Effects of a novel 2,3-oxidosqualene cyclase inhibitor on cholesterol biosynthesis and lipid metabolism in vivo

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Abstract BIBB 515 (1-(4-chlorobenzoyl)-4-((4-(2-oxazolin-2-yl)benzylidene))piperidine) is a potent and selective inhibitor of 2,3-oxidosqualene cyclase (OSC) [EC 5.4.99.7]. In rats and mice BIBB 515 inhibited OSC in vivo in a dose-dependent manner after 1, 3, and 5 h with ED_{50} values from 0.2 to 0.5 mg/kg (1 to 5 h) in rats and 0.36 (1 h) to 15.5 (3 h) and 33.3 (5 h) mg/kg in mice. Inhibition of [^{14}C]acetate incorporation into sterols was found to parallel the effects on OSC when measured after 1 h (mice) or 3 h (rats). ED_{50} calculated were 0.9 mg/kg (mice) and 0.1 mg/kg (rats). Dose-dependent lipid-lowering activity was seen in normolipemic hamsters after 11 days treatment (−19% for total cholesterol and −32% for VLDL + LDL cholesterol at 55 mg/kg BIBB 515 per day) and in hyperlipemic hamsters after 25 days (−25% for total cholesterol and −59% for LDL-cholesterol at 148 mg/kg BIBB 515 per day). Calculation of kinetic parameters revealed no relevant differences between control and treatment groups in LDL clearance or fractional catabolic rates, but significant reductions of LDL production rates (−30% to −54%). Liver LDL receptor mRNA of the treated animals was not or only slightly increased. Liver VLDL secretion as measured by the Triton WR1339 method was reduced after BIBB 515 in rats and hamsters. It is concluded that the lipid-lowering effect of BIBB 515 is mainly the result of an inhibition of LDL production rather than due to an increase in LDL catabolism. OSC inhibitors may offer a novel approach for lipid-lowering therapy.—**Eisele, B., R. Budzinski, P. Müller, R. Maier, and M. Mark.** Effects of a novel 2,3-oxidosqualene cyclase inhibitor on cholesterol biosynthesis and lipid metabolism in vivo. *J. Lipid Res.* 1997. **38**: 564–575.

Supplementary key words HMG-CoA reductase • squalene oxidase • 2,3-monoepoxysqualene • 2,3;22,23-diepoxysqualene • lanosterol • oxysterols • LDL kinetics • VLDL secretion

Interfering with cholesterol biosynthesis has been shown to be an effective therapeutic approach to lower elevated plasma cholesterol levels. The only available class of compounds successfully exploiting that mechanism are the HMG-CoA reductase inhibitors. These compounds (e.g., simvastatin) inhibit the rate-limiting enzyme of the cholesterol synthesis cascade, HMG-CoA reductase (1, 2). The lipid-lowering effect of HMG-CoA reductase inhibitors is mediated via an up-regulation of LDL-receptors thus leading to an enhanced LDL cholesterol catabolism (3–7). On the other hand, HMG-CoA reductase inhibition will result in an up-regulation of HMG-CoA reductase synthesis as is evidenced by in vitro and in vivo results (8–12).

In a recent report we have proposed the enzyme 2,3-oxidosqualene cyclase (OSC) (EC 5.4.99.7) as another attractive target to interfere with the cholesterol biosynthesis path (13). With BIBX 79 as a potent and selective OSC inhibitor it could be demonstrated, that by interfering with the OSC two synergistically acting mechanisms will contribute to the overall inhibition of cholesterol biosynthesis by 1) direct inhibition of OSC, thereby reduction of cyclization of 2,3-monoepoxysqualene (MES) to lanosterol and further to cholesterol; and 2) repression of HMG-CoA reductase activity via the increased formation of 2,3;22,23-diepoxysqualene (DES) and oxysterols (13–17). It was hypothesized in that publication that OSC inhibition will result in a net reduction of cholesterol biosynthesis which might be translated into a net inhibition of lipoprotein production.

Therefore, the aim of the present study was to investigate the in vivo effects of OSC inhibition. Using BIBB 515, another potent and specific OSC inhibitor of a similar chemical class as BIBX 79, the effects on cholesterol biosynthesis in rats and mice as well as effects on lipoprotein levels and lipoprotein kinetics in hamsters were studied.

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.

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EXPERIMENTAL PROCEDURES

Chemicals

Cholesterol was obtained from Serva Heidelberg Germany and digitonin from Merck, Darmstadt, Germany. AG 1-X8, the anion exchange resin, came from Bio-Rad. [2-^{14}C]acetate and DL-3-[^{14}C]hydroxy-3-methylglutaryl coenzyme A and [α-^{32}P]deoxycytidine-5′-triphosphate were obtained from New England Nuclear. Triton WR 1339® was purchased from Serva, Heidelberg, Germany.

BIBB 515

BIBB 515 (1-(4-chlorbenzoyl)-4-((4-(2-oxazolin-2-yl)benzylidene))piperidine) (**Fig. 1**) was designed as a transition state analog of the OSC reaction (18). The synthesis of BIBB 515 was performed by the following steps.

Diethyl 4-cyanobenzylphosphonate. 4-Bromomethylbenzonitrile (98 g) and triethylphosphite (300 ml) were heated to 140°C (bath temperature). After the reaction started the mixture was heated to reflux (bath temperature 150–160°C) for 2 h, the formed ethyl bromide distilled off, and the mixture was heated to 150°C for another hour. Excess triethylphosphite was distilled off in vacuo, the residue was treated with cyclohexane (250 ml) at 0°C, the crystals were filtered off and washed with cyclohexane (150 ml) to give diethyl-4-cyanobenzylphosphonate (125.6 g): mp 41.5–43°C.

N-(4-cholorobenzoyl-4-piperidone. To a suspension of pulverized 4-piperidone-hydrochloride-hydrate (80.6 g) in THF (1.0 l) were added first 4-chlorobenzoylchloride (87.5 g) and then a solution of potassium carbonate (276 g) in water (552 ml), cooled to 5°C. The mixture was stirred for 45 min at room temperature, the organic phase separated, and the aqueous phase was extracted with ethyl acetate (2×). Workup of the combined organic phase gave a residue that was dissolved in ethyl acetate. Addition of petroleum ether gave N-(4-chlorobenzoyl)-4-piperidone (88.6 g): mp 61–63°C.

