Inhibition of Human Sterol Δ7-Reductase and Other Postlanosterol Enzymes by LK-980, a Novel Inhibitor of Cholesterol Synthesis

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ABSTRACT:

Novel potential inhibitors of the postsqualene 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 prospective compound and was characterized further in cultures of human primary hepatocytes from seven donors. In vitro kinetic measurements show that the half-life of LK-980 is at least 4.3 h. LK-980 does not induce CYP3A4 mRNA nor enzyme activity. Target prediction was performed by gas chromatography-mass spectrometry, allowing simultaneous separation and quantification of nine late cholesterol intermediates. Experiments indicated that human sterol Δ7-reductase (DHCR7) is the major target of LK-980 (34-fold increase of 7-dehydrocholesterol), whereas human sterol Δ14-reductase (DHCR14), human sterol Δ24-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 whether indeed more than a single enzyme is inhibited. In silico inhibition of only DHCR7 modifies the flux of cholesterol intermediates, resulting in a 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.

Introduction

The imbalance of cholesterol homeostasis with a high plasma concentration of low-density lipoprotein cholesterol is a risk factor for cardiovascular diseases (atherosclerosis, coronary heart disease, and myocardial infarction); therefore, antihyperlipidemic therapy is essential for prevention of the progression of cholesterol-laden plaques in vessel linings (Maas and Böger, 2003; LaRosa, 2007). Lipid-modifying interventions to decrease elevated cholesterol concentrations constitute inhibition of de novo cholesterol biosynthesis in the liver and a decrease in 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 (HMGR) inhibitors, which are relatively safe and well tolerated drugs for most patients; however, 2 to 3% of patients experience statin-based muscular adverse drug reactions (myopathies and 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, mevalonate kinase, but not in more distal enzymes of cholesterologenesis, are associated with the statin-provoked skeletal

ABBREVIATIONS: HMGR, HMG-CoA reductase; DHCR14, human sterol Δ14-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); DHCR24, human sterol Δ24-reductase; DHCR7, human sterol Δ7-reductase; SC5DL, human sterol C5-desaturase; LK-980, 2-(4-phenethylpiperazin-1-yl)-1-(pyridine-3-yl)ethanol; GC, gas chromatography; MS, mass spectrometry; LC, liquid chromatography; MS/MS, tandem mass spectrometry; RT, reverse transcriptase; PCR, polymerase chain reaction; LSD, least significant difference; Ay 9944, trans-1,4-bis-[2-chlorobenzaminomethyl] cyclohexane dihydrochloride; SR 31747, N-cyclohexyl-N-ethyl-3-(3-chloro-4-cyclohexylyphenyl)propen-2-yamine hydrochloride; SKF 104976, 3j-hydroxylanost-8,15-dien-32-carboxylic acid; CYP51A1, lanosterol 14α-demethylase; MER-29, triparanol; LK-980, 2-(4-phenethylpiperazin-1-yl)-1-(pyridin-3-yl)ethanol.
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 (CoQ-10). This might contribute to muscle-related complications, but data in patients remain controversial (Schaars and Stalenhoef, 2008). Because of the increasing incidence of hyperlipidemias and cardiovascular diseases in the developed world and the risk of severe adverse drug reactions associated with statin-based therapy, developing novel strategies in cholesterol-lowering therapy and searching for potential hypolipidemic drugs with new molecular targets remain important tasks for the pharmaceutical industry.

Several enzymes are involved in postsqualene cholesterol synthesis (Fig. 1). The human sterol \(\Delta^{14}\)-reductase (DHCR14) transforms follicular fluid meiosis activating sterol (FF-MAS) or its \(\Delta^{24}\) analog into testis meiosis activating sterol (T-MAS) or its \(\Delta^{24}\) analog. 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 (Wassif et al., 2007; Rozman and Monostory, 2010). One of the most interesting enzymes is human sterol \(\Delta^{24}\)-reductase (DHCR24), a FAD-dependent oxidoreductase that 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. In addition, reduced expression of the gene occurs in the temporal cortex of patients with Alzheimer’s disease, and overexpression has been observed in adrenal gland cancer cells (Rozman and Monostory, 2010). 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, 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 the 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, 2010). 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 previously (Rozman and Monostory, 2010).

