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SHORT COMMUNICATION

Biochemical Basis for a Cholesterol-lowering Activity of 2-[2″-(1″,3″-dioxolane)]-2-methyl-4-(2′-oxo-1′-pyrrolidinyl)-6-nitro-2*H*-1-benzopyran (SKP-450), a Novel Antihypertensive Agent

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ABSTRACT. Administration (p.o.) of SKP-450, 2-[2″-(1″,3″-dioxolane)]-2-methyl-4-(2′-oxo-1′-pyrrolidinyl)-6-nitro-2*H*-1-benzopyran, a novel antihypertensive agent, to hypercholesterolemic Syrian hamsters led to a significant reduction in plasma lipids in a dose-dependent manner, i.e., a 10.8% to 29% reduction in low-density lipoprotein cholesterol at doses of 0.3 to 10 mg/kg of SKP-450. SKP-450 was found to specifically inhibit the hepatic microsomal lanosterol 14α-methyl demethylase (14α-DM) in a competitive manner (K_i: 2.65 μM). Furthermore, a dose-dependent decrease in the 14α-DM activity by SKP-450 parallelled the cholesterol synthetic rate *in vitro* in both the rat hepatic S_{10} fractions (supernatants at 10,000 *g*; IC_{50}: 20 μM) and Chinese hamster ovary cells (IC_{50}: 23 μM). However, this phenomenon was not seen in AR45 cells, which are deficient in 14α-DM, suggesting that 14α-DM is the major target for the inhibitory action of SKP-450 in regard to cholesterol biosynthesis. BIOCHEM PHARMACOL **57**;5:579–582, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. lanosterol 14α-methyl demethylase; SKP-450; cholesterol-lowering; antihypertension; cardioprotection; AR45 cells

It has been well documented that many of the currently available antihypertensive drugs tend to cause adverse changes in the serum lipid profile, including an increase in TC§, triglyceride, and LDLc, and a decrease in HDLc [1, 2]. Therefore, combined treatment of antihypertensive drugs (e.g., captopril) plus a lipid-lowering drug (e.g., pravastatin) has emerged as a better strategy for treating complex cardiovascular disease, which could otherwise result in tremendous medical costs [3].

SKP-450 (previously known as KR-30450) has been shown to be an orally effective blood pressure-lowering agent through its altering of various cellular functions via modulation of K_{ATP} [4–7]. The major objectives of this study were twofold. First, we wished to know whether SKP-450, an antihypertensive agent (or SKP-451, a stereoisomer), possesses a cholesterol-lowering potential *in vivo*. Second, if so, we wanted to determine what the underlying mechanism by which SKP-450 lowers lipid levels (if any) might be. Here, we describe the newly established cholesterol-lowering activity of SKP-450, which is mainly attributed to its direct inhibition of 14α-DM, a major regulatory enzyme in cholesterol biosynthesis in mammals [8, 9], and implications of dual mechanisms of action by SKP-450 in the treatment of hypertensive dyslipidemia, the leading cause of cardiovascular disease.

MATERIALS AND METHODS

Sources of SKP-450, its stereoisomer SKP-451, and lemakalim were as described [4–7]. The sources of the following drugs or agents have been described previously [10]: AY-9944, cholestyramine, Lovastatin®, miconazole, NADP, NADPH, mevalonolactone, glucose-6-phosphate, glucose 6-phosphate dehydrogenase, Triton WR-1339, lanosterol, and cholesterol. The following isotopes were purchased from Amersham: [1,2-^{14}C]-acetic acid (59.0 mCi/mmol), [^{3}H]cholesterol (46 Ci/mmol), (*R*,*S*)[5-^{3}H] mevalonolactone (33 Ci/mmol), and [^{14}C]-mevalonic acid (72 mCi/mmol). Most other tissue culture media and supplements were from GIBCO. All other reagents were of the best grade available.