1-(4-Chlorobenzoyl)-4-(4-cyanobenzylidene)piperidine. To lithium diisopropyl-amide, prepared from diisopropylamine (3.34 g) in THF (20 ml) and a 1.6 M solution of n-butyl lithium in n-hexane (19 ml), was added at −50°C diethyl 4-cyanobenzylphosphonate (7.6 g) in THF (50 ml). After 20 min at −50°C N-(4-chlorobenzoyl)-4-piperidone (7.13 g) in THF (30 ml) was added. The cooling bath was removed, the mixture was stirred for 2 h at room temperature, poured into ice, and extracted with ethyl acetate. Workup of the organic extract gave 1-(4-chlorobenzoyl)-4-(4-cyanobenzylidene) piperidine (7.6 g): mp 134–135.5°C (ethyl acetate).

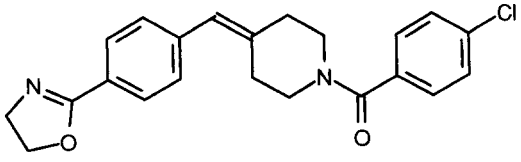

Fig. 1. Chemical structure of BIBB 515.

1-(4-Chlorobenzoyl)-4-(4-ethoximidoyl-benzylidene)piperidine hydrochloride. Gaseous hydrogen chloride (40.7 g) was introduced into a suspension of 1-(4-chlorobenzoyl)-4-(4-cyanobenzylidene)piperidine (10.8 g) in dry ethanol (70 ml). After 16 h at room temperature the solvent was removed (bath temperature 30°C), the residue was dissolved in ethanol (35 ml), and the solvent was again removed. Trituration with ethyl acetate of the residue gave 1-(4-chlorobenzoyl)-4-(4-ethoxyimidoyl-benzylidene)piperidine hydrochloride (13.2 g) as a colorless powder.

1-(4-Chlorobenzoyl)-4-((4-(2-oxazolin-2-yl)benzylidene))piperidine. 1-(4-Chlorobenzoyl)-4-(4-ethoximidoyl-benzylidene) piperidine hydrochloride (13.2 g) was suspended in dry ethanol (59 ml); ethanolamine (2.64 g) and triethylamine (6.5 g) were added and the mixture was heated to reflux for 1.5 h. After cooling in ice the crystals were filtered off and washed with ethanol to give 1-(4-chlorobenzoyl)-4-((4-(2-oxazolin-2-yl)benzylidene)) piperidine (10.4 g): mp 181–183°C.

In vitro studies in HepG2 cells

Sterol synthesis, 2,3-oxidosqualene cyclase activity, HMG-CoA reductase activity, and the specificity of BIBB 515 versus 2,3-oxidosqualene cyclase were measured in intact HepG2 cells or cell homogenates thereof as described recently (13).

Studies in rats and mice: Animals

Male Wistar rats, strain Chbb:THOM with a body weight of 160–180 g or male mice, strain Chbb:NMRI weighing 33–37 g were used. Animals were fed ad libitum with standard pelleted diets. Animals were housed under a 12/12 h light/dark rhythm with 07.00 AM to 07.00 PM being the light phase. Experiments were started, when not otherwise stated, between 08.00 and 09.00 AM.

Blood sampling and liver removal

Blood was sampled from the retrobulbar venous plexus with the animals under light isoflurane (Forene®, Abbott) anesthesia. For liver removal, animals were killed by cervical dislocation and livers were immediately removed and frozen at −30°C until analysis.

Inhibition of OSC

One, 3, and 5 h after oral administration of BIBB 515 to the animals, plasma was obtained (rats) or livers were

removed (mice). Calciferol as internal standard was added to plasma samples or homogenized liver aliquots (0.5 g). Lipid extraction was done according to Bligh and Dyer (19). After purification over Amchro-Bond Elut columns, lipids were extracted into methanol for separation by HPLC on a 2.0 × 150 mm ODS-Hypersil 5 μm using acetonitrile–water 87:13 as the mobile phase at 1 ml/min. The column effluent was monitored by an on-line UV photometer at 210 nm wave length. Values were normalized to the internal standard and are expressed in μg/ml (plasma) or μg/g (liver tissue). Four animals per group were used.

Inhibition of cholesterol biosynthesis

These experiments were started at 01.00 PM to take advantage of a very stable phase of cholesterol biosynthesis within the diurnal rhythm of the animals. Substances were administered by gavage to groups of 5 animals each. After 5 min (mice) or 2 h (rats) all animals were injected intraperitoneally with 740 kBq [^{14}C]acetate. Sixty min later animals were killed and livers were removed. Samples of 0.5 g liver tissue each were saponified in ethanolic KOH (10%) at 60°C for 1 h. Non-saponified materials were extracted with petroleum ether, evaporated, and dissolved in ethanol–acetone 1:1(v/v). Sterols were then precipitated with digitonin. After washing, the precipitate was dissolved in methanol and after addition of scintillation cocktail, radioactivity was counted with a liquid scintillation counter.

Protein in liver samples was determined according to the method described by Lowry et al. (20) with human albumin as standard. Data were expressed as ^{14}C labeled sterols (cpm) per mg liver protein.

Calculation of results

Calculation of IC_{50} values was performed using the curve fit (Solver) function of Microsoft Excel 5® fitting to the equations $y = b + [a*k/(k + x^n)]$ (descending values) and $y = b + [a*(x^n/(k + x^n))]$ (ascending values); 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 the lower asymptotes of the dose response curve; and b is the baseline level.

Studies in hamsters: Animals

Male golden Syrian hamsters (Han: AURA, Central Institute for Laboratory Animal Breeding, Hannover, Germany) weighing ~100 g at the start of the experiment were housed individually on wood shavings. Normolipemic hamsters were fed a standard hamster diet (NAFAG No. 924) ad libitum. Hyperlipemic hamsters were fed the same diet containing 0.12% cholesterol. They were kept on the hyperlipemic diet for 2 months prior to the start of the experiment and the diet was continued throughout the experiment. The animals were subjected to a light cycle of light 08:30 AM to 08:30 PM and dark 08:30 PM to 08:30 AM.

Dosage

The substances were administered as diet admixture. Substances were dissolved in ethanol and distributed over the pelleted diet. Ethanol was evaporated over a period of at least 3 days. Control diet was prepared with ethanol alone. Concentrations of BIBB 515 in the diet were 0.03 and 0.1% (normolipemic animals) or 0.03, 0.1, and 0.3% (hyperlipemic animals).

Study design

Normolipemic hamsters were treated with BIBB 515 for a 20-day period; hyperlipemic hamsters were treated with BIBB 515 for a 40-day period. An exactly weighed amount of the substance-containing diet was supplied to the animals. The daily substance dosage was calculated on the basis of food consumption and body weight for each individual animal.