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

Herein we describe LK-980, a member of a novel class of distal inhibitors of cholesterol biosynthesis that target DHCR7 and to a lesser extent DHCR14 (TM7SF2), DHCR24, and SC5DL, as deduced from studies in human primary hepatocytes and sustained with a mathematical model of cholesterol synthesis. LK-980 is stable in human primary hepatocytes and does not induce CYP3A4.

Materials and Methods

Chemicals. Collagenase, nutrient mixture F-12 (HAM’s), Williams’ medium E, trypan blue, and rifampicin were the products of Sigma Chemie GmbH (Deisenhofen, Germany). EGTA was purchased from Fluka (Buchs, 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 (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco’s modified Eagle’s medium containing 5% iron-supplemented bovine calf serum and 1% l-glutamine at 37°C and 5% CO\(_2\) in 300–cm\(^2\) flasks. The cells were harvested at approximately 90% confluence 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 dithiothreitol, 1 mM phenylmethylsulfonyl

FIG. 1. A scheme of the postsqualene portion of cholesterol synthesis with underlined sterol intermediates that have been measured in our study. Enzyme abbreviations are according to Unigene. SQLE, squalene epoxidase; LSS, lanosterol synthase; NSDHL, 3β-hydroxy Δ⁷-steroid dehydrogenase; HSD17B7, 3β-keto reductase; EBP, sterol Δ⁵,7-isomerase.
fluoride and 1× Complete Protease Inhibitor. For each 300-cm² flask, 0.6 ml of buffer was used. The cell suspension was placed on ice and sonicated for 1 min. The sonicated sample was centrifuged at 12,000g and 4°C for 10 min to obtain clarified homogenate containing microsomes that were used for the HMG-CoA reductase assay activity. The total protein concentration of clarified cell homogenate was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

HMGCR activity was determined using a modification of the method of Beg and Stonick (1982). Assay buffer contained 1 μg/ml 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. Homogenate (25 μl) containing microsomes was preincubated at 37°C with 70 μl of assay buffer at 37°C for 20 min before the addition of NADPH at a final concentration of 2.3 mM, inhibitor, and [3-14C]HMG-CoA (PerkinElmer Life and Analytical Sciences, Waltham, MA) to the reaction. The reaction was allowed to proceed for 30 min at 37°C and terminated with 50 μl of 1 M propanoic acid. The samples were centrifuged (16,000g at 4°C) to pellet any insoluble material. Supernatant was applied to a AG1-X8 anion exchange resin column (Bio-Rad Laboratories, Hercules, CA). Columns were prepared by equilibrating AG1-X8 resin in 1 M acetic acid at room temperature followed by 1 M propanoic acid 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 and then were positioned over liquid scintillation vials. The sample was applied to the column and the columns were washed with 2 ml of distilled water and then were positioned over liquid scintillation vials. The sample was applied to the column and the amounts of 3H and 14C radioactivity were determined by liquid scintillation spectrometry (Ramona2000; Raytest, Straubenhardt, Germany).

Isolation and Culture of Human Hepatocytes. Human livers (HH-089, HH-114, HH-129, HH-150, and HH-272) were obtained from kidney transplant donors or from lobectomy segments (HH-269, HH-270, and HH-271) resected from adult patients for medical reasons unrelated to our research program (Transplantation and Surgical Clinic, Semmelweis University, Budapest, Hungary; Centre Hospitalier Universitaire, 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 human 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 1.7×10⁶ cells/ml at 37°C in a humid atmosphere containing 5% CO₂. The parent compound was added directly to the medium (final concentration of 10 μM) or LK-980 (10 μM) for 48 h. After being washed in phosphate-buffered saline, the cells were harvested for sterol profile analysis.

