Selective Inhibition of Mammalian Lanosterol 14α-Demethylase by RS-21607 in Vitro and in Vivo

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ABSTRACT: The discovery of selective lanosterol 14α-demethylase inhibitors may lead to novel hypolipidemic drugs. RS-21607, (2*S*,4*S*)-*cis*-2[(1*H*-imidazol-1-yl)methyl]-2-[2-(4-chlorophenyl)ethyl]-4-[[(4-aminophenyl)thio]methyl]-1,3-dioxolane, was characterized as a tight-binding, competitive inhibitor of lanosterol 14α-demethylase purified from rat liver. The apparent K_i was determined to be 840 pM and found to be similar in hepatic microsomes from human, rat, and hamster. RS-21607, which contains two chiral centers, was a more effective lanosterol 14α-demethylase inhibitor than its three stereoisomers. In vitro, RS-21607 had a greater affinity for lanosterol 14α-demethylase than the other cytochromes P450 evaluated: CYP7, CYP27, CYP11A1, CYP19, CYP17, CYP11B1, CYP21, CYP3A4, CYP4A, CYP2D6, CYP1A2, CYP2C9, and 27-hydroxycholesterol 7α-hydroxylase. The other stereoisomers were not as selective as RS-21607. Doses of 3–30 mg/kg RS-21607 given orally to hamsters caused a dose-dependent decrease in cholesterol biosynthesis with a corresponding accumulation of 24,25-dihydrolanosterol. RS-21607 inhibited the enzyme and cholesterol biosynthesis in hamster liver by 50% at 18 h following a 30 mg/kg oral dose. This was interpreted to indicate that RS-21607 is able to distribute to the site of action in hamsters and inhibit the target enzyme. In the same dose range, the plasma concentrations of testosterone, corticosterone, and progesterone, the endpoints for the cytochromes P450 involved in steroid biosynthesis, were relatively unaffected. These data show RS-21607 to be an effective and selective inhibitor of lanosterol 14α-demethylase, both in vivo and in vitro. RS-21607 interacted with the purified enzyme to produce a type II binding spectrum, consistent with an interaction between the imidazole moiety and the heme. The electrostatic contribution of the imidazole binding was investigated using the desimidazole analog of RS-21607. The apparent K_i for the desimidazole compound (65 μM) was similar to the apparent K_m for the substrate DHL (79 μM). Together, these data confirm that the ligand attached to the imidazole in RS-21607 is a good non-sterol substitute for DHL, i.e., binding to the enzyme with similar affinity, and that the coordination of the imidazole to the heme provides a major electrostatic contribution for the inhibition of lanosterol 14α-demethylase by RS-21607. RS-21607 was also observed to increase the accumulation of 3β-hydroxy-24,25-dihydrolanost-8-en-32-al, the second intermediate in the multistep oxidation, but not the first intermediate, 24,25-dihydrolanost-8-ene-3β,32-diol. The accumulation of this regulatory oxysterol indicates that RS-21607 inhibits both the first and third steps in the multistep reaction sequence.

Elevated serum cholesterol is implicated in many cardiovascular diseases including atherosclerosis (Anderson et al., 1987). Therapies which reduce serum cholesterol have been shown to reduce the incidence of the disease (Lipid Research Clinics Program, 1984). Blocking the synthesis of cholesterol with HMG-CoA reductase inhibitors has proven to be effective in lowering serum cholesterol (Endo, 1985; Wangworth & Bacon, 1987). There are numerous other enzymatic reactions of cholesterol biosynthesis which have the potential to be therapeutic targets. One such enzyme is lanosterol 14α-demethylase (LDM)[1] which catalyzes the first modification of the sterol nucleus in mammals and yeast. Demethylation of lanosterol by LDM occurs via three successive oxidations at the C-32 methyl group resulting in loss of formic acid and formation of 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol (Figure 1)[2] (Yoshida & Aoyama, 1984; Aoyama et al., 1984; Trzaskos et al., 1986a). Inhibition of any of these three oxidation steps should result in a block of cholesterol biosynthesis.