Normolipemic animals

Study groups consisted of 7 animals each. They were randomized to the groups according to their baseline (day 0) total cholesterol levels. The groups were subjected to the following treatment scheme: 1 control group and 2 substance treatment groups (0.03 and 0.1% BIBB 515 in the diet). Body weight and food consumption of the animals were followed over the experiment. At day 11 blood was collected for examination of beta-lipoprotein cholesterol and HDL cholesterol values. Body weight as well as food consumption were determined. At day 12, and under light isoflurane (Forene®, Abbott) anesthesia, animals were injected iv with tracer amounts of ^{125}I-labeled hamster LDL and 300-μl samples of blood were taken at 10 time points during the next 48 h for investigation of LDL kinetics. At day 21, the animals were killed under isoflurane anesthesia, the abdomen was opened, the livers were removed and immediately frozen in liquid nitrogen for mRNA determination. The animals were fasted for 24 h prior to being killed.

Hyperlipemic animals

Animals were randomized to four groups according to their baseline (day 0) total cholesterol levels. The four groups were subjected to the following treatment scheme: one control group and three substance treatment groups (0.03, 0.1, and 0.3% BIBB 515 in the diet). At day 25, blood was collected for examination of cholesterol values. Body weight as well as food consumption were determined over the first 25 days of the experiment. At day 33 and under light isoflurane anesthesia,

animals were injected iv with tracer amounts of ^{125}I-labeled hamster LDL and 100-µl samples of blood were taken at 10 time points during the next 48 h for the evaluation of LDL kinetics. At day 40, after a 24-h fasting period, the animals were killed under isoflurane anesthesia, the abdomen was opened, the livers were removed and immediately frozen in liquid nitrogen for mRNA determination. Recovery time between lipoprotein profile determination and LDL kinetics was longer in hyperlipemic hamsters to allow for the larger amount of blood taken for complete lipoprotein profile determination in hyperlipemic hamsters (see below).

Blood sampling

Blood was collected from the retrobulbar venous plexus between 08.00 and 09.00 AM with the animals under light isoflurane (Forene®, Abbott) anesthesia.

Determinations

Serum total cholesterol as well as the cholesterol content of the HDL and VLDL/LDL fractions were measured with the cholesterol esterase/cholesterol oxidase method (cholesterol CHOD-PAP test kit No. 236691, Boehringer Mannheim, Germany). The determinations were carried out with an Eppendorf 5040 automatic substrate analyzer. The method used for isolation of lipoprotein fractions was ultracentrifugation (2 h, 4°C, 100000 rpm) with a KBr adjusted density of 1.063 kg/l for flotation of LDL + VLDL. In normolipemic hamsters, due to the low values, only VLDL/LDL and HDL lipoprotein fractions were separated whereas in hyperlipemic animals VLDL and LDL cholesterol values were determined individually. Therefore an additional centrifugation at a density of 1.020 was performed.

Lipoprotein iodination

Hamster LDL was isolated by ultracentrifugation at densities between 1.020 and 1.063 kg/l (21) and was iodinated with Na^{125}I by the iodine monochloride method as described earlier (22). Of the LDL-associated radioactivity, 93% to 97% was precipitable by trichloroacetic acid. (The specific activities of the different preparations are indicated in Table 2.) To protect LDL from radiation damage, 1.2 ml of hamster serum was added to 5 ml of iodinated LDL and the mixture was sterilized by filtration. The preparation was used the day after the iodination.

^{125}I-labeled LDL kinetics

^{125}I-labeled LDL was injected into the femoral vein of the hamsters (with the animals under light isoflurane anesthesia) in the amount indicated in Table 2. Three hundred µl blood was taken at 0.5, 1, 2, 4, 6, 8, 10, 24, and 48 h in normolipemic hamster experiments and 100 µl blood was taken at 0.33, 1, 2, 4, 6, 8, 10, 24, and 48 h in hyperlipemic hamsters. Fifty µl (normolipemic) or 40 µl (hyperlipemic animals) heparin plasma was counted in a scintillation counter. From the specific activity and the composition of ^{125}I-labeled LDL shown in Table 2, cpm-values were transformed into µmol of ^{125}I-labeled LDL-cholesterol.

Data analysis

The ^{125}I-labeled LDL decay data were fitted to an iv two-compartment model by the TopFit nonlinear iteration program (23). The parameters calculated by the program are listed in Table 2. C_0 = extrapolated zero time concentration, and V_c = distribution volume to the central compartment. The LDL production rate ^0k is calculated as $^0k = C_{SS} * Cl$ where C_{SS} is the steady state endogenous (''cold'') serum LDL concentration and Cl is the ^{125}I-labeled LDL-clearance. The fractional catabolic rate is the ratio of clearance and V_c.

Students *t*-test was used for statistical comparison of the data with the conventional $P = 0.05$ as the level of significance. P values below 0.05 were given the following symbols in the figures: * < 0.05, ** < 0.01, *** < 0.001. Nonsignificant changes were marked n.s. or ns.

RNA determination

Total RNA was isolated using an acidic guanidinium thiocyanate/phenol/chloroform method (24). Poly(A)$^+$RNA was prepared either with the Promega PolyATract Kit (normolipemic hamsters) or the Oligotex-dT Kit of Qiagen (hyperlipemic hamsters) according to the manufacturer's instructions. RNA was electrophoresed in 1.2% agarose/6.6% formaldehyde gels followed by capillary transfer to Hybond N membranes (Amersham). The blots were hybridized with [^{32}P]-dCTPlabeled cDNAs of human LDL receptor, rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or hamster HMG-CoA reductase. After autoradiography the relative amount of specific mRNA was determined by densitometric analysis. Values for mRNA of LDL receptor and HMG-CoA reductase were normalized according to the hybridization signal of GAPDH of the same probe. Data are expressed relative to the mean of the corresponding control group.

Triton WR1339 experiments

Triton was injected iv via the tail vein of rats under isoflurane (Forene®, Abbott) anesthesia or via the femoral vein of hamsters under phenobarbital anesthesia at about 5:00 PM at a dose of 600 mg/kg. BIBB 515 was given orally 30 min (rats) or 60 min (hamsters) before the Triton injection. The animals were deprived of food 3 h (rats) or directly (hamsters) before the oral adminis-

tration of BIBB 515. Blood was drawn from the retrobulbar venous plexus immediately before the oral administration of BIBB 515 and 15 h after the Triton injection. Secretion rates were calculated as increase in cholesterol (μmol/ml), triglyceride (μmol/ml) or apoB (ng/ml) per h times ml of plasma volume per 100 g body weight. Plasma volume was taken as 4.0 ml/100 g body weight (rats) or 3.8 ml/100 g body weight (hamsters) (25).