In Vitro Pharmacokinetics of LK-980. Biotransformation of LK-980 was performed in cell suspension (1.7×10⁶ cells/ml) at 37°C in a humid atmosphere containing 5% CO₂. The parent compound was added directly to the medium (final concentration of 10 μM). Aliquots (0.5 ml) of the incubation mixtures were terminated by the addition of 0.5 ml of 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 chromatography (LC) coupled to tandem mass spectrometry (MS/MS) for quantitation of the parent compound. For this purpose, a SB C18 Zorbax column (250×4.6 mm; Agilent Technologies, Santa Clara, CA) was used. Gradient elution was applied with mobile phase A consisting of 10 mM ammonium formate (pH 8.5) and with acetonitrile as 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. MS/MS measurements were performed on a 3200 Q TRAP hybrid quadrupole triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a TurboV ion source. The ionization mode was electrospray in positive mode. The instrument was scanned in enhanced product ion mode for structure identification and in multiple reaction monitoring mode for quantitation. The source conditions were as follows: curtain gas, 20 units; spray voltage, 5000 V; source temperature, 450°C; nebulizing gas, 50 units; and drying gas, 30 units. Two multiple reaction monitoring transitions (the first transition as quantifier and the second as the qualifier channel), 312/105 (collision energy 47 eV) and 312/160 (collision energy 33 eV), were monitored for LK-980 in a dwell time of 300 ms.

Estimation of Clearance. The intrinsic clearance for hepatocytes (CLint, ml/min/1.7×10⁶ cells) was calculated from the decrease in the concentration of LK-980 as follows:

\[
CL_{\text{int}} = \frac{\beta}{t_{1/2}}
\]

For scaling up the CLint value to obtain CLint per whole liver (g)/b. wt. (kg), the following parameters were used: cell concentration in liver, 1.07×10⁶ cells/g of liver (Wilson et al., 2003); average liver weight of human, 1660 g; and average body weight of human, 70 kg. The value for hepatic clearance (CHl) was calculated as follows:

\[
CH_l = \frac{C_{\text{int}} \cdot \text{Liver wt.} \cdot f_u \cdot Q_{\text{plasma}}}{(C_{\text{int}} \cdot \text{Liver wt.} \cdot f_u + Q_{\text{plasma}})
\]

where \(Q_{\text{plasma}} = Q_{\text{b}} \cdot \text{plasma/blood ratio} \). To calculate \(C_{\text{int}} \cdot Q_{\text{b}} = 1140 \text{ ml of blood/(h}\cdot \text{kg})\), the plasma/blood ratio = 0.57, and \(f_u = 1\) values were used. (\(Q_{\text{b}}\) is the hepatic blood low, and \(f_u\) is the unbound fraction of the compound.) The hepatic extraction ratio was defined as \(E = C_{\text{int}} \cdot Q_{\text{b}}\).

CYP3A4 Induction in Human Hepatocytes. Forty-eight hours after serum deprivation, the plated hepatocytes were cultured in the presence or absence of inducers for 12, 24, or 48 h. Hepatocytes were treated with rifampicin (5 μM), atorvastatin (10 μM), or LK-980 (10 μM). After being washed in phosphate-buffered saline, the cells were harvested for CYP3A4 enzyme assay and transcriptase analysis. A microsomal fraction from cultured human hepatocytes was prepared by differential centrifugation (van der Hoeven and Coon, 1974). The protein content of microsomes was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard. A published method was followed to determine nifedipine oxidation activity selective for CYP3A4 (Guengerich et al., 1986). The incubation mixture contained a NADPH-generating system (1 mM NADPH, 10 mM glucose-6-phosphate, 5 mM MgCl₂, and 2 units/ml glucose-6-phosphate dehydrogenase), microsomes, and nifedipine as the substrate for CYP3A4. The rates of enzyme activity were linearly dependent on the amount of microsomal protein added for the 30-min incubation period. The metabolic extraction procedure and high-performance liquid chromatography analysis were performed according to the published method (Guengerich et al., 1986). CYP3A4 enzyme assay was performed in triplicate, and means ± S.D. were calculated. For comparison among untreated and treated groups, statistical analysis of the results was performed using a two-tailed \(t\) test with \(p < 0.05\) as the criterion for significance. Because of the high variation in basic cytochrome P450 expression of human hepatocytes, the entire experiment was repeated in hepatocytes isolated from four to seven 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

