1. Title page

Inhibition of human sterol delta 7-reductase and other post-lanosterol enzymes by 2-(4-phenethylpiperazin-1-yl)-1-(pyridine-3-yl)ethanol (LK-980), a novel inhibitor of cholesterol synthesis

Jure Ačimovič, Tina Korošec, Matej Seliškar, Ingemar Bjorkhem, Katalin Monostory, Pal Szabo, Jean-Marc Pascussi, Ales Belič, Uroš Urleb, Darko Kocjan and Damjana Rozman

Centre for Functional Genomics and Bio-Chips, Institute of Biochemistry, Faculty of Medicine, Zaloska 4, SI-1000 Ljubljana, University of Ljubljana, Slovenia (J.A., D.R.)
Lek Pharmaceuticals d. d., Drug Discovery, Verovskova 57, SI-1000 Ljubljana, Slovenia (T.K., M.S., U.U.)
L14 Laboratory for Biomolecular Structure, National Institute of Chemistry, EN->FIST Center of Excellence, SI-1000 Ljubljana, Slovenia (D.K.)
Division of Clinical Chemistry, Karolinska Institutet, Div. of clinical chemistry, C1-62, SE-14186 Stockholm, Huddinge, Sweden (I.B.)
Chemical Research Center, Hungarian Academy of Sciences, Pusztaszeri út 59-67., III épület, 1.emelet 106, HU-1025, Budapest, Hungary (K.M., P.S.)
Faculty of Electrical Engineering, Trzaska 25, SI-1000 Ljubljana, University of Ljubljana, Slovenia (A.B.)
Institute of Functional Genomics, Centre National de la Recherche Scientifique (CNRS) UMR5203 ; Institut National de la Santé et de la Recherche Médicale (INSERM) U661, University Montpellier 1 and 2, Montpellier, France (J.-M.P.)

2. Running title page

a) Novel inhibitor of the post-squalene cholesterol synthesis

b) Corresponding author

Prof. dr. Damjana Rozman

Centre for Functional Genomics and Bio-Chips

Institute of Biochemistry

Faculty of Medicine University of Ljubljana

Zaloska 4

SI-1000 Ljubljana, Slovenia

E-mail: damjana.rozman@mf.uni-lj.si

Fax: 00386-(0)1-543-7588

c) Number of

text pages: 25

tables: 3

figures: 5

references: 37

words in the Abstract: 218

words in the Introduction: 761

words in Discussion: 1131

d) A list of nonstandard abbrevations in the paper:

LK-980, 2-(4-phenethylpiperazin-1-yl)-1-(pyridine-3-yl)ethanol; DHCR7, human sterol delta 7-reductase; DHCR14, human sterol delta 14-reductase; DHCR24, human sterol delta 24-reductase; SC5DL, human sterol C5-desaturase; GC-MS, gas chromatography-mass spectrometry; CYP3A4, cytochrome P450 3A4; FF-MAS, follicular fluid meiosis

activating sterol; T-MAS, testis meiosis activating sterol; HMGCR, HMG-CoA reductase; LC-MS/MS, liquid chromatography coupled to mass spectrometry; CYP51A1 human lanosterol 14α-demethylase;

3. Abstract

Novel potential inhibitors of the post-squalene portion of cholesterol synthesis were screened in HepG2 cells. 2-(4-phenethylpiperazin-1-yl)-1-(pyridine-3-yl)ethanol (LK-980) was identified as a perspective compound and was characterized further in cultures of human primary hepatocytes from seven donors. *In vitro* kinetic measurements show that half life of LK-980 is at least 4.3 h. LK-980 does not induce cytochrome P450 3A4 (CYP3A4) mRNA nor enzyme activity. Target prediction was performed by gas chromatography-mass spectrometry (GC-MS) allowing simultaneous separation and quantification of nine late cholesterol intermediates. Experiments indicated that human sterol delta 7-reductase (DHCR7) is the major target of LK-980 (34-fold increase of 7-dehydrocholesterol), while human sterol delta 14-reductase (DHCR14), human sterol delta 24-reductase (DHCR24) and human sterol C5-desaturase (SC5DL) represent minor targets. In the absence of purified enzymes, we used the mathematical model of cholesterol synthesis to evaluate if indeed more than a single enzyme is inhibited. *In silico* inhibition of only DHCR7 modifies the flux of cholesterol intermediates, resulting in sterol profile that does not support experimental data. Partial inhibition of the DHCR14, DHCR24 and SC5DL steps, in addition to DHCR7, supports the experimental sterol profile. In conclusion, we provide experimental and computational evidence that LK-980, a novel inhibitor from the late portion of cholesterol synthesis, inhibits primarily DHCR7 and to a lesser extent three other enzymes from this pathway.

4. Introduction

The imbalance of cholesterol homeostasis and high plasma concentration of LDL-cholesterol is a risk factor of cardiovascular diseases (atherosclerosis, coronary heart disease, myocardial infarction); therefore, antihyperlipidemic therapy is essential for prevention of the progression of cholesterol-laden plaques in vessel linings (Maas and Boger, 2003; LaRosa, 2007). Lipid-modifying interventions to decrease elevated cholesterol concentrations constitute the inhibition of *de novo* cholesterol biosynthesis in the liver and the decrease of the dietary cholesterol uptake from intestine (LaRosa, 2007; Bays et al., 2008; Koh et al., 2008; Sanossian and Ovbiagele, 2008). The most commonly prescribed cholesterol lowering agents are the statins, the HMG-CoA reductase (HMGCR) inhibitors, which are relatively safe and well-tolerated drugs for most of the patients; however, 2-3% of patients suffer from the statin-based muscular adverse drug reactions (myopathies, rhabdomyolysis), impaired cognitive function, nephropathies and hepatotoxicity (Tiwari et al., 2006; Alsheikh-Ali et al., 2007; Armitage, 2007; Martin and Krum, 2007; Rallidis et al., 2007). It has been reported that derangements in an early enzyme of the pathways, the mevalonate kinase, but not of more distal enzymes of cholesterologenesis, are associated with the statin-provoked skeletal myopathy (Buhaescu and Izzedine, 2007). By inhibition of the major regulatory enzyme of cholesterol biosynthesis, HMGCR, statins also decrease the level of an essential metabolite, coenzyme Q (Co-Q-10). This might contribute to muscle related complications, but data in patients remain controversial (Schaars and Stalenhoef, 2008). Due to the increasing incidences of hyperlipidemias and cardiovascular diseases in the developed world and the risk of statin-based therapy for severe adverse drug reactions, developing novel strategies in cholesterol lowering therapy and searching for potential hypolipidemic drugs with new molecular targets remains an important task for the pharmaceutical industry.

Several enzymes are involved in post-squalene cholesterol synthesis. The human sterol delta 14-reductase (DHCR14) transforms follicular fluid meiosis activating sterol (FF-MAS) or its delta 24 analogue into testis meiosis activating sterol (T-MAS) or its delta 24 analogue. Two enzymes encoded by two different genes (TM7SF2, known also as DHCR14, and LBR), can perform the same enzymatic reaction and they belong to the same B lamin receptor family (Rozman and Monostory; Wassif et al., 2007). One of the most interesting enzymes is human sterol delta 24-reductase (DHCR24), a FAD-dependent oxidoreductase which catalyzes the reduction of the delta-24 double bond of sterol intermediates during cholesterol biosynthesis. The protein contains a leader sequence that directs it to the endoplasmic reticulum membrane. Missense mutations in this gene have been associated with desmosterolosis. Also, reduced expression of the gene occurs in the temporal cortex of Alzheimer disease patients and overexpression has been observed in adrenal gland cancer cells (Rozman and Monostory). The human sterol delta 7-reductase (DHCR7) is an enzyme that removes the C(7-8) double bond in the B ring of sterols and catalyzes the conversion of 7-dehydrocholesterol to cholesterol. It is ubiquitously expressed and its transmembrane portion localizes to the endoplasmic reticulum membrane and nuclear outer membrane. Mutations in this gene cause Smith-Lemli-Opitz syndrome (SLOS); a disease that is metabolically characterized by reduced serum cholesterol levels and elevated serum 7-dehydrocholesterol levels and phenotypically characterized by mental retardation, facial dysmorphism, syndactyly of second and third toes, and holoprosencephaly in severe cases to minimal physical abnormalities and near-normal intelligence in mild cases. Similarly to DHCR14, DHCR7 also belongs to the B lamin receptor family(Rozman and Monostory). The sterol C5-desaturase (SC5DL), catalyzes the conversion of lathosterol into 7-dehydrocholesterol. Mutations in this gene have been associated with lathosterolosis. Alternatively spliced transcript variants encoding the same protein have been described (Rozman and Monostory).

