and II (contained in the column "Further sterols").

The medium (Fig. 2) showed the same pattern as for cells in the controls. The qualitative composition of the patterns for medium and hepatocytes were comparable in the cultures inhibited with BM 15.766. The lower total sterol content compared with controls (minimum at 48 hr) and the relatively low fraction of 7-dehydrocholesterol in the total sterol fraction was readily visible.

In the female rats (Table 1), 7-dehydrocholesterol was accumulated to a considerably lesser degree, but the proportion of fraction III was much higher. Fractions I and II also increased somewhat. The inhibition of the cholesterol synthesis regressed much earlier and, at 48 hr, cholesterol contributed 18%.

Dose–response relationship of BM 15.766

The BM 15.766 dose related inhibition of Δ^7-reductase and the associated accumulation of cholesterol precursors in the hepatocytes was investigated. The incubation period was 48 hr. The maximum dose was 2×10^{-5} M and could not be increased any further because of the poor solubility of BM 15.766 without producing solvent effects.

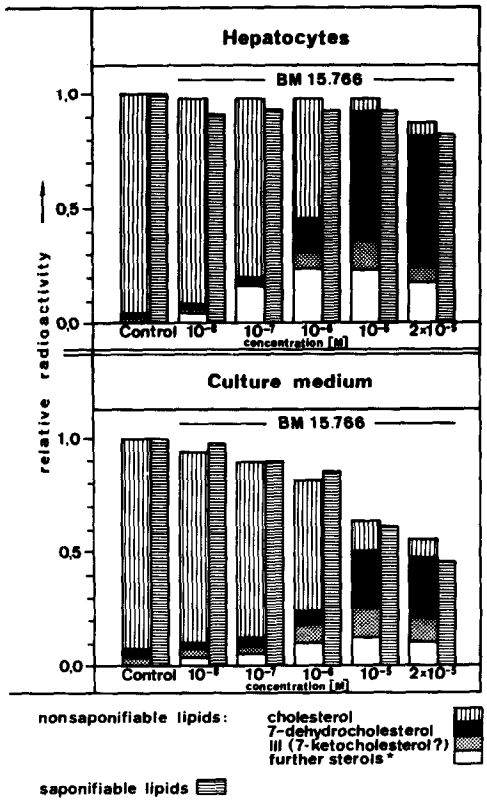


Fig. 3. BM 15.766 dose-related inhibition of ^{14}C-acetate incorporation in lipids in male rat hepatocytes monolayer cultures. The incubation time in serum-free MEM-Dulbecco with [2-^{14}C]acetate and BM 15.766 was 48 hr. The saponifiable lipid fraction contained all sterols and their esters as well as the triglycerides. All values are related to the respective controls = 1.0. Three independent experiments with three rats were carried out. One column represents the mean of 9 experiments; standard deviation <10% (not plotted). * Lanosterol, squalene, desmosterol, sterol I and sterol II.

The relative distribution of cholesterol and its precursors in comparison with controls is given in Fig. 3. In the hepatocytes no inhibition of the ^{14}C-acetate incorporation could be shown over a wide dose range from 10^{-8} to 10^{-5} M in the non-saponifiable and in the saponifiable neutral lipids. A slight inhibition only appeared at 2×10^{-5} M. In contrast, there was already a marked shift in the

Table 1. Relations between individual sterol fractions in hepatocytes from female rats. Same experimental conditions as for Fig. 2. The female rats were in the oestrus phase

Incubation time (hr)	2	6	10	24	48	72
Ratio: 7-DHC_{15766} : $\text{Cholesterol}_{15.766}$	1.1	2.2	4.7	2.0	1.6	1.1
Ratio: $\text{Cholesterol}_{15766}$: $\text{Cholesterol}_{\text{Controls}}$	0.04	0.04	0.04	0.09	0.18	0.26
Ratio: III_{15766} : $\text{Cholesterol}_{\text{Controls}}$	—	—	0.16	0.15	0.15	0.17