Apolipoprotein B determination

Chicken anti-rat apoB was purified from egg-yolk of chicken immunized with rat apoB purified from rat VLDL. The antibody does not discriminate between B-48 and B-100 and also crossreacts with hamster apoB. ApoB in rat plasma was determined by a competitive ELISA. Maxisorb microtiter plates (Greiner) were coated overnight at 4°C with rat VLDL (0.32 μg/ml in 50 mM Na-bicarbonate, pH 9.6) followed by 3 washings with phosphate-buffered saline (PBS). Probes in suitable dilution were preincubated for 1 h at room temperature with anti-rat apoB (in PBS containing 0.1% Triton X-100, 10% bovine serum albumin, and 1 mM EDTA) and transferred to the microtiter plates. After 2 h incubation at room temperature, plates were washed 3 times with PBS containing 0.1% Triton followed by 1 h incubation at 37°C with horseradish peroxidase-conjugated anti-chicken antibody (in 0.5% Triton containing PBS) and color development with ortho phenylene diamine (OPD) and quantitation in a Molecular Devices plate reader. All buffers contained 0.01% NaN_3. Working range was 5–500 ng/ml final antigen concentration. ApoB in hamster β-lipoproteins centrifuged from hamster plasma (d 1.063) was determined by a saturation ELISA. Probes were incubated on Maxisorb microtiter plates for 1 h at 37°C. Then the plates were washed 3 times with PBS followed by incubation for 1 h with saturation buffer (SB = 1% BSA + 1 mM EDTA in PBS), 3 washings with PBS containing 0.5% Triton (PBST), incubation with chicken anti-rat apoB (in 0.5% Triton SB) for 1 h, 3 washings with PBST, and incubation at 37°C with horseradish peroxidase-conjugated anti-chicken antibody and color development and quantitation as described above.

RESULTS

In vitro studies

Concentration-dependent inhibition of cholesterol biosynthesis by BIBB 515 as monitored by [^{14}C]-acetate incorporation into digitonin precipitable sterols could be demonstrated in HepG2 cells (ED_{50} = 4.11 * 10^{-9} ± 1.71 * 10^{-9} M). A similar inhibition of OSC activity (ED_{50} = 8.69 * 10^{-9} ± 2.72 * 10^{-9} M) was seen in HepG2 cell homogenates. No inhibition of HMG-CoA reductase could be measured in HepG2 cell homogenates at concentrations of BIBB 515 up to 1 and 10 μM (activity = 105% and 98% of control respectively). The ED_{50} values shown are means of 4 experiments ± SD.

Specificity of BIBB 515 versus 2,3-oxidosqualene cyclase

As was demonstrated for BIBX 79, an earlier compound of this series (13), BIBB 515 also proved to be a specific inhibitor of OSC. In a radio HPLC system, a concentration-dependent decrease in [^{14}C]cholesterol formation was seen. No significant [^{14}C]acetate incorporation into cholesterol intermediates other than MES and DES could be detected (**Table 1**). The formation of the regulatory 24,25-epoxycholesterol (14) which results from the cyclization and transformation of 2,3; 22,23-diepoxysqualene (13, 26) could be seen at 10^{-7} and 10^{-8} M BIBB 515.

Studies in rats and mice: Inhibition of 2,3-oxidosqualene cyclase

Inhibition of OSC in vivo was measured by the increase of monoepoxysqualene (MES) in serum or liver 1, 3, and 5 h after oral administration of BIBB 515. In rats, measurement was performed in plasma because in this species, due to the high cholesterol biosynthesis rate, MES levels in plasma are above the detection limit. On the other hand, it is difficult to get plasma material from mice in an amount that is sufficient to measure MES levels. In the latter species MES levels had to be determined in liver samples where MES levels (as in other species) are about 10-fold the levels measured in plasma into which MES is secreted via VLDL (unpublished results). Due to the high turnover of VLDL, changes in plasma levels closely reflect changes in liver levels.

At all time points measured, a dose-dependent increase of MES levels in plasma (rats) or liver (mice) could be demonstrated. The dose–response curves for the 3-h period in rats (panel A) and the 1-h period in mice (panel B) are shown in **Fig. 2.** The ED_{50} values calculated from the curves at different time points are nearly independent of the sampling time in rats, but show a sharp increase with time in mice (**Fig. 3**). This is due to much higher clearance of BIBB 515 in mice than in rats (148 ml * min^{-1} * kg^{-1} in mice versus 24 ml * min^{-1} * kg^{-1} in rats).

Inhibition of cholesterol biosynthesis

Inhibition of cholesterol biosynthesis was determined at the optimal time as determined in the experiments

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
BIBB 515							
IE-9 M	87.7	bql	bql	bql	bql	bql	bql
IE-8 M	28.2	8.3	3.4	3.2	bql	bql	bql
IE-7 M	9.0	36.4	21.2	3.1	bql	bql	bql
IE-6 M	bql	60.1	28.7	bql	bql	bql	bql

HepG2 cells were incubated with BIBB 515 dissolved in dimethyl sulfoxide (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 Bligh and Dyer method. After purification over Amchro Bond-Elut columns, lipids were separated by HPLC. The column effluent was monitored by an on-line radioactivity detector. 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).

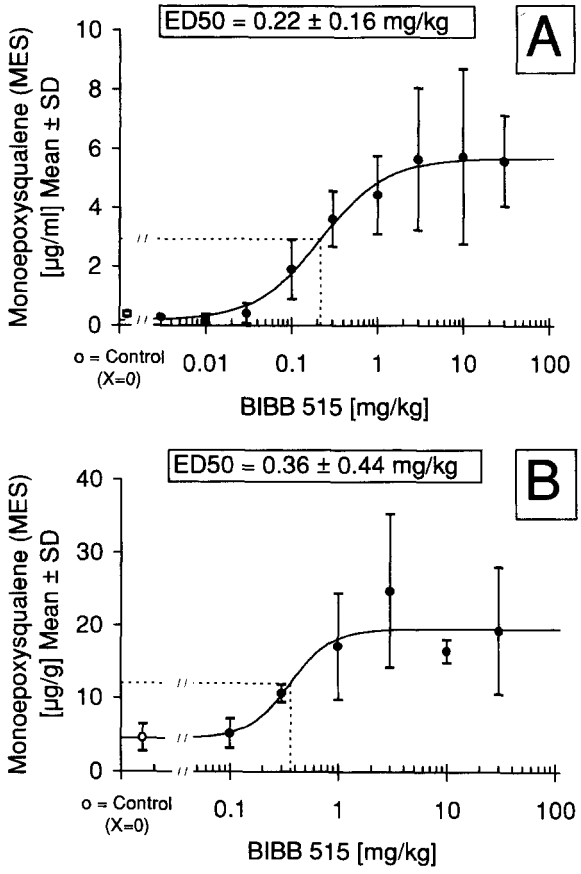

Fig. 2. Inhibition of OSC in rats (panel A) and mice (panel B). Plasma was obtained three h after oral administration of BIBB 515 to rats, 1 h after oral administration to mice livers were removed. To plasma samples or homogenized liver aliquots (0.5 g) calciferol as internal standard was added. Lipid extraction was performed according to Bligh and Dyer. After purification over Amchro-Bond Elut columns, lipids were extracted into methanol for determination of MES by HPLC. Values were normalized to the internal standard and are expressed in µg/ml (plasma) or µg/g (liver tissue). Values shown are the mean ± SD of 4 animals.

on OSC inhibition in the both species (Fig. 2), i.e., 3 h for rats and 1 h for mice, after administration of BIBB 515. In both species a dose-dependent inhibition of sterol biosynthesis in the livers of the animals could be demonstrated. ED_{50} values were calculated as 0.1 mg/kg (rats) and 0.9 mg/kg (mice). Maximal inhibition of sterol biosynthesis was observed with doses > 1 mg/kg (rats) and >10 mg/kg (mice) (**Fig. 4**).