The novel class of cholesterol lowering drugs has been discovered recently, blocking cholesterol biosynthesis after the farnesyl pyrophosphate branchpoint leaving the isoprene pathways untouched in order to avoid statin side effects (Urleb et al., 2006). 2-(4-phenethylpiperazin-1-yl)-1-(pyridine-3-yl)ethanol (LK-980) was selected as a lead compound upon sterol profiling experiments in HepG2 cells (data will be published elsewhere). Unfortunately, while the majority of human drug metabolizing cytochrome P450 transcripts are present in HepG2 cells, their mRNA levels are usually low as compared to primary human hepatocytes. Consequently, the enzyme activities and drug-metabolizing capacity of immortal hepatocytes differ substantially from the primary hepatocytes (Westerink and Schoonen, 2007). Thus, LK-980 was tested as well in primary cells

Herein we describe LK-980, a novel class of distal inhibitors of cholesterol biosynthesis that targets the human delta 7-reductase (DHCR7) and to a lesser extent human delta 14-reductase (DHCR14, LBR), human delta 24-reductase (DHCR24) and human sterol C5-desaturase (SC5DL), as deduced from studies in human primary hepatocytes and sustained with mathematical model of cholesterol synthesis. LK-980 is stable in human primary hepatocytes and does not induce CYP3A4.

5. Materials and Methods

Chemicals. Collagenase, nutrient mixture F-12 (HAM), Williams' medium E, trypan blue and rifampicin were the products of Sigma Chemie GmbH (Deisenhofen, Germany). EGTA was purchased from Fluka AG (Buchs SG, Switzerland). All other chemicals for hepatocyte isolation and acetonitrile were purchased from Merck (Darmstadt, Germany). Atorvastatin and LK-980 were provided by Lek d.d. (Ljubljana, Slovenia). All chemicals for sterol extraction and consecutive GC-MS analysis reagents were purchased as described previously (Acimovic et al., 2009).

HMGCR activity assay. Human hepatoma cell line (HepG2-ATCC No. HB-8065) was cultured in Dulbecco's Modified Eagle medium containing 5% of iron supplemented bovine calf serum and 1% of L-glutamine at 37°C and 5% CO2 in 300 cm2 flasks. The cells were harvested at approximately 90% confluency and resuspended in cold buffer A containing 100 mM potassium phosphate, pH 7.2, 100 mM sucrose, 50 mM KCl, 1 mM EDTA, 200 mM NaCl, 3 mM DTT, 1 mM phenylmethylsulfonyl fluoride and 1 x Complete protease inhibitor. For each 300 cm^2 flask 0.6 ml of buffer was used. The cell suspension was placed on ice and sonicated for 1 min. Sonicated sample was centrifuged at 12000xg and 4^oC for 10 min to obtain clarified homogenate containing microsomes that was used for the HMG-CoA reductase activity assay. Total protein concentration of clarified cell homogenate was determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA).

HMGCR activity was determined using a modification of the method of (Beg and Stonik, 1982). Assay buffer contained 1 μg/μl bovine serum albumin, 150 mM potassium phosphate, pH 6.9, 200 mM KCl, 6 mM sodium EDTA, 0.4 mM sodium azide and 1 mM dithiothreitol. 25 μl of cell homogenate containing microsomes was pre-incubated at 37^oC with 70 μl of

assay buffer at 37^{o}C for 20 min prior to the addition of NADPH at final concentration of 2.3 mM, inhibitor and [3-^{14}C] HMG-CoA (PerkinElmer) to the reaction. The reaction was allowed to proceed for 30 min at 37^{o}C and terminated with 50 µl of 10 M propionic acid. To correct for the amount of product recovered, 2 µl (approximately 20,000 cpm) of [^{3}H] mevalonate (PerkinElmer) was added to each assay as internal standard. Samples were incubated for 15 min at room temperature to allow the lactonisation of mevalonate to mevalonolactone. The samples were centrifuged (16000xg, 4^{o}C) to pellet any insoluble material. Supernatant was applied to a AG1-X8 anion exchange resin column (Bio-Rad, Hercules, CA). Columns were prepared by equilibrating AG1-X8 resin in 1 M acetic acid at room temperature followed by 1 M propionic acid for four times. Resin was pipetted to a height of 2 cm in plastic columns. To remove excess acetic acid, the columns were washed with 2 ml of distilled water then positioned over liquid scintillation vials. The sample was applied to the column and washed with 2 ml of double-distilled water. Mevalonolactone was eluted from the column while the substrate HMG-CoA was not eluted from the anion exchange column. 4 ml of scintillation cocktail was added to the vials and the amounts of ^{3}H and ^{14}C radioactivity were determined by liquid scintillation spectrometry (Raytest, Ramona 2000).

Isolation and culture of human hepatocytes. Human livers (HH-089, HH-114, HH-129, HH-150, HH-272) were obtained from kidney transplant donors or from lobectomy segments (HH-269, HH-270, HH-271) resected from adult patients for medical reasons unrelated to our research program (Transplantation and Surgical Clinic, Semmelweis University, Budapest, Hungary; CHU, Saint-Eloi, Montpellier, France). Permissions of the Hungarian and French Regional Committee of Science and Research Ethics were obtained to use human tissues. Clinical histories of the donors are shown in Table 1. Liver cells were isolated by the method

of (Bayliss and Skett, 1996). Hepatocytes having viability better than 90% as determined by trypan blue exclusion, were used in the experiments. The cells were plated at a density of $1.7x10^5$ cells/ cm^2 in plastic dishes precoated with collagen in medium described by (Ferrini et al., 1998). After overnight culture, the medium was replaced by serum-free medium. Forty-eight hours after serum deprivation, cells were treated with atorvastatin (10 μM) or LK-980 (10 μM) for 48 hr. After washing in phosphate buffered saline (PBS), the cells were harvested for sterol profile analysis.

In vitro pharmacokinetics of LK-980. Biotransformation of LK-980 was performed in cell suspension ($1.7x10^6$ cells/ml) at 37°C in a humid atmosphere containing 5% CO_2. The parent compound was added directly to the medium (final concentration of LK980: 1 μM). Aliquots (0.5 ml) of the incubation mixtures were terminated by the addition of 0.5 ml ice-cold acetonitrile at 0, 30, 60, 120, 240 and 360 min and the samples were stored at -80°C. Cell debris was separated by centrifugation and 10 μl of the supernatant was analyzed by liquid chromtatography coupled to mass spectrometry (LC-MS/MS) for quantitation of the parent compound. For this purpose, a SB C18 Zorbax, 250 x 4.6 mm column (Agilent, Santa Clara, CA) was used. Gradient elution was applied with the mobile phase A consisting 10 mM ammonium-formate (pH 8.5) and with acetonitrile as the mobile phase B. The column was eluted at a rate of 1 ml/min at 40°C and the effluent was analyzed by mass spectrometry. Tandem mass spectrometry (MS/MS) measurements were performed on a 3200 Qtrap hybrid (quadrupole-linear ion trap) mass spectrometer (Applied Biosystems, Foster City, Canada) equipped with TurboV ion source. The ionisation mode was electrospray in positive mode. The instrument was scanned in enhanced product ion (EPI) mode for structure identification and in multiple reaction monitoring (MRM) mode for quantitation. The source conditions were: curtain gas: 20 units, spray voltage: 5000 V, source temperature: 450°C, nebulising gas:

50 units, drying gas: 30 units. Two MRM transitions (the first transition as quantifier and the second as the qualifier channel), were 312/105 (CE: 47 eV) and 312/160 (CE: 33 eV), were monitored for LK-980 with dwell time of 300 msec.

Estimation of clearance. The intrinsic clearance for hepatocytes (Cl_{int}) [ml/(min 1.7x10^{6}cells)] was calculated from the decrease in the concentration of LK-980 as follows: $Cl_{int} = \beta = \frac{\ln 2}{t_{1/2}}$. For scaling up the Cl_{int} value to obtain $Cl_{int\ per\ whole\ liver\ (g)/bw\ (kg)}$, the following parameters were used: cell concentration in liver: 1.07x10^8 cells per 1 g of liver (Wilson et al., 2003), average liver weight of human: 1660 g, average body weight of human: 70 kg. The value for hepatic clearance (Cl_H) was calculated as follows: $Cl_H = \frac{Cl_{int\ liver/bw} * fu * Q_{plasma}}{(Cl_{int\ liver/bw} * fu) + Q_{plasma}}$ where $Q_{plasma}=Q_H*plasma/blood\ ratio$. To calculate Cl_H, Q_H=1140 ml blood/hr/kg, plasma/blood ratio=0.57 and fu=1 values were used. (Q_H is the hepatic blood flow, while *fu* is the unbound fraction of the compound.) The hepatic extraction ratio was defined as $E=Cl_H*Q_H$.