![TABLE 1](https://example.com/table1.png)

**TABLE 1**

Clinical histories of human donors

<table>
<thead>
<tr>
<th>Donor</th>
<th>Age</th>
<th>Sex</th>
<th>Race</th>
<th>COD/Disease State</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH-089</td>
<td>44</td>
<td>Female</td>
<td>White</td>
<td>COD: subarachnoidal hemorrhage</td>
</tr>
<tr>
<td>HH-114</td>
<td>57</td>
<td>Female</td>
<td>White</td>
<td>COD: rupture of aneurism</td>
</tr>
<tr>
<td>HH-129</td>
<td>49</td>
<td>Female</td>
<td>White</td>
<td>COD: stroke</td>
</tr>
<tr>
<td>HH-150</td>
<td>53</td>
<td>Female</td>
<td>White</td>
<td>COD: subarachnoidal hemorrhage</td>
</tr>
<tr>
<td>HH-269</td>
<td>46</td>
<td>Male</td>
<td>White</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HH-270</td>
<td>57</td>
<td>Male</td>
<td>White</td>
<td>Metastasis of colon cancer</td>
</tr>
<tr>
<td>HH-271</td>
<td>56</td>
<td>Female</td>
<td>White</td>
<td>Metastasis of colon cancer</td>
</tr>
<tr>
<td>HH-272</td>
<td>20</td>
<td>Male</td>
<td>White</td>
<td>COD: subarachnoidal hemorrhage</td>
</tr>
</tbody>
</table>

COD, cause of death.
μg) was reverse-transcribed into single-stranded cDNA using a 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) and a UPL probe for CYP3A4 (Roche Diagnostics GmbH). The quantity of target RNA relative to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was determined. The sequences of primers and probes used for RT-PCR analyses have been described previously (Kohalmy et al., 2007). 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 performed 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- and atorvastatin-treated (positive control) and nontreated primary hepatocytes from seven donors (HH-114, HH-129, HH-150, HH-272, HH-269, HH-270, and 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 analyzed with a novel GC-MS method, which enables quantitative measurements of several postquealene 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 seven donors was normalized on the total protein content of the cells. Treatment with atorvastatin served as the positive control. Statistical analysis was performed using R (version 2.9.0). An appropriate statistical method was used, depending on the data being analyzed. A two-tailed t test was used for comparison of two groups (control and treatment) and a multiple range test was used for more than two treatments. Levene’s test of homogeneity of variances between three treatments (control, atorvastatin, and LK-980) with α = 0.05 was used to establish further parametric or nonparametric statistical tests. Levene’s test was repeated on logarithmic data if the raw data showed statistically significant differences between variances. One-way analysis of variance (α = 0.05) was used for comparison of treatments. If there was a statistically significant difference (α = 0.05), least significant difference (LSD) post hoc tests were performed with α = 0.05 for pairwise comparison between treatments.

**Mathematical Model of Cholesterol Synthesis.** The model was designed as a kinetic model with relations between molecules as illustrated in Fig. 2. Every enzyme reaction (thick arrows in Fig. 2) is described with four differential equations:

\[
\frac{dS}{dt} = \Phi_1 - k_C \cdot E \cdot S + k_C \cdot C
\]

\[
\frac{dC}{dt} = k_C \cdot E \cdot S + k_P \cdot E \cdot P - k_C \cdot C - k_C \cdot C
\]

\[
\frac{dP}{dt} = k_P \cdot C - \Phi_0 - k_P \cdot E \cdot P
\]