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§ *Abbreviations:* AY-9944, *trans*-1,4-bis(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride; CL, 5% cholestyramine plus 0.1% lovastatin; 14α-DM, lanosterol 14α-methyl-demethylase; HDL, high-density lipoprotein; lanosterol, 4,4′,14α-trimethyl-5α-cholesta-8,24-dien 3β-ol; LDL, low-density lipoprotein; SKP-450, 2-[2″-(1″,3″-dioxolane)]-2-methyl-4-(2′-oxo-1′-pyrrolidinyl)-6-nitro-2*H*-1-benzopyran; lemakalim, (−)-*trans*-3,4-dihydro-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-2*H*-1-benzopyran-6-carbonitrile; TC, total cholesterol; CHO-KI, Chinese hamster ovary cells; HDLc, high-density lipoprotein cholesterol; and LDLc, low-density lipoprotein cholesterol.

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For the *in vivo* test of SKP-450, hamsters (90–110 g body weight, 5–10 hamsters per group) that had been maintained on 0.5% cholesterol in normal chow for 14 days were administered drugs (e.g. SKP-450) orally by gavage in 0.25% methyl cellulose once daily (11 p.m. for animals) for 14 days. At the end of the treatment, hamsters were fasted for 24 hr and killed by CO_2 inhalation. Blood was drawn by heart puncture as described [10] and analyzed for serum cholesterol. The plasma serum cholesterol was determined by a Hitachi 7150 automatic analyzer. HDLc was analyzed using the isolated HDL as previously described [11]. LDLc was calculated from the method of Friedewald formulation [11]. For the preparation of enzyme sources, hamsters or rats that had been maintained on 5% cholestyramine plus 0.1% lovastatin (CL diet group; for enzyme sources) [10] for 7 days were decapitated at midpoint of the dark period, and their tissues including liver were excised and processed for microsome preparation, as previously described [10]. This CL diet has been shown to induce the most cholesterogenic enzymes in the distal pathway as well as in 3-hydroxy-3-methyglutaryl coenzyme A reductase [10, 14]. The rat or hamster lanosterol 14α-DM assay was performed using lanosterol as the substrate according to the methods previously described [12]. Protein assay was carried out using BSA as a standard according to the method of Lowry *et al.* [13].

For measurement of the cholesterol synthetic rate *in vitro*, CHO-K1 or AR45 cells were plated at 5×10^4 cells on a 60-mm plate in an RPMI-1640 medium containing 10% (v/v) fetal bovine lipoprotein-deficient serum [14, 15]. On day 3, cells were incubated with SKP-450 at the final concentrations indicated (0.01 to 1000 μM) in 95% air/5% CO_2 at 37° for 1 hr and then pulsed with 2 μCi [^{3}H]-mevalonolactone (33 Ci/mmol), and cells were further incubated at 37° for 2 hr. At the end of the incubation period, each dish was washed with cold PBS twice, and then carrier cholesterol (50 μg), lanosterol (50 μg), and [^{3}H]-cholesterol (30,000 dpm, internal control) were added; thereafter, nonsaponifiable radiolabeled sterols were extracted and subjected to TLC analysis [10]. To measure the sterol synthetic rate in rat hepatic tissues, 10 mg of proteins of S_{10} fractions (supernatants at 10,000 *g*) and [^{14}C]-mevalonic acid (72 mCi/mmol) were used and processed according to a previously described method [10]. The relative cytotoxicity of SKP-450 was determined in CHO-K1 cells on 96-well microtitration plates at the density of 2×10^3 cells per well, and the cells were incubated in the presence of each drug which had been dissolved in DMSO at the final concentration indicated in 95% air/5% CO_2 at 37° for 48 hr. At the end of incubation, viable cells were counted as described [16].