ED_{50} values of OSC inhibition and decrease in sterol biosynthesis were comparable within the species. The dosage needed to reach the plateau effects in the two different experimental approaches was also within the same range.

Studies in hamsters: Lipid lowering activity in normolipemic hamsters

BIBB 515 was well tolerated by the animals. There was no relevant difference in food consumption or body weight development between treatment and control

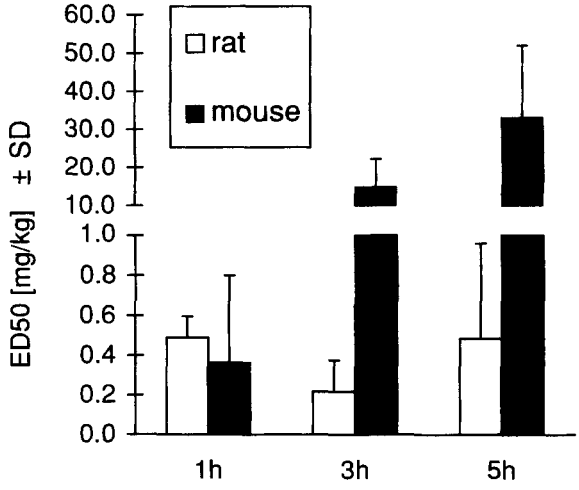

Fig. 3. ED_{50} values from MES level dose—response curves in mice and rats. ED_{50} values were calculated from dose response curves (see Fig. 2) at the indicated time points after oral administration of BIBB 515. The values shown are the mean ± SD of 4 animals.

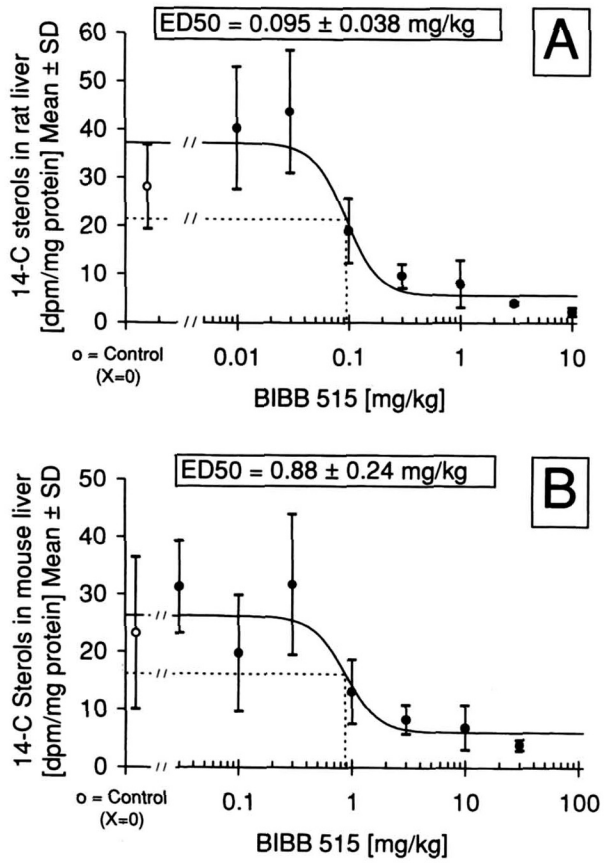

Fig. 4. Effect of BIBB 515 on [^{14}C]acetate incorporation in sterols. Panel A: Rat livers 3 h after oral administration. Panel B: Mouse livers 1 h after oral administration of BIBB 515. One h before killing animals were injected intraperitoneally with 740 kBq of [^{14}C] acetate. Samples of liver tissue, 0.5 g each, were saponified in ethanolic KOH (10%) at 60°C for 1 h. Nonsaponified material was extracted with petroleum ether, evaporated, and dissolved in ethanol–acetone 1:1 (v/v). ^{14}C-labeled sterols were isolated by digitonin precipitation and counted in a liquid scintillation counter. Data were expressed as ^{14}C-labeled sterols (dpm) per mg liver protein. Values shown are the mean ± SD of 5 animals.

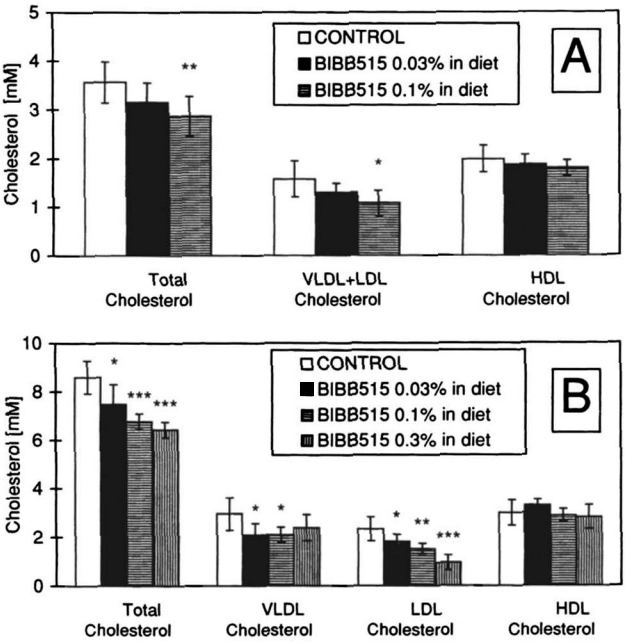

Fig. 5. Lipoprotein profile of hamster after treatment with BIBB 515. Panel A: Normolipemic hamsters; treatment time 11 days. Mean daily dose at 0.03% drug in the diet: 16.5 mg/kg; at 0.1% in the diet: 55.2 mg/kg. Lipoprotein fractions were isolated by ultracentrifugation (2 h, 4°C, 100000 rpm) with a KBr-adjusted density of 1.063 kg/l for flotation of LDL + VLDL. Due to the low level of VLDL cholesterol in normolipemic hamsters, VLDL and LDL fractions were combined. Panel B: Hyperlipemic hamsters; treatment time 25 days. Mean daily dose at 0.03% drug in the diet: 16.0 mg/kg; at 0.1% in the diet: 49.7 mg/kg; at 0.3% in the diet: 148.2 mg/kg. The values shown are means ± SD of groups of 7 animals. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for changes versus control.