Many antifungal azole compounds including ketoconazole, clotrimazole, fluconazole, and miconazole have been found to interact with fungal LDM (Vanden Bossche, 1988). It has been determined that these compounds cause fungal cell death by blocking the biosynthesis of ergosterol at the lanosterol 14α-demethylation step. Upon the approval of the orally active antifungal agent ketoconazole, the interaction with lanosterol 14α-demethylase in humans could be investigated. Keto-

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[1] Abbreviations: LDM, lanosterol 14α-demethylase; DHL, 24,25-dihydrolanosterol; 8,14-diene, 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol; 32-alcohol, 24,25-dihydrolanost-8-ene-3β,32-diol; 32-aldehyde, 3β-hydroxy-24,25-dihydrolanost-8-en-32-al; RS-21607, (2*S*,4*S*)-*cis*-2-[(1*H*-imidazol-1-yl)methyl]-2-[2-(4-chlorophenyl)ethyl]-4-[[(4-aminophenyl)-thio]methyl]-1,3-dioxolane; desimidazole or *trans*-2*R*,4*S*-DI, (2*R*,4*S*)-*trans*-[2-(4-chlorophenyl)ethyl]-2-methyl-4-[[(4-aminophenyl)thio]methyl]-1,3-dioxolane; *cis*-2*S*,4*S*-DI, (2*S*,4*S*)-*cis*-[2-(4-chlorophenyl)ethyl]-2-methyl-4-[[(4-aminophenyl)thio]methyl]-1,3-dioxolane; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; BHT, butylated hydroxytoluene; ^TV = tritium isotope effect upon V_{max}; $^T(V/K)$ = tritium isotope effect upon V_{max}/K_m.

[2] Dihydrolanosterol is used instead of lanosterol as the enzyme substrate in this study because it is similar to lanosterol in enzyme activity (Trzaskos et al., 1986b) and dihydrolanosterol, not lanosterol, primarily accumulates after RS-21607 treatment to hamsters. The accumulation of DHL is thought to result from the shunting of lanosterol to the side-chain reductase pathway when oxidation by LDM is blocked.

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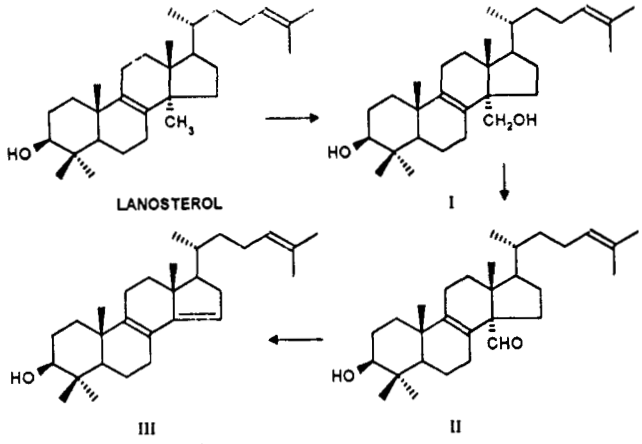

FIGURE 1: Mechanism of lanosterol demethylation.

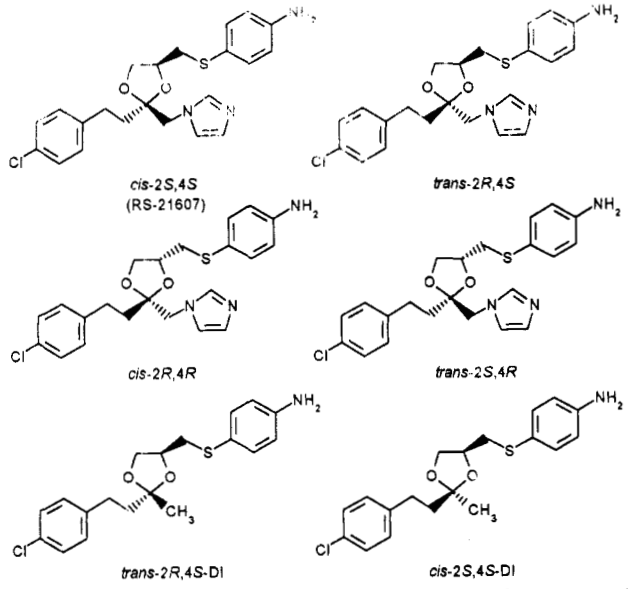

FIGURE 2: Structures of the stereoisomers of RS-21607 and desimidazoles.

conazole has been shown to reduce serum total cholesterol by approximately 30% in humans, with a corresponding increase in serum lanosterol concentrations (Kraemer & Pont, 1986; Miettinen, 1988; Gylling et al., 1991). However, ketoconazole is a relatively nonspecific cytochrome P450 inhibitor in that at therapeutic doses it interferes with the synthesis of adrenal steroid hormones and testosterone (Feldman, 1986).