CYP3A4 induction in human hepatocytes. Forty-eight hours after serum deprivation, the plated human hepatocytes were cultured in the presence or absence of inducers for 12, 24 or 48 hr. Hepatocytes were treated with rifampicin (5 μM), atorvastatin (10 μM), or LK-980 (10 μM). After washing in PBS, the cells were harvested for CYP3A4 enzyme assay and transcriptome analysis. Microsomal fraction from cultured human hepatocytes was prepared by differential centrifugation (van der Hoeven and Coon, 1974). Protein content of microsomes was determined by the method of (Lowry et al., 1951), with bovine serum albumin as the standard. Published method was followed to determine nifedipine oxidation activity selective for CYP3A4 (Guengerich et al., 1986). The incubation mixture contained NADPH-generating system (1 mM NADPH, 10 mM glucose 6-phosphate, 5 mM $MgCl_2$ and

2 units/ml glucose 6-phosphate dehydrogenase), microsomes and nifedipine as the substrate for CYP3A4. The rates of enzyme activity were linearly dependent upon the amount of microsomal protein added for the 30 min incubation period. The metabolic extraction procedure and high-performance liquid chromatography (HPLC) analysis were performed according to the published method (Guengerich et al., 1986). CYP3A4 enzyme assay was performed in triplicate and means±standard deviations were calculated. For comparison among untreated and treated groups, statistical analysis of the results was carried out using a two-tailed *t*-test with $p<0.05$ as the criterion for significance. Due to high variation in basic cytochrome P450 expression of human hepatocytes, entire experiment was repeated in hepatocytes isolated from 4 to 7 donors to confirm the results.

RNA extraction and quantitative RT-PCR. Total RNA was isolated from human hepatocytes using TRIzol reagent (Invitrogen, Carlsbad, CA). Ten million liver cells were homogenized in 1 ml of TRIzol reagent, and total RNA was extracted according to the manufacturer's instructions. The RNA was precipitated using ethanol and stored at -80˚C for further analyses. RNA (3 μg) was reverse transcribed into single stranded cDNA using Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) and then real time PCR with human cDNA was performed using FastStart Taq DNA polymerase (LightCycler TaqMan Master, Roche Diagnostics GmbH, Mannheim, Germany) and UPL probe for CYP3A4 (Roche Diagnostics GmbH, Mannheim, Germany). The quantity of target RNA relative to that of housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined. CYP3A4 mRNA levels were quantified by RT-PCR measurements in the same human hepatocytes in which CYP3A4 activities were measured. Statistical analysis of the results obtained by RT-PCR was carried out similarly to that of cytochrome P450 activities.

Sterol extraction and GC-MS analysis of human primary hepatocytes. Sterol extraction and GC-MS analysis of LK-980, atorvastatin treated (positive control) and non-treated primary hepatocytes from 7 donors (HH-114, HH-129, HH-150, HH-272, HH-269, HH-270, HH-271) were performed as described previously (Acimovic et al., 2009). Sterol contents of primary human hepatocytes were normalized by the total protein content of the cells. Treatment with atorvastatin served as the positive control. The lipids (sterols) were extracted from human primary hepatocytes and analysed with a novel GC-MS method which enables quantitative measurements of several post-squalene cholesterol biosynthesis intermediates in a single run (lanosterol, FF-MAS, T-MAS, zymosterol, desmosterol, 7-dehydrocholesterol, lathosterol, squalene and cholesterol) (Acimovic et al., 2009). The quantity of total (free and esterified) sterols of 7 donors was normalized on the total protein content.

Mathematical model of cholesterol synthesis. The model was designed as kinetic model with relations between molecules as described in Fig. 1. Every enzyme reaction (thick arrows in Fig. 1) is described with four differential equations:

$$\frac{dS}{dt} = \Phi_I - k_C \cdot E \cdot S + k_{CR} \cdot C$$

$$\frac{dC}{dt} = k_C \cdot E \cdot S + k_{PR} \cdot E \cdot P - k_P \cdot C - k_{CR} \cdot C$$

$$\frac{dP}{dt} = k_P \cdot C - \Phi_O - k_{PR} \cdot E \cdot P$$

$$\frac{dE}{dt} = \Phi_{EI} + k_P \cdot C + k_{CR} \cdot C - k_C \cdot E \cdot S - k_{PR} \cdot E \cdot P - \Phi_{EO},$$

where S denotes the concentration of the substrate, C the concentration of the complex, P the concentration of the product, E the concentration of the enzyme, Φ_I the flux of the substrate

into the reactor, Φ_O the flux of the product out of the reactor, Φ_{EI} the flux of the enzyme into the reactor, Φ_{EO} the flux of the enzyme out of the reactor, k_C the rate constant of the complex formation, k_{CR} the rate constant of decomposition of the complex into the substrate and the enzyme, k_P the rate constant of the product formation, and k_{PR} the rate constant of the complex formation from the enzyme and the product. For batch reactors, where finite quantities of the substrate and the enzyme are mixed, each enzyme reaction would contribute four independent rate constants to the pool of model parameters. However, metabolic networks normally operate in a mode with continuous supply of substrates and enzymes which introduces some limitations on the choice of the model parameters values. Operation in continuous metabolic flux mode requires that the concentrations of the involved substances settle at a non-zero steady-state. Further specifications of the model are explained in Supplemented data.

Statistical analysis. Statistical analysis was performed using R version 2.9.0. Levene's test of homogeneity of variances between 3 treatments (control, atorvastatin and LK-980) with α = 0.05 was used to establish further parametric or non-parametric statistical tests. Levene's test was repeated on logarithmic data if raw data showed statistically significant differences between variances. One-way analysis of variance (ANOVA) (α = 0.05) was used for comparison of treatments. If there was statistically significant difference (α = 0.05), Least Significant Difference (LSD) posthoc tests were performed with α = 0.05 for pair wise comparison between treatments.

6. Results

***In vitro* kinetics of LK-980 in cultures of human primary hepatocytes.** To establish the biological stability of LK-980, the biotransformation of LK-980 (1 μM) was determined in suspension of primary human hepatocytes isolated from three donors (Fig. 3). The consumption of LK-980 measured by LC-MS/MS was found to be low (HH-114) or negligible (HH-089 and HH-129) within 6 hr of incubation. The elimination half life was estimated to be 4.32 hours in the hepatocytes of HH-114 and more than 6 hr in the liver cells of HH-089 and HH-129 (Table 2), indicating slow hepatoc metabolic degradation of LK-980. These results suggest that LK-980 is a drug candidate with advantageous pharmacokinetic properties for further development.

CYP3A4 induction in human primary hepatocytes. To determine whether LK-980 treatment results in changed expression of the main drug-metabolizing cytochrome P450 CYP3A4, we investigated the effect of LK-980 in primary cultures of human hepatocytes from several donors (as indicated in Fig. 4) because of high individual variance in basic activities or CYP expression of the cells. Since both atorvastatin and LK-980 efficiently inhibited *de novo* cholesterol biosynthesis at 10 μM, we applied this concentration in further experiments. Rifampicin was used as a reference compound at the concentration of 5 μM. Rifampicin binds to and strongly activates pregnane X receptor (PXR), leading to induction of CYP3A4 (Luo et al., 2002). To evaluate the significant inducibility of human hepatocytes by atorvastatin and LK-980, a paired *t*-test was performed with $p<0.05$ as the criterion for significance. Fig. 4 presents CYP3A4 activities and mRNA levels relative to control hepatocytes (0.1% dimethyl sulfoxide treated cells).

The reference compound, rifampicin increased nifedipine oxidation of CYP3A4 by more than 30-fold and also produced strong transcriptional activation of the *CYP3A4* gene (more than 60-fold after 48-hr exposure) (Fig. 4). Treatments with atorvastatin resulted in about 10-fold induction of CYP3A4 activity in hepatocytes isolated from various donors (Fig. 4A). The elevation in the enzyme activity was confirmed by the changes in levels of CYP3A4 mRNA as a consequence of atorvastatin treatment (Fig. 4B). The maximal increase in the transcription of the *CYP3A4* gene was generated after 24-hr exposure to atorvastatin. On the other hand, LK-980 induced neither the activity nor mRNA level of CYP3A4.

Identification of LK-980 enzyme targets in human primary hepatocytes. To evaluate the inhibitory potential and enzyme target for LK-980 in human primary hepatocytes, the cells from 7 liver donors were prepared (HH-114, HH-129, HH-150, HH-272, HH-269, HH-270, HH-271) as described in Materials and methods. Treatment with atorvastatin, a well known HMGCR inhibitor, served as a positive control. Sterol extracts were analyzed by GC-MS with a novel method that enables quantitative measurements of nine intermediates of the post-squalene cholesterol synthesis (lanosterol, FF-MAS, T-MAS, zymosterol, desmosterol, 7-dehydrocholesterol, lathosterol, squalene and cholesterol) in a single run (Acimovic et al., 2009).

Different statistical tests have been used to evaluate the significant changes for each sterol in human hepatocyte cultures, comparing atorvastatin-treated, LK-980-treated and the non-treated control cells. Sterols that pass the statistical threshold ($p < 0.05$) are shown in Fig. 5. One-way ANOVA showed no statistically significant difference between treatments for cholesterol ($p = 0.9640$), lanosterol ($p = 0.8922$), squalene ($p = 0.7786$), T-MAS ($p = 0.3660$) and zymosterol ($p = 0.6774$). However, a statistically significant difference between

treatments was observed for 7-dehydrocholesterol (p = 0.0000), desmosterol (p = 0.0039), FF-MAS (p = 0.0062) and lathosterol (p = 0.0002) (Fig. 5). Furthermore, 95% Least Significant Difference (LSD) post-hoc tests were performed to show statistically significant difference between all pairs of treatments. We have to take into account that the extracts of primary human hepatocytes contain total (esterified and free) sterols of the cells.