groups. Lipid-lowering activity determined after 11 days treatment was dose-dependent with maximal effects in β-lipoprotein cholesterol (−32% versus control at 0.1% in the diet = 55 mg/kg). Total cholesterol was decreased by 19%; HDL cholesterol showed a slight but insignificant decrease **(Fig. 5, panel A).**

Lipid-lowering activity in hyperlipemic hamsters

BIBB 515 was well tolerated by the hyperlipemic animals. There was a slight decrease in food consumption in the two higher dose groups (7% and 8%, respectively) which did not lead to significant changes in body weight development. Lipid-lowering activity determined after 25 days treatment was dose-dependent with maximal effects in LDL-cholesterol (−23%, −35%, and −59% versus control at 0.03%, 0.1%, and 0.3% in the diet (16.0, 49.7, and 148.2 mg/kg)). Total cholesterol was decreased by 13%, 21%, and 25%. The decrease in VLDL cholesterol was not dose-dependent (29%, 29%, and 20% (n.s), respectively). There was no change in the HDL cholesterol levels (Fig. 5, panel B).

^{125}I-labeled LDL kinetics

The time-decay curves of ^{125}I-labeled LDL indicated as μmol ^{125}I-labeled LDL-cholesterol/l plasma are shown in **Fig. 6.** The data of each animal were analyzed using the TopFit program (23). The means of the parameters calculated by the program are listed in **Table 2.** For definition of the parameters see Methods section. There were no relevant changes observed in LDL clearance or fractional catabolic rates after treatment with BIBB 515 in both normal as well as in hyperlipemic hamsters. However, a dose-dependent decrease in normo- and hyperlipemic animals was seen when the LDL production rate was calculated (Table 2).

Clearance in hyperlipemic hamsters was lower than in normolipemic hamsters. This may be due to a down-regulation of LDL receptors by the hyperlipemic diet.

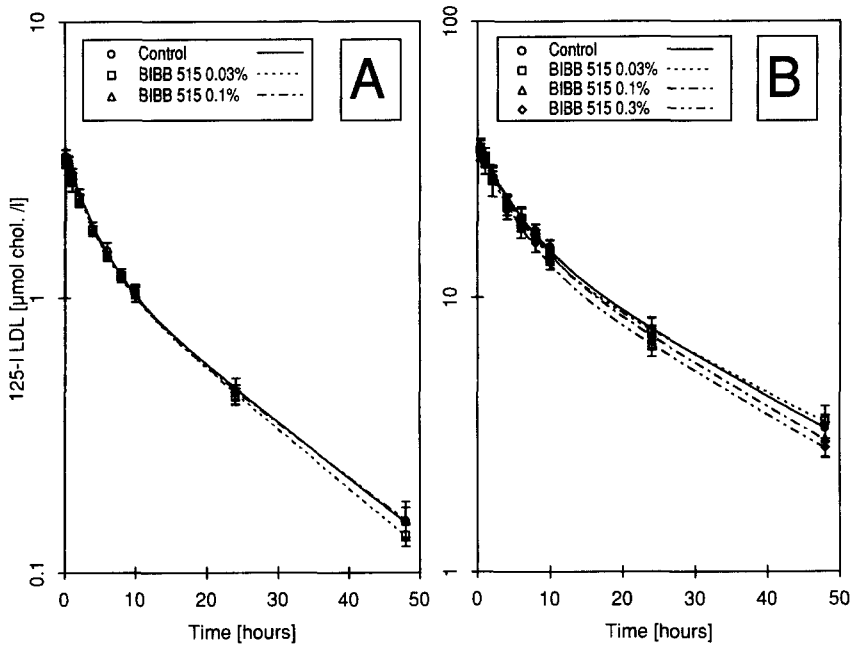

Fig. 6. Decay curves of ^{125}I-labeled hamster LDL injected iv into pretreated hamsters. Panel A: Normolipemic hamsters; treatment time 12 days. Panel B: Hyperlipemic hamsters; treatment time 32 days. Dosage as indicated in legend to Fig. 5. ^{125}I-labeled LDL was injected into the femoral vein of the hamsters in the amount indicated in Table 2. Blood (300 µl in normolipemic hamster experiments and 100 µl in hyperlipemic hamsters) was taken at the time points indicated. Fifty µl (normolipemic) or 40 µl (hyperlipemic animals) heparin plasma was counted in a scintillation counter. From the specific activity and the composition of ^{125}I-labeled-LDL shown in legend to Table 2, cpm values were transformed into µmol of LDL-cholesterol. The values shown are means ± SD of groups of 7 animals.

TABLE 2. ^{125}I-labeled LDL kinetic parameters of normo- and hyperlipemic hamsters treated with BIBB 515

Hamsters	V_c	Clearance	C_0	LDL Chol	LDL Prod.	FCR
	ml	*µl/min*	*µmol/l*	*mmol/l*	*µmol/h*	*1/day*
Normolipemic						
Control	38.6 ± 1.6	57.3 ± 3.8	3.33 ± 0.14	1.58 ± 0.37	5.43 ± 1.36	2.14 ± 0.12
BIBB 515, 0.03% in diet	39.6 ± 2.9	60.1 ± 2.5	3.27 ± 0.27	1.29 ± 0.19	4.66 ± 0.71	2.19 ± 0.10
% Change	2.4 ns	4.8 ns	−1.8 ns	−17.9 ns	−14.2 ns	2.5 ns
BIBB 515, 0.1% in diet	41.6 ± 2.0	57.9 ± 3.5	3.08 ± 0.15	1.08 ± 0.26	3.70 ± 0.77	2.00 ± 0.10
% Change	7.7[a]	1.0 ns	−7.5[b]	−31.8[a]	−31.9[a]	−6.2[a]
Hyperlipemic						
Control	43.9 ± 3.6	42.0 ± 2.6	33.86 ± 2.75	2.33 ± 0.49	5.82 ± 0.95	1.38 ± 0.07
BIBB 515, 0.03% in diet	43.3 ± 3.3	42.0 ± 3.7	34.24 ± 2.89	1.81 ± 0.30	4.55 ± 0.88	1.40 ± 0.14
% Change	−1.2 ns	−0.1 ns	1.1 ns	−22.5[a]	−21.8[a]	1.2 ns
BIBB 515, 0.1% in diet	42.7 ± 3.2	44.6 ± 4.5	35.01 ± 2.14	1.51 ± 0.14	4.06 ± 0.59	1.50 ± 0.08
% Change	−2.6 ns	6.1 ns	3.4 ns	−35.0[b]	−30.3[b]	8.5[a]
BIBB 515, 0.3% in diet	44.4 ± 4.8	47.6 ± 2.7	33.59 ± 3.28	0.95 ± 0.21	2.69 ± 0.59	1.55 ± 0.12
% Change	1.2 ns	13.3[b]	−0.8 ns	−59.4[c]	−53.7[c]	12.4[b]