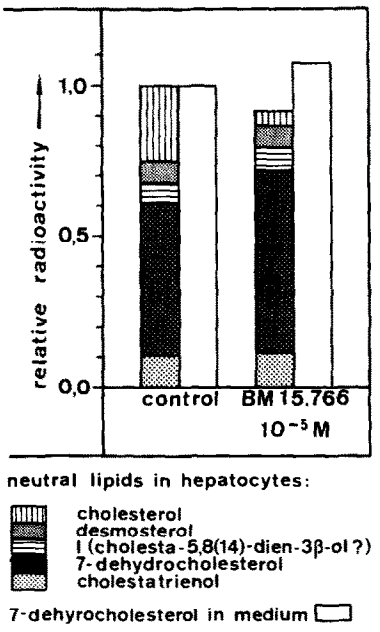


Fig. 4. Incorporation of [U-^{14}C]7-dehydrocholesterol in the sterol fractions of saponifiable neutral lipids in the absence and presence of BM 15.766 (10^{-5} M) in rat hepatocytes monolayer cultures (incubation time: 48 hr, two independent experiments with two different male rats).

sterol pattern in favour of 7-dehydrocholesterol above 10^{-6} M, while the fraction of cholesterol at 10^{-5} M BM 15.766 was insignificant. The fraction of intermediate product III rose slightly with increasing doses. In the medium, there was a dose-dependent decrease of the nonsaponifiable neutral lipids up to 45% at 2×10^{-5} M BM 15.766 and of the saponifiable neutral lipids of 54%. Here, too, the shift in sterol pattern from cholesterol to 7-dehydro-cholesterol was clearly seen and corresponded to that of the hepatocytes. Only the relative fraction of 7-dehydrocholesterol in the total sterols in the medium was lowered compared with that in the cells.

Conversion of 7-dehydrocholesterol to cholesterol

The aim of this experiment was to show the direct effect of BM 15.766 on the conversion of [U-^{14}C] 7-dehydrocholesterol to cholesterol. The results are shown in Fig. 4. The fraction of newly synthesized cholesterol contributing to total labelled sterols in the cells after 25 hr incubation was 25% on controls. In addition, 7-dehydrocholesterol was converted to other cholesterol precursors, i.e. desmosterol, cholestatrienol and substance I. Under inhibition with BM 15.766, only 16% of the cholesterol formed in the controls was synthesized, while the 7-dehydrocholesterol fraction in the cells increased. The other fractions remained practically unchanged.

DISCUSSION

The cholesterol synthesis was almost totally inhibited by BM 15.766 at 10^{-5} M. The ratio of 7-dehydrocholesterol to cholesterol rose during incubation in favour of 7-dehydrocholesterol. At 48 hr there was almost 30 times more 7-dehydrocholesterol than cholesterol.

The experiments with [U-^{14}C]7-dehydrocholesterol show the direct action of BM 15.766 on the conversion of 7-dehydrocholesterol to cholesterol.

The BM 15.766-dependent intermediates I and II, which give indications in mass spectroscopy for cholesta-5,8-dienol and cholesta-5,8(14)-dienol, can be explained according to Goad [19] by a desaturation of the Δ^5-double bond which is only possible in the presence of Δ^7-double bonds, i.e. after isomerisation of Δ^8 to Δ^7. Otherwise an inversion of the reactions of the enzymes is described too [20, 21].

The intermediate III (probably 7-ketocholesterol) is known as an autoxidation product of cholesterol and has a high inhibitory effect on HMG-CoA reductase [22]. The dose-dependence of the increase of 7-ketocholesterol would be an indication for a direct role of BM 15.766 in its biosynthesis and not for an artificial formation. An inhibition of the overall cholesterol biosynthesis by 7-keto-cholesterol could, however, be causally associated.

Relatively little 7-dehydrocholesterol accumulates in the hepatocytes of female rats. The considerably higher content of newly synthesized cholesterol indicates that the inhibition was not maximum. A possible explanation is a rapid metabolism of the inhibitory substance, and/or that the female hepatocytes require a higher active dose to produce the same inhibition.

It is notable that a dose related reduction of lipid content is found in the medium in comparison with controls during incubation with BM 15.766, while the hepatocytes do not show any such dependence. The relative fraction of 7-dehydrocholesterol in the total lipids in the medium is less than that in the hepatocytes. This could be an indication for secretion of 7-dehydrocholesterol and not for leakage.

The results obtained with BM 15.766 in hepatocytes are comparable with those obtained in *in vivo* experiments [5, 23] and those obtained with rat livers perfused with AY 9944 [24] with respect to dose-dependence and incubation time.

The therapeutic value of BM 15.766 or of any substance that interferes with the later stages of sterol biosynthesis is more than doubtful. In contrast to AY 9944 BM 15.766 was tolerated well in different animals [7] in long term applications. Therefore, BM 15.766 provides an interesting tool for the further investigation of sterol biosynthesis.

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