The average FF-MAS content of LK-980 treated hepatocytes (2.9 μg/g total proteins) was 3.17 times higher (p=0.026) than that of control cell population (0.7 μg/g total proteins). These results indicated either the inhibition of DHCR14 or one of the later enzymes. The average FF-MAS content of atorvastatin treated hepatocytes (0.3 μg/g total proteins) was, as expected, lower (2.18 times) than that of the control (p=0.167).

The average desmosterol content in LK-980 treated (10.1 μg/g total proteins), was 1.84 times higher compared to non-treated cells (5.5 μg/g total proteins), which indicated a weak inhibition of DHCR24 by LK-980 (p=0.085). The desmosterol content of atorvastatin treated cells (2.7 μg/g total proteins) was 2.01 times lower compared to controls (p=0.051) which is in accordance with atorvastatin inhibiting HMGCR that is before DHCR24 in the cholesterol synthesis pathway.

LK-980 (p=0.006) and atorvastatin (p=0.048) treatments caused statistically significant changes in lathosterol of primary hepatocytes as compared to the control cells. The average lathosterol content in LK-980 treated cells (118.9 μg/g total proteins) was 2.50 times higher than in non-treated cells (47.7 μg/g total proteins), suggesting that LK-980 might inhibit SC5DL or later enzymes in the pathway. Atorvastatin slightly reduced lathosterol of the human hepatocytes (25.8 μg/g total proteins) as compared to non-treated cells.

The average content of 7-dehydrocholesterol after LK-980 treatment (556 μg/g total proteins) was, surprisingly, 34.16 times higher (p=0.000) than in non-treated cells (16.3 μg/g total proteins). This indicated that DHCR7 is the major target of LK-980 in human primary hepatocytes. Atorvastatin resulted in negligible changes of 7-dehydrocholesterol (15.8 μg/g total proteins).

Since the results of GC-MS analysis indicate that LK-980 might inhibit several sterol reductases (DHCR7, DHCR14, DHCR24), its inhibitory potential has been tested also for HMGCR. HMGCR activities were measured in triplicates and expressed as pmol of product (mevalonate) formed per mg of total proteins per minute. HMGCR enzyme activity in the absence of inhibitor was (69 ± 9) pmol*mg^{-1}*min^{-1}. Addition of LK-980 at the concentration of 100 μM did not lower the activity (76 ± 12 pmol*mg^{-1}*min^{-1}), while the addition of rosuvastatin at the concentration of 0.1 μM substantially inhibited HMGCR activity (2.8 ± 0.5 pmol*mg^{-1}*min^{-1}). These results suggest that LK-980 is not a HMGCR inhibitor.

Identification of LK-980 enzyme targets in human primary hepatocytes with mathematical model simulations. In support to experimental results, we applied a mathematical model of cholesterol synthesis. The aim of the model simulations was to describe as accurately as possible the experimentally measured concentrations of sterol intermediates that have shown statistical significance after LK-980 and atorvastatin treatments. The model shows that for a linear pathway, only the concentrations of substrates and complexes that are directly involved in reactions with the disturbed enzyme are changed, while concentrations of other metabolites remain almost identical. However, the effect of a single enzyme change is spread wider across the network, with several interconnected

pathways. *In silico* experimenting with the model showed that it is possible to obtain the same relative changes in metabolite concentrations as have been measured in laboratory experiments (Table 3). Table 4 explains changes in enzyme concentrations that were introduced into the mathematical model, to simulate experimental results, as shown in Table 3. In both experimental cases, the effect of cholesterol on the cholesterogenic genes expressions is negligible, since the levels of cholesterol remain the same.

Table 4 shows that after inhibition by LK-980 the experimental sterol data can be simulated if concentration of DHCR7 is reduced majorly in addition to minor reduction of DHCR14, SC5DL and DHCR24. *In silico* inhibition of DHCR7 only was not sufficient to describe experimentally measured sterol concentrations (not shown).

7. Discussion

High plasma concentration of LDL-cholesterol is a major risk factor of cardiovascular diseases (atherosclerosis, coronary heart disease, myocardial infarction), massively increasing in the developed world. Antihyperlipidemic therapy is thus essential for prevention of the progression of cholesterol-laden plaques in vessel linings (Maas and Boger, 2003; LaRosa, 2007). Currently mostly used drugs on the market for lowering cholesterol are statins, the HMGCR inhibitors, which are relatively safe and well-tolerated drugs for majority of the patients; however, 2-3% of patients suffer from the statin-based muscular adverse drug reactions (myopathies, rhabdomyolysis), impaired cognitive function, nephropathies and hepatotoxicity (Tiwari et al., 2006; Alsheikh-Ali et al., 2007; Armitage, 2007; Martin and Krum, 2007; Rallidis et al., 2007).

Our aim was to determine the enzymes targeted of a novel cholesterol synthesis inhibitor LK-980. Several compounds structurally not related to LK-980 were already shown to inhibit the post-squalene cholesterol synthesis. Trans-1,4-bis(2-chlorobenzaminomethyl) cyclohexane dihydrochloride (AY 9944) inhibits several human post-squalene enzymes: two sterol reductases, DHCR14 and DHCR7, and the human sterol delta 8-7-isomerase (EBP). N-cyclohexyl-Nethyl-3-(3-chloro-4-cyclohexylphenyl)propen-2-ylamine hydrochloride (SR 31747) was shown to be a selective EBP inhibitor (Fernandez et al., 2005; Suarez et al., 2005). SKF 104976, an analog of lanosterol (Mayer et al., 1991), inhibits human lanosterol 14α-demethylase (CYP51A1). Our previous work showed compounds also targeting CYP51A1 (Korosec et al., 2008). Nevertheless, triparanol (MER-29), a cholesterol lowering agent, was marketed in USA from 1959, but was withdrawn by US Food and Drug Administration in 1962 because the patients receiving triparanol developed cataracts as a

result of notable accumulation of a cholesterol intermediate desmosterol (Flaxman, 1963; Perdriel, 1964; Kirby, 1967). Accumulation of desmosterol was reported several decades ago when mass spectrometry was not yet available. Thus it is questionable if desmosterol is indeed the sterol being accumulated.

Sterol profiling with radio-HPLC on immortal human hepatocytes HepG2 exposed LK-980 as a leading compound, based on negligible accumulation of FF-MAS and incomplete inhibition of cholesterol (data will be published elsewhere). Even if immortal hepatocytes are a very efficient tool for drug screening and toxicology, their major limitation is their non efficient drug metabolizing system. The low levels of phase I and II enzymes in HepG2 cells might have been responsible for the fact that 30% of compounds scored negative in toxicology tests (Westerink and Schoonen, 2007). Some of the cholesterol lowering statins (i.e. atorvastatin) and other potential cholesterol lowering drugs (LK-935) (Monostory et al., 2009) have been reported to increase the expression of CYP3A4 (Kocarek et al., 2002) and other drug metabolizing cytochromes P450. CYP enzyme induction is an undesirable drug interaction, especially for CYP3A4, the major drug metabolizing enzyme of the human liver, that transforms up to 70% of lipophylic drugs. Development of novel drugs suggests avoiding compounds that induce CYP3A4 (Smith, 2000). This is the case of LK-980 that induced neither the CYP3A4 enzyme activity nor the mRNA level of CYP3A4 (Fig. 4). Additionally, LK-980 is biologically stable in human primary hepatocytes, showing a slow metabolic degradation.

To characterize further properties of LK-980, additional experiments were performed in cultures of primary hepatocytes isolated from three human donors. On the basis of pharmacological efficiency and promising pharmacokinetic properties, LK-980 inhibitory

potential and enzyme targets was evaluated by original GC-MS, following 9 post-squalene cholesterol biosynthesis intermediates in a single run (Acimovic et al., 2009). However, only the total (and not the *de novo* synthesized) sterols have been measured, since the deuterium or ^{13}C labelling of human primary hepatocytes was not possible.

Several enzymes of the late part of cholesterol synthesis are still poorly characterized and are thus difficult to express in an active form. Thus, we determined the targets of the new cholesterol lowering compound by combination of sterol measurements and mathematical modelling. The four sterols that showed statistically significant difference between treatments (FF-MAS, lathosterol, desmosterol, 7-dehydrocholesterol) were applied to predict which enzymes are inhibited. The quantity of the four sterols increased after addition of LK-980 to human primary hepatocytes, indicating that several enzymes might be inhibited.

The quantities of lathosterol and desmosterol diminished after atorvastatin treatment, which is in accordance with inhibition of HMGCR, an early enzyme of this pathway. The reason for no change in FF-MAS and 7-dehydrochoelsterol after addition of atorvastatin might lie in the fact that we measured total sterols and not only the non-esterified ones.

FF-MAS is produced from lanosterol in a CYP51A1-mediated demethylation reaction (Fink et al., 2005). It serves as a substrate for DHCR14, an enzyme with poorly characterized properties, that removes the double bond at position C14 of the sterol ring D. LK-980 treatment resulted in a statistically significant increase (three times) in FF-MAS as compared to control or atorvastatin treated hepatocytes. This identified DHCR14 or a later enzyme in the pathway as targets of LK-980.