\[
\frac{dE}{dt} = \Phi_0 + k_P \cdot C + k_C \cdot C - k_C \cdot E \cdot S - k_P \cdot E \cdot P - \Phi_E
\]

where \( S \) denotes the concentration of the substrate, \( C \) the concentration of the enzyme, \( P \) the concentration of the product, \( E \) the activity (concentration) of the enzyme, \( \Phi_1 \) the flux of the substrate into the reactor, \( \Phi_0 \) the flux of the product out of the reactor, \( \Phi_E \) the flux of the enzyme into the reactor, \( k_C \) the rate constant of the complex formation, \( k_C \) the rate constant of decomposition of the complex into the substrate and the enzyme, \( k_P \) the rate constant of the product formation, and \( k_P \) 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 a 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 substances involved settle at a nonzero steady state. Further specifications of the model are explained in the supplemental data.

**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 a 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 h of incubation. The elimination half-life was estimated to be 4.32 h in the hepatocytes of HH-114 and more than 6 h in the liver cells of HH-089 and HH-129 (Table 2), indicating slow
hepatic 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 cytochrome P450 expression of the cells. Because 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, 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. Figure 4 presents CYP3A4 activities and mRNA levels relative to that of 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 induction of CYP3A4 activity in hepatocytes isolated from various donors (HH-089, HH-129, and HH-114 are independent liver donors). The consumption of LK-980 as a function of time was determined by LC-MS/MS as described under Materials and Methods. HH-089, HH-129, and HH-114 were independent liver donors.

**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 seven liver donors were prepared (HH-114, HH-129, HH-150, HH-272, HH-269, HH-270, and HH-271) as described under 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 postsqualene 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 nontreated control cells. Sterols that pass the statistical threshold (p < 0.05) are shown in Fig. 5. One-way analysis of variance 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% LSD post hoc tests were performed to show statistically significant differences between all pairs of treatments. We have to take into account the fact that the extracts of primary human hepatocytes contain total (esterified and free) sterols of the cells.

Figure 5 shows that the average FF-MAS content of LK-980-treated hepatocytes (2.9 µg/g total proteins) was 3.17 times higher.

![Graph showing induction of CYP3A4 activity and mRNA expression](image)

**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 under Materials and Methods. HH-089, HH-129, and HH-114 are 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).

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HH-089</th>
<th>HH-114</th>
<th>HH-129</th>
</tr>
</thead>
<tbody>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>&gt;6.0</td>
<td>4.32</td>
<td>&gt;6.0</td>
</tr>
<tr>
<td>C&lt;sub&gt;Lin&lt;/sub&gt; (ml/h · kg)</td>
<td>&lt;183</td>
<td>254.5</td>
<td>&lt;183</td>
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<tr>
<td>C&lt;sub&gt;Ls&lt;/sub&gt; (ml/h · kg)</td>
<td>&lt;143</td>
<td>182.9</td>
<td>&lt;143</td>
</tr>
<tr>
<td>E (extraction ratio)</td>
<td>&lt;0.220</td>
<td>0.281</td>
<td>&lt;0.220</td>
</tr>
</tbody>
</table>

**TABLE 2**

Kinetic parameters for LK-980 (1 µM) metabolism by primary human hepatocytes. HH-089, HH-129, and HH-114 were independent liver donors.
Figure 5 also shows that LK-980 \((p = 0.006)\) and atorvastatin \((p = 0.048)\) treatments caused statistically significant changes in lathosterol of primary hepatocytes compared with that of the control cells. The average lathosterol content in LK-980-treated cells \((118.9 \mu g/g\) total proteins\) was 2.50 times higher than that in nontreated cells \((47.7 \mu g/g\) total proteins\), suggesting that LK-980 might inhibit SC5DL or later enzymes in the pathway. Atorvastatin slightly reduced lathosterol of the human hepatocytes \((25.8 \mu g/g\) total proteins\) compared with that of nontreated cells.