V_c, distribution volume to the central compartment; C_0, extrapolated zero time concentration of ^{125}I-labeled LDL; FCR, fractional catabolic rate. For definition and calculation of kinetic parameters, see Methods. Normolipemic hamster: specific activity of ^{125}I-labeled LDL, 1.31×10^9 cpm/mg protein; 2.89×10^8 cpm/µmol LDL cholesterol. Total amount ^{125}I-labeled LDL injected: 3.71×10^7 cpm/kg = 0.128 µM ^{125}I-labeled LDL cholesterol/kg. LDL composition: 0.220 mg protein/µmol cholesterol. Hyperlipemic hamster: specific activity of ^{125}I-labeled LDL, 1.60 × 108 cpm/mg protein; 2.52×10^7 cpm/µmol LDL cholesterol. Total amount ^{125}I-labeled LDL injected: 3.73×10^7 cpm/kg = 1.477 µmol ^{125}I-labeled LDL/kg. LDL composition: 0.158 mg protein/µmol cholesterol. % change is calculated versus control animals; ns, $P > 0.05$; [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$.

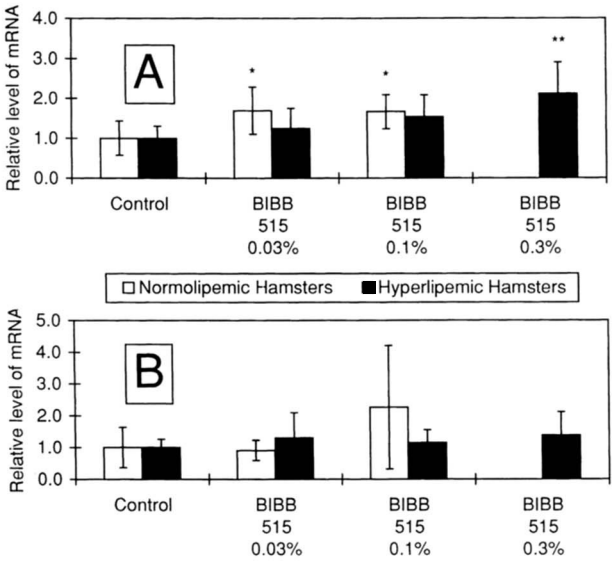

Fig. 7. LDL receptor mRNA (panel A) and HMG-CoA reductase mRNA (panel B) in liver of hamsters treated with BIBB 515. Treatment conditions as indicated in legend to Fig. 6 plus 5 days recovery time, with continuation of treatment after the end of the lipoprotein kinetics experiment. mRNAs were determined by Northern blot. After autoradiography the relative amount of specific mRNA was quantified by densitometric analysis. Values for mRNA of LDL receptor and HMG-CoA reductase was normalized according to the hybridization signal of GAPDH of the same probe. Data are expressed relative to the mean of the corresponding control group. The highest dose used in normolipemic hamsters was 0.1% BIBB 515 in the diet. Values are given as mean ± SD of 4 to 6 livers. * $P < 0.05$; ** $P < 0.01$ for changes versus control.

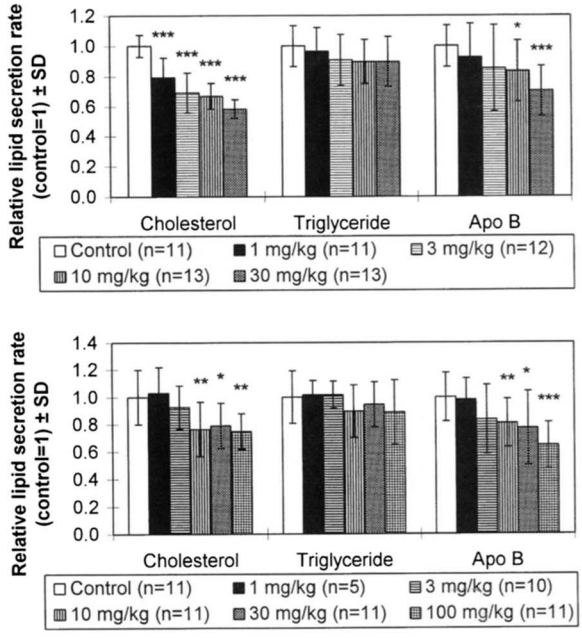

Fig. 8. Inhibition of VLDL secretion in Triton WR 1339 treated rats (upper panel) and hamsters (lower panel). Triton was injected iv into the tail vein (rats) or femoral vein (hamsters) at about 7:00 PM at a dose of 600 mg/kg. BIBB 515 was given orally 30 min (rats) or 60 min (hamsters) before the Triton injection. The animals were deprived of food 3 h (rats) or directly (hamsters) before the oral administration of BIBB 515. Blood was drawn immediately before the oral application of BIBB 515 and 15 h after the Triton injection. Upper panel: rats: control secretion rates ± SD were: cholesterol: 2.2 ± 0.4 μmol/h/100 g body weight; triglycerides: 9.3 ± 1.3 μmol/h/100 g body weight; apoB: 252 ± 34 μg/h/100 g body weight. Lower panel: hamsters: control secretion rates ± SD were: cholesterol: 1.5 ± 0.3 μmol/h/100 g body weight; triglycerides: 13.0 ± 2.3 μmol/h/100 g body weight; apoB: 41 ± 7 μg/h/100 g body weight; n.s., $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Effects on mRNA of LDL receptor and HMG-CoA reductase

BIBB 515 showed only slight effects in doses up to 0.1% in diet admixture in normo- and hyperlipemic animals. A more than 2-fold increase of LDL receptor mRNA was only seen in the highest dose group (0.3% in diet) (**Fig. 7, panel A**).

Hamsters treated with BIBB 515 had very low levels of HMG-CoA reductase mRNA that were similar to control (Fig. 7, panel B).