Lathosterol is formed from zymosterol in two consecutive enzymatic steps (delta 24,25 reduction and delta 7,8 isomerization), the order of which is not possible to determine from our analysis. Lathosterol is converted to 7-dehydrocholesterol by the SC5DL enzyme. The

average lathosterol content in LK-980 treated cells was 2.5 times higher than in non-treated cells, suggesting that LK-980 might inhibit SC5DL or a later enzyme.

Desmosterol and 7-dehydrocholesterol both represent the last intermediates before cholesterol, depending on the order of enzymatic reactions. Desmosterol differs from cholesterol in the unsaturated delta 24,25-double bond, while the difference between cholesterol and 7-dehydrocholesterol is in the unsaturated delta 7,8-double bond. Comparison of desmosterol in LK-980 treated and non-treated cells (about 2 times higher) indicated a weak inhibition of DHCR24 by LK-980. However, 7-dehydrocholesterol significantly increased (34-fold) as a consequence of LK-980 treatment, indicating that DHCR7 is the major target of this compound.

Our sterol measurements suggested that in addition to DHCR7, other late enzymes of the pathways might represent LK-980 targets. To confirm these predictions with an independent method, and to get additional insights into the inhibitory action of LK-980 in comparison to statins, we developed and applied a mathematical model of cholesterol synthesis. To our surprise, just by modifying concentrations of the crucial post-lanosterol enzymes, the model was able to completely simulate the experimentally measured sterols in drug-treated and untreated cells (shown as ratios of each individual sterol in treated/untreated samples). The mathematical simulation has helped importantly in providing the final conclusion regarding the inhibitory potential of LK-980. This novel cholesterol synthesis inhibitor indeed affects several enzymes of the pos-lanosterol portion of the pathway. The major target of LK-980 is DHCR7, one of the last two enzymes of cholesterol synthesis. LK-980 has also three minor targets, two sterol reductases DHCR14, DHCR24 and a sterol desaturase SC5DL. Since knowledge regarding enzymes of the late portion of cholesterol synthesis remains limiting, LK-980 can be a useful tool to determine potential structural similarities of the affected sterol metabolizing enzymes.

8. Acknowledgments

Thanks to Anne-Grete Byskov, Rikhospitalitet, Univ. of Copenhagen, for providing FF-MAS and T-MAS sterol standards and Anja Kersiek from karolinska Institute for technical assistance.

9. References

Acimovic J, Lovgren-Sandblom A, Monostory K, Rozman D, Golicnik M, Lutjohann D and Bjorkhem I (2009) Combined gas chromatographic/mass spectrometric analysis of cholesterol precursors and plant sterols in cultured cells. *J Chromatogr B Analyt Technol Biomed Life Sci* **877:**2081-2086.

Alsheikh-Ali AA, Maddukuri PV, Han H and Karas RH (2007) Effect of the magnitude of lipid lowering on risk of elevated liver enzymes, rhabdomyolysis, and cancer: insights from large randomized statin trials. *J Am Coll Cardiol* **50:**409-418.

Armitage J (2007) The safety of statins in clinical practice. *Lancet* **370:**1781-1790.

Bayliss KM and Skett P (1996) Isolation and culture of human hepatocytes, in: *Human cell culture protocols* (Jones GE ed), pp 369-390, Human Press, Totowa.

Bays HE, Neff D, Tomassini JE and Tershakovec AM (2008) Ezetimibe: cholesterol lowering and beyond. *Expert Rev Cardiovasc Ther* **6:**447-470.

Beg ZH and Stonik JA (1982) Reversible inactivation of 3-hydroxy-3-methylglutaryl coenzyme A reductase: reductase kinase and mevalonate kinase are separate enzymes. *Biochem Biophys Res Commun* **108:**559-566.

Buhaescu I and Izzedine H (2007) Mevalonate pathway: a review of clinical and therapeutical implications. *Clin Biochem* **40:**575-584.

Fernandez C, Martin M, Gomez-Coronado D and Lasuncion MA (2005) Effects of distal cholesterol biosynthesis inhibitors on cell proliferation and cell cycle progression. *J Lipid Res* **46:**920-929.

Ferrini JB, Ourlin JC, Pichard L, Fabre G and Maurel P (1998) Human hepatocyte culture. *Methods Mol Biol* **107:**341-352.

Fink M, Acimovic J, Rezen T, Tansek N and Rozman D (2005) Cholesterogenic lanosterol 14alpha-demethylase (CYP51) is an immediate early response gene. *Endocrinology* **146:**5321-5331.

Flaxman N (1963) Mer-29 (Triparanol) and Cataract. *Med Trial Tech Q* **10:**11-32.

Guengerich FP, Martin MV, Beaune PH, Kremers P, Wolff T and Waxman DJ (1986) Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J Biol Chem* **261:**5051-5060.

Kirby TJ (1967) Cataracts produced by triparanol. (MER-29). *Trans Am Ophthalmol Soc* **65:**494-543.

Kocarek TA, Dahn MS, Cai H, Strom SC and Mercer-Haines NA (2002) Regulation of CYP2B6 and CYP3A expression by hydroxymethylglutaryl coenzyme A inhibitors in primary cultured human hepatocytes. *Drug Metab Dispos* **30:**1400-1405.

Koh KK, Quon MJ, Rosenson RS, Chung WJ and Han SH (2008) Vascular and metabolic effects of treatment of combined hyperlipidemia: focus on statins and fibrates. *Int J Cardiol* **124:**149-159.

Korosec T, Acimovic J, Seliskar M, Kocjan D, Tacer KF, Rozman D and Urleb U (2008) Novel cholesterol biosynthesis inhibitors targeting human lanosterol 14alpha-demethylase (CYP51). *Bioorg Med Chem* **16:**209-221.

LaRosa JC (2007) Low-density lipoprotein cholesterol reduction: the end is more important than the means. *Am J Cardiol* **100:**240-242.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265-275.

Luo G, Cunningham M, Kim S, Burn T, Lin J, Sinz M, Hamilton G, Rizzo C, Jolley S, Gilbert D, Downey A, Mudra D, Graham R, Carroll K, Xie J, Madan A, Parkinson A,

Christ D, Selling B, LeCluyse E and Gan LS (2002) CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug Metab Dispos* **30:**795-804.

Maas R and Boger RH (2003) Old and new cardiovascular risk factors: from unresolved issues to new opportunities. *Atheroscler Suppl* **4:**5-17.

Martin JH and Krum H (2007) Statins and clinical outcomes in heart failure. *Clin Sci (Lond)* **113:**119-127.

Mayer RJ, Adams JL, Bossard MJ and Berkhout TA (1991) Effects of a novel lanosterol 14 alpha-demethylase inhibitor on the regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in Hep G2 cells. *J Biol Chem* **266:**20070-20078.

Monostory K, Pascussi JM, Szabo P, Temesvari M, Kohalmy K, Acimovic J, Kocjan D, Kuzman D, Wilzewski B, Bernhardt R, Kobori L and Rozman D (2009) Drug interaction potential of 2-((3,4-dichlorophenethyl)(propyl)amino)-1-(pyridin-3-yl)ethanol (LK-935), the novel nonstatin-type cholesterol-lowering agent. *Drug Metab Dispos* **37:**375-385.

Perdriel G (1964) [Cataract and Triparanol.]. *Bull Soc Ophtalmol Fr* **64:**699-701.

Rallidis LS, Lekakis J and Kremastinos DT (2007) Current questions regarding the use of statins in patients with coronary heart disease. *Int J Cardiol* **122:**188-194.

Rozman D and Monostory K Perspectives of the non-statin hypolipidemic agents. *Pharmacol Ther* **127:**19-40 (published ahead of print).

Sanossian N and Ovbiagele B (2008) Drug insight: translating evidence on statin therapy into clinical benefits. *Nat Clin Pract Neurol* **4:**43-49.

Schaars CF and Stalenhoef AF (2008) Effects of ubiquinone (coenzyme Q10) on myopathy in statin users. *Curr Opin Lipidol* **19:**553-557.

Smith DA (2000) Induction and drug development. *Eur J Pharm Sci* **11:**185-189.

Suarez Y, Fernandez C, Ledo B, Martin M, Gomez-Coronado D and Lasuncion MA (2005) Sterol stringency of proliferation and cell cycle progression in human cells. *Biochim Biophys Acta* **1734:**203-213.

Tiwari A, Bansal V, Chugh A and Mookhtiar K (2006) Statins and myotoxicity: a therapeutic limitation. *Expert Opin Drug Saf* **5:**651-666.

Urleb U, Kocjan D, Korosec T, Rozman D, Acimovic J, Tomazi A and Rode B (2006) HETEROCYCLIC COMPOUNDS, Lek pharmaceuticals d.d.

van der Hoeven TA and Coon MJ (1974) Preparation and properties of partially purified cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase from rabbit liver microsomes. *J Biol Chem* **249:**6302-6310.