We report here the details of the interactions of another azole, RS-21607, and its three stereoisomers (Figure 2) with mammalian lanosterol 14α-demethylase. The results obtained show the 2*S*,4*S*-diastereomer, RS-21607, to be a potent and selective non-steroidal inhibitor of mammalian lanosterol 14α-demethylase. This compound has also been found to effectively lower cholesterol in hamster (Walker et al., 1993) and man (Dr. K. Schwartz, personal communication).

MATERIALS AND METHODS

Materials. RS-21607 and its stereoisomers were prepared as previously described (Walker et al., 1993) as were [32-$^3H_3$]DHL, the 8,14-diene (DeKeczer et al., 1993), the 32-alcohol (Takano & Morisaki, 1991), and nafimidone (Walker et al., 1980; Rush et al., 1987). The radiochemicals with the exception of [24,25-$^3H_2$]DHL and [7β-^{3}H]-7α-hydroxycholesterol were purchased from New England Nuclear (Boston, MA). [16,22-^{3}H]-27-Hydroxycholesterol was prepared from krypotogenin by the method of Scheer et al. (1956). Steroids and sterols were purchased from Steraloids (Wilton, NH) or Research Plus (Bayonne, NJ). Debrisoquine and 4-hydroxydebrisoquine were obtained from Biomol (Plymouth. Meeting, PA), and 1-heptanesulfonic acid sodium salt, 1-hydrate was obtained from Eastman Kodak (Rochester, NY). All other chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI) and Sigma Chemical Co. (St. Louis, MO). RIA kits for progesterone and testosterone were supplied by Diagnostic Products Corporation (Los Angeles, CA), and the kits for corticosterone were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA).

Enzyme Source. Tissues were minced with a Brinkman polytron and then homogenized in 3–5 mL/g of tissue of 50 mM Tris buffer (0.25 M sucrose, 1.15% KCl, pH 7.5, at 4 °C). The homogenate was centrifuged at 1000*g* for 10 min at 5–10 °C. The pellet was discarded, and the supernatant was centrifuged at 9000*g* for 20 min at 5–10 °C. The resulting mitochondrial pellet was resuspended in at least 10 pellet volumes of homogenizing buffer and recentrifuged at 9000*g* for 10 min. The final mitochondrial pellet was suspended in one to two pellet volumes of 0.25 M sucrose and stored at −80 °C. Microsomes were prepared from the initial 9000*g* supernatant which was centrifuged at 100000*g* for 80 min. The resulting microsomal pellet was washed by suspension in 10 mM EDTA (pH 7.4, 1.15% KCl) and recentrifugation at 100000*g* for 80 min. The final microsomal pellet was suspended and stored as described for mitochondria. Samples stored at −80 °C retained catalytic activity for up to 3 years. When microsomes were prepared without concurrent mitochondria preparation, the 0.25 M sucrose was omitted from the homogenization buffer.

NADPH–cytochrome P450 reductase was purified from phenobarbital-treated rats to a specific activity of >65 000 units/mg of protein by a combination of the methods of Dignam and Strobel (1975) and Yasukochi and Masters (1976). One unit of reductase catalyzes the reduction of 1 nmol of cytochrome *c*/min at 22 °C in 0.3 M potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA and 0.1 mM NADPH. Cytochrome b_5 was purified from rat liver microsomes by the method of Spatz and Strittmatter (1971) to a specific activity of 19.8 nmol/mg of protein.