RESULTS AND DISCUSSION

Hamsters were used to determine the role of SKP-450 *in vivo* in cholesterol metabolism, because they have shown excellent responses to cholesterol diet and exhibited lipoprotein profiles very similar to those of humans [17]. In the first set of experiments, hypercholesterolemic hamsters that had been maintained on 0.5% cholesterol (w/w) for 14 days were fed 0.5% (w/w) SKP-450 or its chemical analogues (SKP-451 and lemakalim) in regular chow for another 14 days. From this experiment, it was found that the diet containing 0.5% SKP-450 in chow lowered TC by 15.7 ± 1.4% (N = 5) and LDLc by 21.7 ± 2.6% (N = 5) as compared to the untreated group, while HDLc was increased about 3.9 ± 0.4% (N = 5). However, in the hypercholesterolemic hamsters fed either 0.5% lemakalim or SKP-451, there was no significant change in any lipid levels.* Therefore, we focused on the use of SKP-450 for further studies *in vivo*. To determine whether SKP-450 can exert its cholesterol-lowering activity in a dose-dependent manner *in vivo*, hypercholesterolemic hamsters were administered (p.o.) with various doses of SKP-450. Data summarized in Fig. 1 suggest that SKP-450 does, indeed, lower plasma cholesterol *in vivo* in a dose-dependent manner. For example, at a lower dose (2.5 mg/kg body weight), reductions in TC and LDLc were 9.3% (246.4 ± 24.4 [control] vs 223.7 ± 18.9 mg/dL, N = 5) and 17.2% (116.0 ± 11.3 [control] vs 96.0 ± 18.2 mg/dL, N = 5), respectively. The maximum reduction in TC (15.0%) and LDLc (29.1%) was reached at 10 mg/kg of SKP-450, while HDLc remained relatively constant (+2.4% to −4.0%) under this condition. To elucidate the underlying mechanism whereby SKP-450 lowers plasma cholesterol level *in vivo* (Fig. 1), we examined changes in the overall rate of sterol synthesis by measuring the incorporation of [^{14}C]-mevalonolactone into cholesterol in CHO-K1 cells as well as AR45 cells, which are deficient in 14α-DM [15], in the presence of various doses of SKP-450. As shown in Fig. 2, a steady decrease in the rate of cholesterol biosynthesis along with lanosterol accumulation (inset) was observed with increasing amounts of SKP-450 in CHO-K1 cells. In contrast, there was

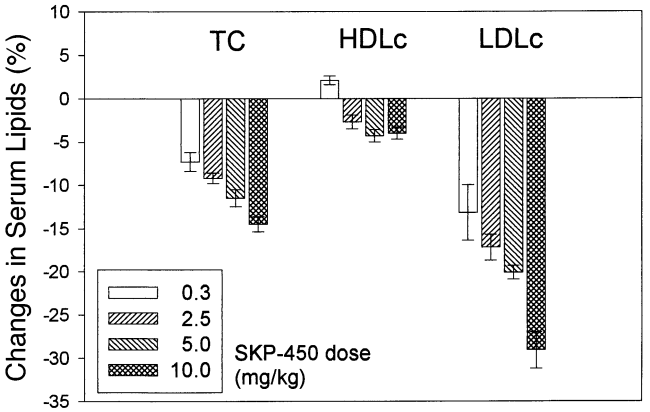

FIG. 1. Changes in serum lipid levels in hypercholesterolemic hamsters administered SKP-450. Results are shown as average reduction (%) in TC, HDLc, and LDLc of the treated groups (N = 5) obtained from one of the two separate experiments.

* Lee E-Y and Paik Y-K, unpublished data.