The average content of 7-dehydrocholesterol after LK-980 treatment \((556 \mu g/g\) total proteins\) was, surprisingly, 34.16 times higher \((p = 0.000)\) than that in nontreated cells \((16.3 \mu g/g\) total proteins\). This finding indicated that DHCR7 is the major target of LK-980 in human primary hepatocytes. Atorvastatin resulted in negligible changes in 7-dehydrocholesterol \((15.8 \mu g/g\) total proteins\) (Fig. 5).

Because the results of GC-MS analysis indicate that LK-980 might inhibit several sterol reductases \((DHCR7, DHCR14,\) and \(DHCR24\)), its inhibitory potential was also tested for HMGCR. HMGCR activities were measured in triplicate and are expressed as picomoles of product (mevalonate) formed per milligram of total proteins per minute. HMGCR enzyme activity in the absence of inhibitor was \(69 \pm 9\) pmol \(\cdot g^{-1}\) min\(^{-1}\). Addition of LK-980 at the concentration of 100 \(\mu M\) did not lower the activity \((76 \pm 12\) pmol \(\cdot g^{-1}\) mm\(^{-1}\)), whereas the addition of rosuvastatin at the concentration of 0.1 \(\mu M\) substantially inhibited HMGCR activity \((2.8 \pm 0.5\) pmol \(\cdot g^{-1}\) 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.** To support the experimental results, a mathematical model of cholesterol synthesis was used. The model shows that for a linear pathway, only the concentrations of substrates and the complexes that are directly involved in the enzyme reactions are changed, whereas the concentrations of other metabolites remain almost identical. However, the effect of a single enzyme activity change is spread wider across the network, with several interconnected pathways. Laboratory experiments with the cholesterol biosynthesis network provided data with measured sterol concentrations in primary human hepatocytes treated with atorvastatin and LK980. 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 (Fig. 6). In both experimental cases, the effect of cholesterol on the cholesterogenic gene expressions is negligible, because the levels of cholesterol remain unchanged. Figure 6 shows that after inhibition by LK-980 the experimental sterol data can be simulated if activity of DHCR7 is reduced greatly in addition to a minor reduction in DHCR14, SC5DL, and DHCR24. To simulate the experimental sterol data after inhibition of atorvastatin, which inhibits HMGCR at the start of cholesterol synthesis, concentrations of many of the postsqualene enzymes are increased or close to normal.

**Discussion**

A high plasma concentration of low-density lipoprotein cholesterol is a major risk factor for cardiovascular diseases \((atherosclerosis, coronary heart disease, and myocardial infarction\), which are increasing greatly in the developed world. Antihyperlipidemic therapy is thus essential for prevention of the progression of cholesterol-laden plaques in vessel linings (Maas and Böger, 2003; LaRosa, 2007). The most used drugs for lowering cholesterol that are currently on the market are statins. However, evidence suggests that up to 3% of patients suffer from adverse reactions (Tiwari et al., 2006; Alsheikh-Ali et al., 2007; Armitage, 2007; Martin and Krum, 2007; Vallidis et al., 2007).
Fig. 6. Experimental and simulated results (relative change compared with untreated hepatocytes) for hepatocyte treatment with LK980 (LK) and atorvastatin (AT). A value of 1.0 is normal concentration or activity; values less than 1.0 mean reduced concentrations/activities, and values greater than 1.0 mean elevated concentrations/activities, and — means that the measurements were not performed. For sterols, the results were calculated from GC-MS sterol measurements, whereas enzyme activity was only simulated. Results of mathematical model simulations are shown in parentheses. The sterols that were not measured are presented as black circles. NSDHL, 3β-hydroxy Δ7-sterol dehydrogenase; HSD17B7, 3β-keto reductase; EBP, sterol Δ7,9-isomerase.