Purification of Lanosterol 14α-Demethylase. LDM was purified from the livers of cholestyramine-treated rats by a modification of the procedures of Trzaskos et al. (1986a). Male rats (strain Crl:CD-BR VAF+, Charles River Labs, Wilmington, DE, 200 g) on a reverse-light cycle were treated with 4% cholestyramine resin in the diet for 1 week and livers removed at the middle of the dark cycle. Approximately 6.5 g of microsomal protein was diluted to 4 mg/mL with PEDG buffer (100 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, 0.1 mM DTT, 20% glycerol) containing 0.6% sodium cholate (w/v) and stirred for 2 h. The supernatant isolated by centrifugation (105000*g* for 1 h) was treated with 50% polyethyleneglycol to a final percentage of 16%. The pellet was isolated (0–16% PEG) and dissolved in PEDG, pH 7.25, containing 0.6% sodium cholate. This was applied to four *n*-octylamine columns (2.6 cm × 30 cm) (Sigma Co., St. Louis). Each column was previously equilibrated with 300 mL of PEDG, pH 7.25, containing 0.6% sodium cholate and washed with 340 mL of the equilibration buffer. The protein eluted with PEDG, pH 7.25, containing 0.2% Triton N-101. The pooled *n*-octylamine fractions were concentrated against an Amicon YM-30 membrane to a final volume of 100 mL. This was dialyzed overnight against 4 L of 5 mM PEDGT,

pH 7.8, diluted to 260 mL with a solution of 0.1 mM EDTA, 0.1 mM DTT, 20% glycerol, and 0.2% Triton N-101, and applied to a DEAE Sephacel column (2.6 × 25 cm) (Pharmacia, Uppsala, Sweden) previously equilibrated with 250 mL of 5 mM PEDGT, pH 7.8. The column was washed with 100 mL of 5 mM PEDGT, pH 7.8, followed by 200 mL of 10 mM PEDGT, pH 7.8. The column was eluted with a 600-mL linear gradient of 10 mM PEDGT to 100 mM PEDGT, both pH 7.8. Fractions containing lanosterol demethylase activity were pooled and dialyzed against 10 mM PEDGT, pH 6.8, overnight. These fractions (approximately 200 mL) were applied to an S-Sepharose fast flow column (1.5 cm × 7 cm) (Pharmacia, Uppsala, Sweden) previously equilibrated with the same buffer. The column was washed with 50 mL of equilibration buffer and eluted with a 300-mL linear gradient from 0 to 0.2 M KCl in equilibration buffer. Fractions showing a single band on 7.5% SDS-PAGE gel and having lanosterol demethylase activity were pooled, concentrated with an Amicon centricon-10 concentrator, and dialyzed against 50 mM PEDG, pH 7.4. Triton N-101 was removed using a course Sephadex G-25 gel filtration column (1.5 × 10 cm) (Pharmacia, Uppsala, Sweden). The column was equilibrated with 200 mL of 50 mM PEDG, pH 7.4, and the concentrated protein was loaded on to the column and eluted with the same buffer. The fractions absorbing at 280 nm were pooled and concentrated with an Amicon centricon-10 concentrator.

Enzymatic Activities. Unless stated otherwise, inhibitors and substrates were added in methanol to a final concentration of methanol of not greater than 2%. Control incubations not containing inhibitor contained 2% methanol. Final incubation volumes were 1 mL, and all reactions were carried out at 37 °C.

Lanosterol 14α-Demethylase. Microsomal activity was determined in 30-min incubations containing potassium phosphate buffer (0.2 M, pH 7.0), dithiothreitol (0.3 mM), EDTA (0.1 mM), magnesium chloride (3 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 unit), NADPH (1 mM), microsomal protein (1 mg/mL, unless otherwise stated), and [32-$^3H_3$]DHL (40 μM, unless otherwise stated). Both tritium-labeled and unlabeled DHL were purified by HPLC prior to use. Substrate was added in tyloxapol (1:75 w/w ratio) as described by Trzaskos et al. (1986a). The incubations were terminated using 250 μL of a 40% trichloroacetic acid solution. The activity was determined from the radioactivity not retained on activated 1-mL C-18 Bond-Elut extraction columns (Varian, Harbor City, CA). The columns were activated with methanol (1 × 1 mL) followed by deionized water (2 × 1 mL). In incubations using [24,25-$^3H_2$]DHL as substrate, AY-9944 (50 μM) and potassium cyanide (5 mM) were included to inhibit the further metabolism of 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol to cholesterol. These reactions were stopped with 1 mL of 15% potassium hydroxide in 95% methanol and the mixtures heated at 60 °C for 30 min and extracted with petroleum ether. The product of the reaction, 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol, was separated from substrate with a Jones analytical 5-μm Apex ODS column (4.6 cm × 25 cm), eluted with a 45/45/10 mixture of isopropyl alcohol/acetonitrile/water isocratically at a flow rate of 1 mL/min (Trzaskos et al., 1984), and quantitated by radiochemical detection. The product eluted at 17 min and substrate at 21.5 min. The products of the studies investigating the accumulation of 32-oxylanosterols were separated isocratically at 1 mL/min with a 65/10/25 mixture of isopropyl alcohol/acetonitrile/water. The 32-alcohol eluted at 15 min, the 32-aldehyde at 17 min, the diene at 33 min, and DHL at 40 min. Experiments with reconstituted enzyme used 10 000 units/mL cytochrome P450 reductase. Additional lipid was omitted as dilauroylphosphatidylcholine did not stimulate catalytic activity (1–20 μg/mL).