Wassif CA, Brownson KE, Sterner AL, Forlino A, Zerfas PM, Wilson WK, Starost MF and Porter FD (2007) HEM dysplasia and ichthyosis are likely laminopathies and not due to 3beta-hydroxysterol Delta14-reductase deficiency. *Hum Mol Genet* **16:**1176-1187.

Westerink WM and Schoonen WG (2007) Cytochrome P450 enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. *Toxicol In Vitro* **21:**1581-1591.

Wilson ZE, Rostami-Hodjegan A, Burn JL, Tooley A, Boyle J, Ellis SW and Tucker GT (2003) Inter-individual variability in levels of human microsomal protein and hepatocellularity per gram of liver. *Br J Clin Pharmacol* **56:**433-440.

10. Footnotes

a) Unnumbered footnote providing the source of financial support.

This paper and work it concerns was generated in the context of the FP6 STEROLTALK project funded by the European Community as contract No. LSHG-CT-2005-512096. The study was supported also by the Slovenian-Hungarian Intergovernmental S&T Cooperation Programme (Sl-5/2008), by the Reseach Agency of the Republic Slovenia grants J1-9438 and P1-0104 and by funds of Lek pharmaceuticals.

b) The name and full address of person to receive reprint request.

Prof. dr. Damjana Rozman

Centre for Functional Genomics and Bio-Chips

Institute of Biochemistry

University of Ljubljana, Faculty of Medicine

Zaloska 4

SI-1000 Ljubljana, Slovenia

E-mail: damjana.rozman@mf.uni-lj.si

Fax: 00386-(0)1-543-7588

11. Legends for figures

Fig. 1. Structure of the mathematical model describing cholesterol synthesis. The meaning of the symbols: black circles – metabolites, gray circles – enzymes, white circles – mRNA, thick black lines – metabolic flux under normal conditions (arrows show the normal direction of reactions), thin black lines – involvement of enzymes, gray lines – the effect of metabolites on enzyme degradation, broken lines – enzyme formation and degradation, dotted lines – the effect of metabolites on gene expression, % - division of metabolic flux at branching points.

Fig. 2. A scheme of the post-squalene portion of cholesterol synthesis with underlined sterol intermediates that have been measured in our study and the enzyme abbreviations according to Unigene. SQLE - squalene epoxidase, LSS - lanosterol synthase, CYP51A1 - lanosterol 14α-demethylase, DHCR24 - sterol delta 24-reductase, DHCR14 - sterol delta 14-reductase, SC4MOL - sterol C4 methyl-oxidase, NSDHL - 3β-hydroxy delta 5-steroid dehydrogenase, HSD17B7 - 3β-keto reductase, EBP - sterol 8,7-isomerase, SC5DL - sterol C5-desaturase, DHCR7 - sterol delta 7-reductase. FF-MAS - follicular fluid meiosis activating sterol (4,4-dimethyl-5a-cholesta-8,14,24-triene-3b-ol), T-MAS - testis meiosis activating sterol (4,4-dimethyl-5a-cholesta-8,24-diene-3b-ol).

Fig. 3. *In vitro* kinetic analysis of LK-980 in primary human hepatocytes. LK-980 at the concentration of 1 μM was incubated in hepatocytes isolated from three donors. The consumption of LK-980 as a function of time was determined by LC-MS/MS as described in Materials and Methods. HH-089, HH-129, HH-114 – independent liver donors.

Fig. 4. Induction of CYP3A4 activity (A) and mRNA expression (B) in primary human hepatocytes. The cells were treated with rifampicin (Rif, 5 μM), atorvastatin (ATO, 10 μM), and LK-980 (10 μM).

Fig. 5. Logarithmic values of FF-MAS, lathosterol, 7-dehydrocholesterol and desmosterol expressed as log(mg of sterol per g of total proteins) for 3 different treatments (n = 7); non-treated control (ctrl), atorvastatin (ato) and LK-980. Each treatment represents the average and 95% LSD confidence intervals of human primary hepatocytes from 7 different donors.

13. Tables

Table 1.

Clinical histories of human donors.

Donor	Age (year)	Sex	Race	COD / Disease state
HH-089	44	Female	Caucasian	COD: subarachnoidal hemorrhage
HH-114	57	Female	Caucasian	COD: rupture of aneurism
HH-129	49	Female	Caucasian	COD: stroke
HH-150	53	Female	Caucasian	COD: subarachnoidal hemorrhage
HH-269	46	Male	Caucasian	Hepatocellular carcinoma
HH-270	57	Male	Caucasian	Metastasis of colon cancer
HH-271	56	Female	Caucasian	Metastasis of colon cancer
HH-272	20	Male	Caucasian	COD: subarachnoidal hemorrhage

COD: cause of death

Table 2.

Kinetic parameters for LK-980 (1μM) metabolism by primary human hepatocytes.

HH-089, HH-129, HH-114 – independent liver donors.

Parameter	HH-089	HH-114	HH-129
$t_{1/2}$ (hr)	>6.0	4.32	>6.0
Cl_{int} (ml/hr*kg)	<183	254.5	<183
Cl_H (ml/hr*kg)	<143	182.9	<143
E (extraction ratio)	<0.220	0.281	<0.220

Table 3.

Experimental and simulated results (relative change compared to untreated hepatocytes) for hepatocyte treatment with LK-980 and atorvastatin. 1 is normal concentration - no change.

Exp. – ratio of sterols (treated *versus* untreated) as calculated form GC-MS sterol measurements. Sim – simulated data from mathematical model. Abbreviations of sterols: chol – cholesterol, 7-dhc – 7-dehydrocholesterol, desmo – desmosterol, latho – lathosterol, lano – lanosterol, zymo – zymosterol, T-MAS - testis meiosis activating sterol, FF-MAS - follicular fluid meiosis activating sterol.

		chol	7-dhc	desmo	latho	lano	zymo	T-MAS	FF-MAS
LK-980	exp.	-	34.2	1.8	2.5	-	-	-	3.2
	sim.	-	34.6	1.8	2.5	-	-	-	3.2
Atorvastatin	exp.	-	-	0.5	0.5	-	-	-	0.5
	sim.	-	-	0.5	0.5	-	-	-	0.5

Table 4.

Relative changes in enzyme concentrations as the effect of drug treatment. 1 is normal concentration - no change.

	CYP51A1	DHCR14	E3[a]	EBP	SC5DL	DHCR7	DHCR24
LK980	-	0.3	-	-	0.3	0.04	0.4
atorvastatin	0.9	1.9	0.9	0.9	2.3	1.2	1.7

[a]E3 – combined enzymes: SC4MOL, NSDHL and HSD17B7

14. Supplemental data

Detailed description of the mathematical model of cholesterol synthesis

The measurements that were used in the study provided only information on the steady-state relations, so the model (see Materials and methods) was developed and analysed for the steady-state conditions. Steady-state of the system is reached when all the time derivatives in the above equations are equal to zero and when $\Phi_I = \Phi_O$, and $\Phi_{EI} = \Phi_{EO}$. The steady state equations of the enzyme reaction form the following system of equations:

$$0 = \Phi_I - k_C \cdot E \cdot S + k_{CR} \cdot C$$

$$0 = k_C \cdot E \cdot S + k_{PR} \cdot E \cdot P - k_P \cdot C - k_{CR} \cdot C$$

$$0 = k_P \cdot C - \Phi_I - k_{PR} \cdot E \cdot P$$

$$0 = k_P \cdot C + k_{CR} \cdot C - k_C \cdot E \cdot S - k_{PR} \cdot E \cdot P .$$

The concentrations of S, E, C, and P can be independently normalised to the dimensionless quantities by dividing their values by their steady-state values. At the same time also the reaction rates are scaled by the steady-state values of S_N, E_N, C_N, and P_N. The normalised reaction rates now represent the steady-state contributions of the specific reaction steps on the whole metabolic flux of the enzyme reaction. Using the steady-state normalised values of S_N, E_N, C_N, P_N, and the normalised reaction rates the equations above can be rewritten. Let us here present only the equation describing the steady-state of C_N since it represents the relation between all the free model parameters:

$$0 = k_{CN} \cdot E_N \cdot S_N + k_{PRN} \cdot E_N \cdot P_N - k_{PN} \cdot C_N - k_{CRN} \cdot C_N$$

To make the relation between the forward and the reverse metabolic fluxes more transparent, the following ratios can be defined:

$$\frac{k_{PRN}}{k_{CN}} = r_1$$

$$\frac{k_{CRN}}{k_{PN}} = r_2$$

Thus the equations can be rewritten in a new form,

$$0 = k_{CN} \cdot E_N \cdot S_N + k_{CN} \cdot r_1 \cdot E_N \cdot P_N - k_{PN} \cdot C_N - k_{PN} \cdot r_2 \cdot C_N ,$$

which yields the final form

$$0 = k_{CN} \cdot E_N \cdot (S_N + r_1 \cdot P_N) - k_{PN} \cdot C_N \cdot (1 + r_2) .$$