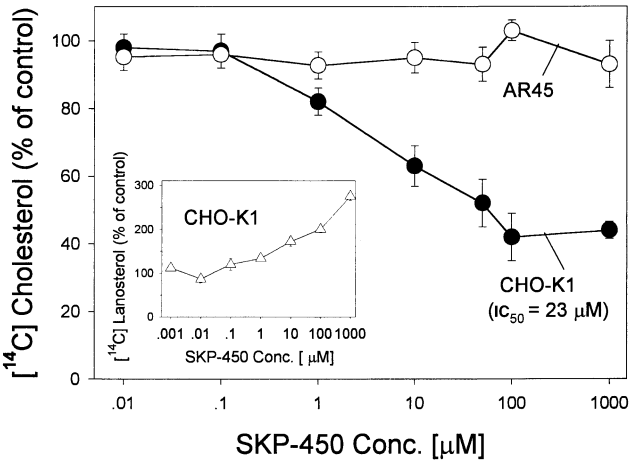

FIG. 2. Differential inhibition of the cholesterol synthetic rate by SKP-450 in CHO and AR45 (14α-DM-deficient) cells. Cells were incubated with SKP-450 at the final concentrations indicated (0.1 to 1000 μM) in 95% air/5% CO_2 at 37° for 1 hr and then pulsed with 1 μCi *(R,S)*[2-^{14}C] mevalonolactone per dish for 2 hr. After cold washing with PBS, carrier cholesterol (50 μg), lanosterol (50 μg), and [^{3}H]-cholesterol (30,000 dpm) were added, and nonsaponifiable, [^{14}C]-labeled sterols resolved on TLC plates were isolated and counted. The absolute values of control point for AR45 and CHO-K1 cells were 8420 dpm (mean) and 53,200 dpm (mean), respectively. Shown in the inset is the dose–response curve of lanosterol at different concentrations of SKP-450 in CHO-K1 cells. The absolute value for control point in the inset was 17,710 dpm (mean) for [^{14}C]-lanosterol.

virtually no change in cholesterol synthesis with respect to varying doses of SKP-450 in AR45 cells. This result clearly suggests that 14α-DM, a major rate-limiting enzyme in the conversion of lanosterol to cholesterol, is the main target for SKP-450's cholesterol-lowering action. In addition, the specificity (or the preferential inhibitory action) of SKP-450 for 14α-DM was also evidenced by the fact that SKP-450 did not show any inhibitory effect (i.e., mostly less than 5% inhibition) on other cholesterol biosynthetic enzymes *in vitro* at the dose of 200 μM: these include sterol 14-reductase, sterol 7-reductase, sterol 8-isomerase, sterol 24-reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and squalene synthase.* The IC_{50} of SKP-450 in regard to biosynthesis of [^{14}C]-cholesterol in CHO-K1 cells was estimated to be 23 μM, a concentration at which cells remained viable. Similar results were also observed in HepG2 cells (23% inhibition at 10 μM, 79% inhibition at 100 μM, data not shown). However, lemakalim and SKP-451, chemical analogues of SKP-450, again did not show any inhibitory effects on sterol synthesis at 100 μM (results not shown). No significant inhibition (i.e. <5%) on the microsomal 14α-DM was seen in the five nonhepatic tissues (heart, testis, intestine, spleen, and kidney) under these conditions, suggesting that SKP-450's inhibitory activity may have some degree of tissue specificity (results not shown).

To verify that the cells were viable while the rate of cholesterol synthesis in cells was being measured (e.g., for 3 to 4 hr) in the presence of different concentrations of SKP-450, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based cytotoxicity assay was carried out [16]. SKP-450 did not show any significant cytotoxicity when cells were exposed to 100 μM SKP-450 (viability 83.3% ± 4, N = 6) for 4 hr as compared to other cholesterol-lowering agents such as AY-9944 (viability 76 ± 7%, N = 6) and miconazole (<1.0%, N = 6). Cells remained quite viable even when they were exposed to 100 μM SKP-450 for a longer period of time (48 hr) (IC_{50} 45 μM, in CHO-K1 cells) (data not shown). This result implies that the rate of cholesterol synthesis was decreased by SKP-450's direct inhibitory effect on the target enzyme (14α-DM), and not by its cytotoxicity.

After establishing optimal assay conditions for 14α-DM in rat liver microsomes (1.0 mg of protein and 30 min of incubation for the measurement of the initial velocity), we examined whether SKP-450's inhibition of 14α-DM leads to cholesterol-lowering in the cells by measuring changes in both enzymic activity and the cholesterol synthesis rate *in vitro* in the presence of SKP-450 or its structural analogues (SKP-451 or lemakalim) [6, 7]. We used rat liver microsomes because previous results on the rate of sterol biosynthesis *in vitro* and enzyme kinetics of 14α-DM have been well documented [12, 14, 18]. As shown in Fig. 3, SKP-450 significantly inhibited not only the microsomal 14α-DM activity (IC_{50}:3.5 μM) but also the rate of cholesterol biosynthesis (IC_{50}:20 μM) in rat liver S_{10} fractions (supernatants at 10,000 *g*) in a dose-dependent manner. Thus, the decrease in 14α-DM activity by SKP-450 paralleled that in cholesterol synthesis in rat liver microsomes. The apparent K_m and V_{max} values of SKP-450 against lanosterol (for 14α-DM that had been fed the CL diet) were determined to be 151 μM and 32.69 nmol/min/mg protein, respectively.† Although the K_m value for lanosterol was similar (151 vs 165 μM for lanosterol), the V_{max} value was almost 10 times that previously reported (32.69 vs 3.39 nmol/min/mg) for rat enzyme [18]. This may be due to the influence of the CL diet-mediated enzyme induction used here. The mode of inhibition of 14α-DM by SKP-450 was competitive with the K_i value of 2.56 μM, indicating that its affinity for the microsomal 14α-DM is about 60-fold higher than that of lanosterol substrate (K'_m 165 μM) (inset, Fig. 3). There appeared to be an excellent correlation between the IC_{50} (3.5 μM) and K_i (2.56 μM) values of SKP-450 for the microsomal 14α-DM. It is not yet known whether a cholesterol-lowering effect of SKP-450 through a competitive inhibition of 14α-DM is mediated via the increased formation of oxysterols as previously suggested [9, 18, 19]. Recently, a potent inhibitor for 14α-DM termed Azalanstat and its mechanism of action have been reported [20], suggesting its potential therapeutic value as a lipid-lowering drug.