VLDL secretion in rats and hamsters

VLDL secretion rates were measured in vivo in acute experiments in rats and hamsters using the Triton WR 1339 method. This nonionic detergent is known to completely block the conversion of VLDL to LDL by inhibition of lipoprotein lipase and has been used to study VLDL secretion in several species including rat and hamster (27–31). After a dose of 600 mg/kg of Triton, triglycerides rose linearly for 6 h with only slow deviation from linearity up to 13 h (27). To allow the OSC inhibitor to display its full activity and to cover the main cholesterol biosynthesis phase of the animals during the night phase, an overnight interval of 15 h was chosen to monitor VLDL secretion. The per hour secretion rates calculated from this time period do not necessarily represent the initial secretion rates but are in good agreement with secretion rates published for shorter time periods (27, 30, 31). In both rats and hamsters, VLDL cholesterol secretion was dose-dependently reduced after a single administration of BIBB 515 (**Fig. 8**). A somewhat lower inhibition was seen with apoB and there was a small but nonsignificant decrease in triglyceride secretion.

DISCUSSION

BIBB 515 is a potent and selective inhibitor of OSC as shown in experiments with human hepatoma cells, HepG2. The inhibitory effect of BIBB 515 on enzymes of the bile acid synthesis pathway, i.e., 7α-hydroxylase,

of rat liver homogenates was 4 orders of magnitude lower than the OSC inhibition (IC_{50} = 5.3 * 10^{-5} mol/l) and the lithogenic index in hamsters at the doses reported in this study was not influenced (B. Eisele, unpublished data).

Interaction of BIBB 515 with OSC results in decreased cholesterol biosynthesis. Inhibition of OSC leads to an increase in MES and in DES levels. Furthermore, partial inhibition of OSC with BIBB 515 leads to an increased formation of regulatory oxysterols as measured as 24,25-epoxycholesterol. These findings support the earlier findings with BIBX 79 (13) on the dual role of OSC in the cholesterol biosynthesis. Inhibition of OSC not only leads to the reduced formation of lanosterol but also leads to a regulation of the cholesterol biosynthesis by the cyclization of DES to regulatory active oxysterols (13, 26). Thus, an overshooting up-regulation of HMG-CoA reductase as seen with the HMG-CoA reductase inhibitors (7, 12, 32) is avoided.

BIBB 515 is also able to inhibit OSC in vivo very effectively after oral administration as evidenced by an increase in MES levels of rat plasma or mice liver. The level of DES in both species was found to be roughly 5 to 10% of the MES levels (data not shown). In none of the species examined in vivo (rat mouse or hamster) could a change in sterol intermediates other than MES and DES after treatment with BIBB 515 be detected by our HPLC system.

In both rats and mice inhibition of OSC is paralleled by an inhibition of cholesterol biosynthesis. The different profile of ED_{50} values in mice and rats can be discussed as the result of a different pharmacokinetic behavior of BIBB 515 in these two species. Whereas in rats the duration of action seems to be long (ED_{50} values after 1, 3, and 5 h within the same range) the substance seems to be inactivated and/or excreted quite effectively in mice (ED_{50} values after 3 and 5 h several-fold higher than after 1 h).

In summary, BIBB 515 is a potent inhibitor of OSC in mice and rats. By this mechanism cholesterol biosynthesis in both species is decreased. Whereas in rats the duration of action of BIBB 515 seems to be longer than 5 h, in mice the maximal effect is seen after 1 h and disappears after longer periods.

Cholesterol biosynthesis inhibitors like HMG-CoA reductase inhibitors (9, 11) or OSC inhibitors (our results) do not lead to a lipid-lowering effect in normal rats or mice. The golden Syrian hamster was chosen as an experimental animal to show lipid-lowering activity because its lipoprotein profile is more similar to humans than that of other rodents (33) and because this animal responds to treatment with agents like HMG-CoA reductase inhibitors or bile acid sequestrants (5, 33).

Lipid-lowering activity of BIBB 515 was dose-dependent in both normo- and hyperlipemic animals. There was some degree of food intake inhibition at the two highest doses of BIBB 515 in hyperlipemic animals. This is not considered to have seriously influenced the results as there is no difference in this parameter between the two groups mentioned whereas a clear cut difference is observed in the lipid-lowering effect. With both animal models, normo- as well as hyperlipemic animals, dose-dependent decreases of total cholesterol as well as of β-lipoprotein cholesterol levels were detected. It is important to note that the HDL cholesterol fraction was unaffected by BIBB 515 treatment.

LDL concentration in the plasma compartment is governed by LDL clearance and LDL production rate (34). Because under steady state conditions the LDL production must equal its clearance, the production rate can be calculated as product of the clearance of ^{125}I-labeled tracer LDL and the steady state LDL concentration under the assumption of identical behavior of tracer and endogenous LDL. The fractional catabolic rate is the fraction of the total pool metabolized per day where the pool is the central compartment distribution volume.

In neither normo- nor hyperlipemic hamsters does BIBB 515 show an influence on clearance or fractional catabolic rates that is relevant for the explanation of the lipid-lowering effects. Clearance and fractional catabolic rate are only slightly increased at the highest doses of BIBB 515 in hyperlipemic animals. This is probably due to a beginning counter regulation as a consequence of nearly complete inhibition of OSC (13, 35). On the other hand, the LDL production rates show a dose-dependent decrease in both hyperlipemic and normolipemic animals.

The level of LDL receptor mRNA determined in the same animals after recovery from the LDL kinetics experiment is in good agreement with the kinetic data. BIBB 515 does not show a substantial up-regulatory effect on HMG-CoA reductase mRNA. Slight alterations in the amount of LDL receptor mRNA at lower doses of BIBB 515 seem to have no relevance for the clearance of LDL. The slight increase in LDL clearance at the highest dose of BIBB 515 corresponds to a 2-fold up-regulation of LDL receptor mRNA in the liver.

In Triton-treated animals, a single dose of BIBB 515 leads to diminished apoB secretion and therefore to a reduction in VLDL particle number. Correspondingly, VLDL cholesterol is also reduced. The only marginal effects on triglycerides may reflect an increase in VLDL particle size. However, in non-Triton-treated animals, VLDL triglyceride is rapidly catabolized by lipoprotein lipase. The data show that cholesterol biosynthesis inhibition by OSC inhibitors leads to a diminished LDL pro-

duction via a decreased input of cholesterol and apoB into the VLDL to LDL conversion cascade.

From all the data in hamster and rat models it is concluded that the lipid-lowering activity of BIBB 515 is due to inhibition of LDL production. The slight increase of LDL receptor mRNA seen with the higher doses of BIBB 515 does not seem to be sufficient to markedly change the catabolic rate of LDL and does not explain the lipid-lowering effects seen with BIBB 515.

Therefore, OSC inhibitors act mainly by interfering with VLDL cholesterol and apoB secretion and, in consequence, with LDL production rate, and may offer a novel and alternative approach for lipid-lowering therapy.

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