However, E_N, S_N, C_N, and P_N are in the steady-state of the undisturbed system equal 1, which further simplifies the relation:

$$0 = k_{CN} \cdot (1 + r_1) - k_{PN} \cdot (1 + r_2) .$$

As explained above, normalised reaction rates represent steady-state metabolic fluxes, therefore the values of r_1 and r_2 are smaller than 1, since in the undisturbed system all enzyme reactions proceed mostly in the forward direction. If r_1 and r_2 are selected equal this significantly simplifies the relation:

$$r_1 = r_2 = r$$

$$0 = k_{CN} \cdot (1 + r) - k_{PN} \cdot (1 + r) .$$

Since the factors (1+r) can be found in both terms, the relation yields:

$$0 = k_{CN} - k_{PN} ,$$

By selecting the values for r and Φ_I all the model parameters can be uniquely calculated by using the steady-state equations of the model. Thus it is clear that it is possible to reduce the number of independent model parameters to two by assuming that the steady-state backward/forward metabolic flux ratio is smaller than 1 and that it is equal for the complex and the product formation. The description of the rate constants with the parameters r and Φ_I

allows more intuitive experimenting with the model. The following equations for the steady-state values of S_N and C_N can be obtained from the steady-state equations:

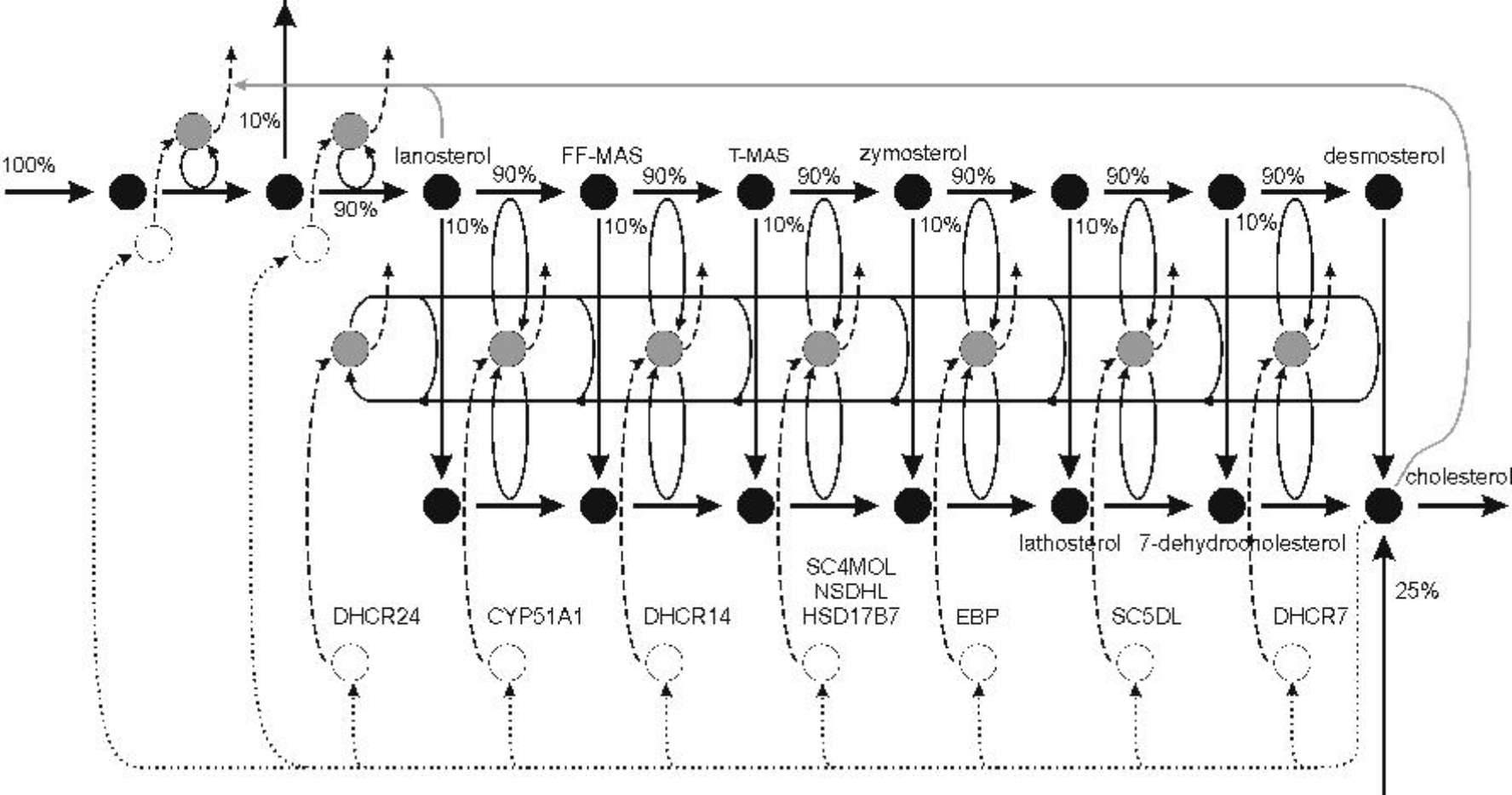
$$S_N = \frac{1-r^2}{E_N} + r^2 \cdot P_N ,$$

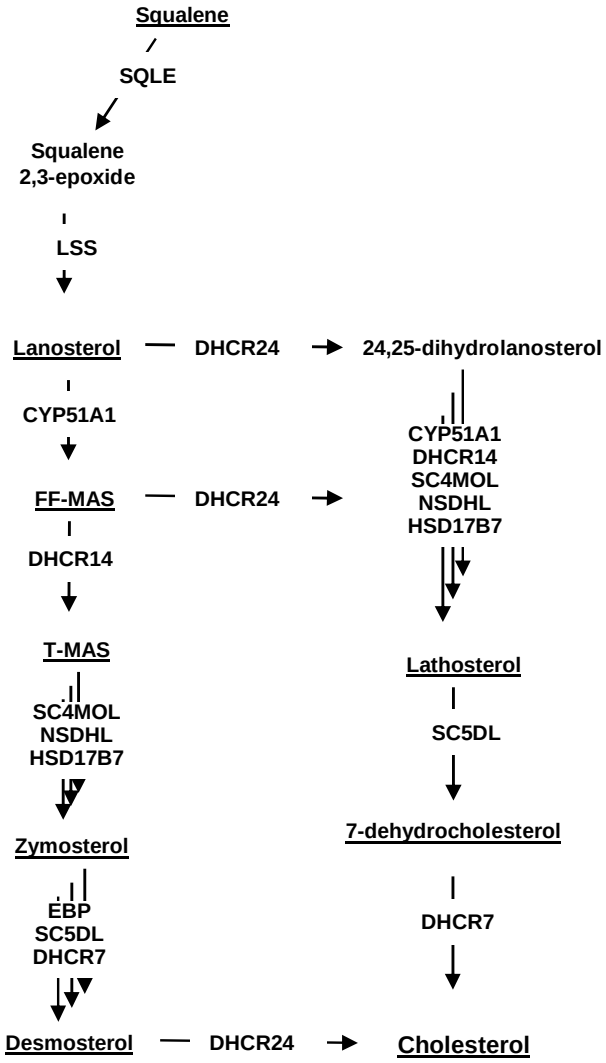
$$C_N = 1 - r \cdot (1 - E_N \cdot P_N) .$$

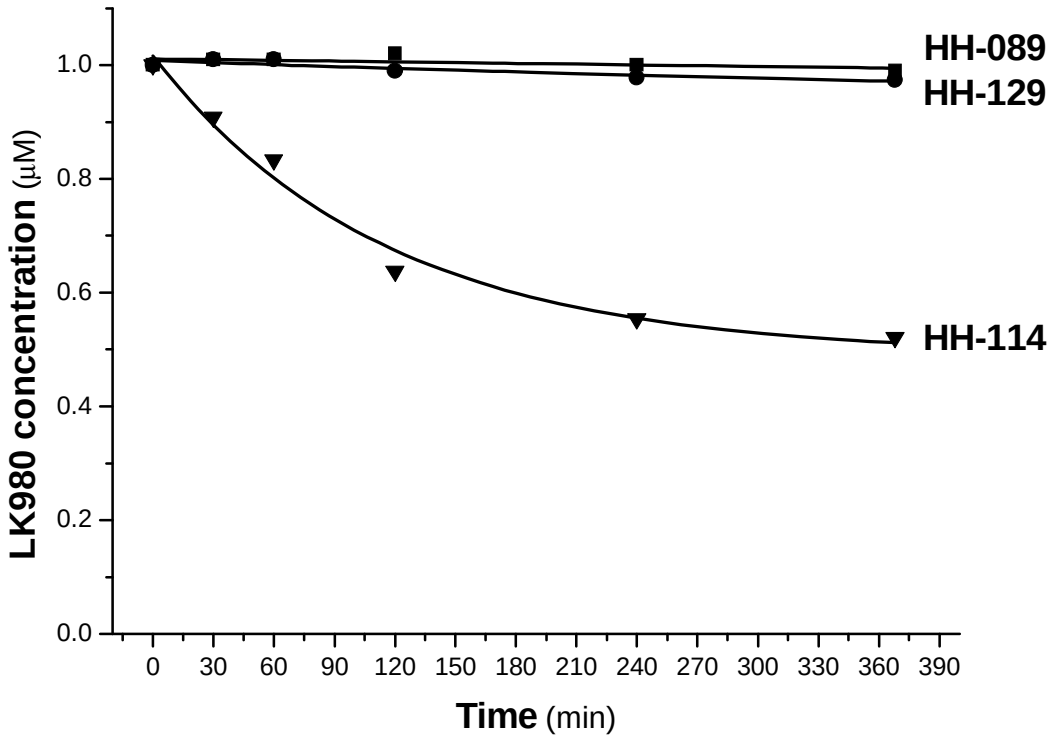
The equations show how the steady-state values of S_N and C_N will change if the new steady-state levels of E_N and P_N are forced. Interestingly, the metabolic flux Φ_I has no effect on the steady-state values of the metabolites, which suggests that for the analysis of the steady-state conditions it is not necessary to exactly know the metabolic flux of the metabolic network. However, the backward/forward metabolic flux ratio has some effect on the steady-state values, especially if r is relatively large. From the equations we can see that the normalised concentrations of the substrate are in reciprocal relation to the normalised concentrations of the enzyme, the value of r is present with its squared value, which shows that for small values of r the steady-state values of the substrate primarily depend on the concentrations of the enzyme; the effect of the enzyme concentrations on the concentrations of the complex is much smaller. The only problem for a unique calculation of the steady-state values of S_N and C_N represents the usually unknown level steady-state value of P_N after perturbation; however, if r is very small, the influence of changed levels of P_N is also small.