* Lee E-Y and Paik Y-K, unpublished data.

† Lee E-Y and Paik Y-K, unpublished data.

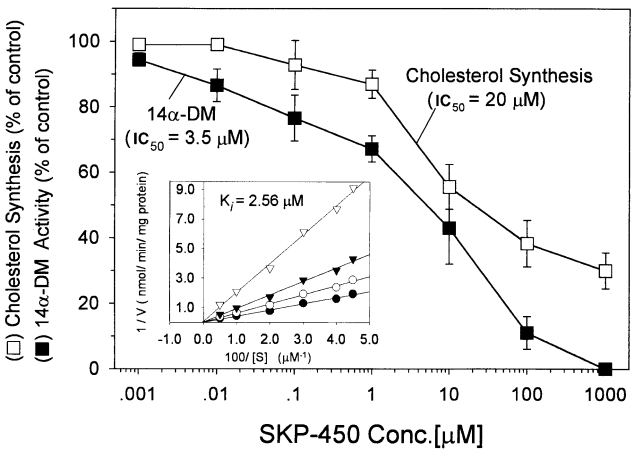

FIG. 3. Parallel reduction in both 14α-DM activity with competitive inhibition and the cholesterol synthetic rate by SKP-450 in rat liver tissues. Changes in cholesterol synthesis (in S_{10} fractions) (□) and the microsomal 14α-DM activity (■) by SKP-450. For the measurement of the cholesterol synthesis rate, the reaction mixture contained [^{14}C]-mevalonate (1.2×10^6 dpm), 10 mg of S_{10} fractions, and cofactors [10] were incubated at 37° for 2 hr. The reaction products were processed for TLC analysis in the presence of [^{3}H]-cholesterol (30,000 dpm) as an internal control. Each value represents the mean of triplicate determinations (± SD) obtained from two separate experiments. The absolute values of control points were 44,125 dpm (mean) for [^{14}C]-cholesterol, and 4.0 nmol/mg/protein for 14α-DM activity. (Inset) Mode of inhibition of 14α-DM by SKP. Lineweaver–Burk plot of the microsomal 14α-DM activity in the presence of 0 (●), 1 (○), 5 (▼), and 10 μM (▽) of SKP-450. Each value represents the average of duplicate assays of two separate experiments. The apparent K_i value was obtained from the intercept at the abscission on the plot of the slopes of lines versus inhibitor concentrations.

In conclusion, with its cholesterol-lowering activity, SKP-450 may be the first example of the cardiac ATP-sensitive K^+-channel activator [6] and nonazole therapeutic drug for inhibiting 14α-DM in mammals. Although there are few antihypertensive drugs with modest lipid-modulating effects, none of them thus far has been known to directly inhibit a major regulatory enzyme (e.g., 14α-DM) in cholesterol biosynthesis with high specificity. Therefore, SKP-450 could be a drug of choice or a better therapeutic means for treating complex hypertensive dyslipidemia, one of the most common types of cardiovascular disease. SKP-450 is currently undergoing a Phase I clinical trial in Korea.

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