Our aim was to determine the enzymes targeted by a novel cholesterol synthesis inhibitor, LK-980. Several compounds structurally not related to LK-980 were already shown to inhibit the postsqualene cholesterol synthesis. Trans-1,4-bis(2-chlorobenzenaminomethyl) cloclohexane dihydrochloride (AY 9944) inhibits several human post-squalene enzymes: two sterol reductases, DHCR14 and DHCR7, and the human sterol Δ7,9-isomerase. N-Cyclohexyl-N-ethyl-3-(3-chloro-4-cyclohexylyl)propen-2-ylamine hydrochloride (SR 31747) was shown to be a selective sterol Δ7,9-isomerase inhibitor (Fernández et al., 2005; Suárez et al., 2005). 3β-Hydroxylanost-8,15-dien-3-carboxylic acid (SKF 104976), an analog of lanosterol (Mayer et al., 1991), inhibits human lanosterol 14α-demethylase (CYP51A1). Our previous work also showed compounds targeting CYP51A1 (Korosec et al., 2008). Another cholesterol-lowering agent, triparanol (MER-29), was marketed in United States from 1959 but was withdrawn by the U.S. 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 before mass spectrometry was available. Thus, it is questionable whether desmosterol was indeed the sterol being accumulated.

Sterol profiling with radio-high-performance liquid chromatography on immortal human hepatocytes, HepG2, identified LK-980 as a lead compound, based on negligible accumulation of FF-MAS and incomplete inhibition of cholesterol (data will be published elsewhere). Even if immortal hepatocytes are an efficient tool for drug screening and toxicology, their major limitation is their inefficient 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 as negative in toxicology tests (Westerinck and Schoonen, 2007). Some of the cholesterol-lowering statins (i.e., atorvastatin) and another potential cholesterol-lowering drug [2-((3,4-dichlorophenethyl)(propyl)amino)-1-(pyridin-3-yl) ethanol (LK-935)] (Monostory et al., 2009) have been reported to increase the expression of CYP3A4 (Kocarek et al., 2002) and that of other drug-metabolizing cytochromes P450. Cytochrome P450 enzyme induction is an undesirable drug interaction, especially for CYP3A4, the major drug-metabolizing enzyme of the human liver, which transforms up to 70% of lipophilic drugs. Avoidance of compounds that induce CYP3A4 is suggested in development of novel drugs (Smith, 2000). This is the case for LK-980, which induced neither CYP3A4 enzyme activity nor the mRNA level of CYP3A4 (Fig. 4). In addition, LK-980 is biologically stable in human primary hepatocytes, showing 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, the LK-980 inhibitory potential and enzyme targets were evaluated by original GC-MS, following nine postsqualene 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, because the deuterium or 13C labeling 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 a combination of sterol measurements and mathematic modeling. The four sterols that showed a statistically significant difference among treatments (FF-MAS, lathosterol, desmosterol, and 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-dehydrocholesterol after addition of atorvastatin might lie in the fact that we measured total sterols and not only the nonesterified 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 compared with control or atorvastatin-treated hepatocytes. This result identified DHCR14 or a later enzyme in the pathway as targets of LK-980.

Lathosterol is formed from zymosterol in two consecutive enzymatic steps (Δ24,25 reduction and Δ7,9 isomerization), the order of which cannot be determined 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 that in nontreated 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 Δ24,25 double bond, whereas the difference between cholesterol and 7-dehydrocholesterol is in the unsaturated Δ7,8 double bond. Comparison of desmosterol in LK-980-treated and nontreated cells (approximately 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 with statins, we developed and applied a mathematical model of cholesterol synthesis. To our surprise, just by modifying concentrations of the crucial post-anostero 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 significantly in providing the final conclusion regarding the inhibitory potential of LK-980. This novel cholesterol synthesis inhibitor indeed affects several enzymes of the postlanosterol portion of the pathway. The major target of LK-980 is DHCR7, one of the last two enzymes of cholesterol synthesis. LK-980 also has three minor targets, two sterol reductases DHCR14 and DHCR24 and a sterol desaturase 5C5D. Because knowledge regarding enzymes of the late portion of cholesterol synthesis remains limited, LK-980 can be a useful tool to determine the potential structural similarities of the sterol-metabolizing enzymes that are affected.

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References

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