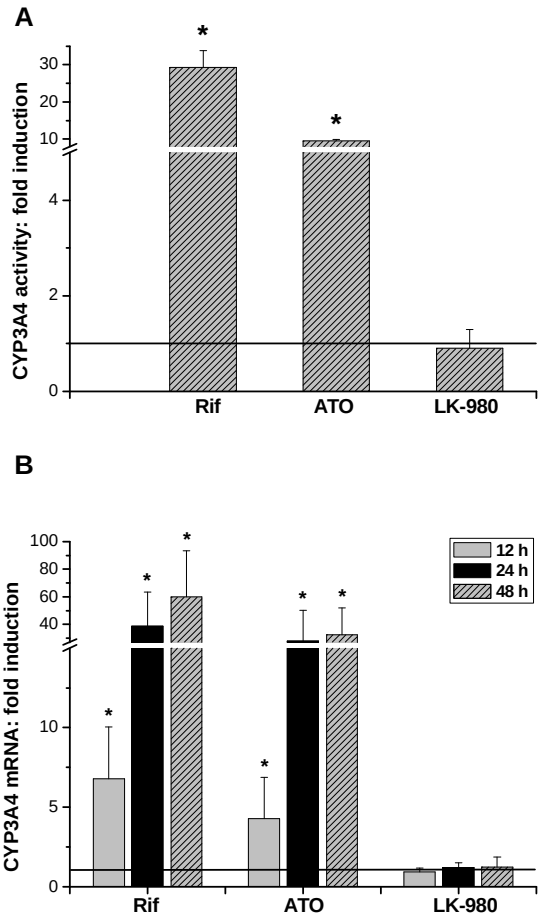
A metabolic network can be described by combining the enzymatic reaction models. If the network consists of a single linear pathway, the metabolic flux through the network in steady-state remains equal to the initial metabolic flux. However, if the network consists of several interconnected pathways the steady-state ratio of the metabolic fluxes at each crossing of the metabolic pathways (branching points) has to be introduced as an additional model parameter.

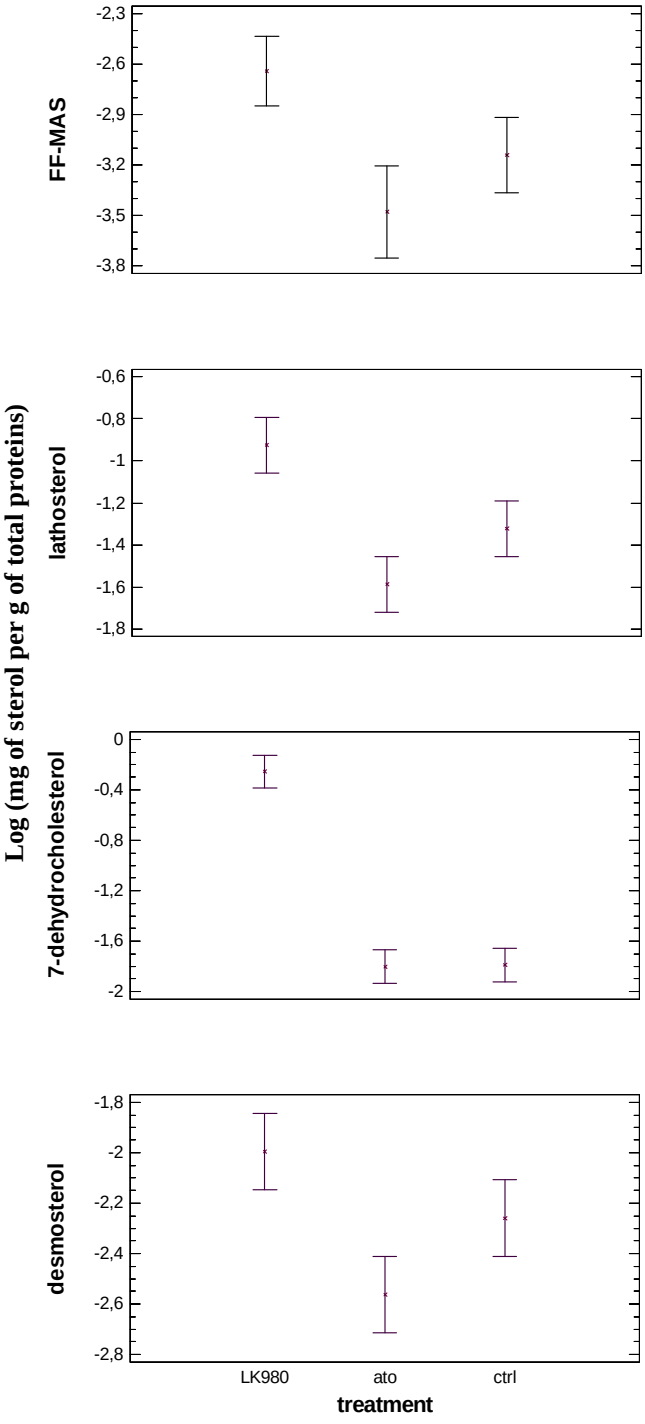
For the tests performed in this study, the value for r was varied and was finally chosen 0.01 for all the reactions in the network while the chosen metabolic flux ratios at branching points are shown Fig. 1. The branching ratios affect the steady-state of the branching point significantly if the concentrations of one or more enzymes immediately after the branching point are changed, since flux ratios can change significantly after the perturbation. The metabolic flux at the beginning was set to 100, however, as shown above, its choice has no effect on the steady-state values of metabolite concentrations. In any metabolic network, there are also irreversible steps, at the latest, when a metabolite is eliminated from an organism. This solves the problem of the unknown value of the steady-state of P_N after perturbation. Since the last step of the network is irreversible, r becomes equal to 0 and the new steady-states of all the metabolites are uniquely set. Without irreversible reactions the network has an infinite number of possible steady-states.


100%
10%
90%
lanosterol
90%
FF-MAS
90%
T-MAS
90%
zymosterol
90%
90%
90%
desmosterol
10%
10%
10%
10%
10%
10%
cholesterol
lathosterol
7-dehydrocholesterol
25%
DHCR24
CYP51A1
DHCR14
SC4MOL
NSDHL
HSD17B7
EBP
SC5DL
DHCR7


Squalene
SQLE
Squalene 2,3-epoxide
LSS
Lanosterol
DHCR24
24,25-dihydrolanosterol
CYP51A1
FF-MAS
DHCR24
CYP51A1
DHCR14
SC4MOL
NSDHL
HSD17B7
DHCR14
T-MAS
Lathosterol
SC4MOL
NSDHL
HSD17B7
SC5DL
Zymosterol
7-dehydrocholesterol
EBP
SC5DL
DHCR7
DHCR7
Desmosterol
DHCR24
Cholesterol


HH-089
HH-129
HH-114
LK980 concentration (μM)
1.0
0.8
0.6
0.4
0.2
0.0
0
30
60
90
120
150
180
210
240
270
300
330
360
390
Time (min)


A
CYP3A4 activity: fold induction
30
20
10
4
2
0
*
*
Rif
ATO
LK-980
B
CYP3A4 mRNA: fold induction
100
80
60
40
10
5
0
12 h
24 h
48 h
Rif
ATO
LK-980


Log (mg of sterol per g of total proteins)
FF-MAS
-2,3
-2,6
-2,9
-3,2
-3,5
-3,8
lathosterol
-0,6
-0,8
-1
-1,2
-1,4
-1,6
-1,8
7-dehydrocholesterol
0
-0,4
-0,8
-1,2
-1,6
-2
desmosterol
-1,8
-2
-2,2
-2,4
-2,6
-2,8
LK980
ato
ctrl
treatment