CYP7—Cholesterol 7α-Hydroxylase. Incubations containing hepatic microsomes from cholestyramine-treated rats (2 mg/mL protein; 0.14 μmol of endogenous cholesterol substrate), magnesium chloride (3 mM), EDTA (0.1 mM), cysteamine hydrochloride (20 mM), and potassium phosphate buffer, pH 7.4 (0.1 M), were agitated for 10 min following addition of NADPH (1 mM). Inhibitors were added to the incubation tubes, and solvent was evaporated prior to addition of the other components. After the 10-min incubation period, the NADPH-dependent reactions were stopped by the addition of sodium cholate (5 mg) to solubilize the membranes and products were converted to their respective 4-cholesten-3-ones by the addition of cholesterol oxidase (0.23 unit dissolved in 100 μL of 10 mM potassium phosphate buffer containing 20% glycerol and 1 mM dithiothreitol) and agitation for 20 min. All reactions were terminated by addition of 1 mL of methanol followed by 5 mL of petroleum ether. The extracts were analyzed using HPLC and the products detected at 240 nm. Separation of products was achieved with two 5-μm, 25-cm silica columns (Dupont Zorbax Sil or Beckman Ultrasphere Sil) preceded by a silica-packed 3-cm guard column. The columns were eluted with hexane/isopropyl alcohol at 1 mL/min under the following conditions: 12 min isocratic at 95/5; 5 min with a linear gradient to 70/30; and 23 min isocratic at 70/30. 7α-Hydroxy-4-cholesten-3-one eluted at 26 min.

27-Hydroxycholesterol 7α-Hydroxylase. Incubations containing hepatic microsomes from male hamsters (0.1 mg/mL protein), [16,22-$^3H_2$]-27-hydroxycholesterol, NADPH (2 mM), magnesium chloride (3 mM), calcium chloride (2 mM), EDTA (0.1 mM), and potassium phosphate buffer, pH 7.4 (100 mM), were agitated for 10 min. The reactions were stopped by the addition of 7.5 mL of acetone (containing 0.02% BHT). Following separation and evaporation, the residues were dissolved in acetonitrile:isopropyl alcohol (50:50, containing 0.02% BHT, 200 μL), sonicated for 5 min, filtered through 4.5-μm filters (Gelman Acrodisc 3CR), and analyzed by HPLC (75-μL injection volume). Separation of products was achieved on a 5-μm, 25-cm C-18 column (Jones Apex or Partisil-ODS 3) preceded by a Newguard C18 guard column. The column was eluted at 1 mL/min with acetonitrile/isopropyl alcohol/water under the following conditions: 2 min isocratic at 32.5/24/43.5; 10 min with a linear gradient to 48/32/20; 15 min isocratic at 48/32/20; 5 min with a linear gradient to 9/90/1; and 15 min isocratic at 9/90/1. 7α,27-Dihydroxycholesterol eluted at 16 min and 27-hydroxycholesterol at 25 min. The kinetic constants associated with product formation were V_{max}, 260 pmol/min/mg, and K_m, 8.9 μM.

CYP27—Cholesterol 27-Hydroxylase. Incubations contained hepatic mitochondria from hamster (2 mg/mL protein), [^{3}H]cholesterol in tyloxapol (1:75 w/w ratio) (diluted by endogenous cholesterol), NADPH (2 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 unit/mL), magnesium chloride (3 mM), calcium chloride (2 mM), EDTA (1 mM), nafimidone (25 μM), and potassium phosphate buffer, pH 7.4 (100 mM). Following a 1-h incubation, the reaction products were processed and analyzed as described for 27-hydroxycholesterol 7α-hydroxylase.

CYP11A1—Cholesterol Side-Chain Cleavage. [26-^{3}H]-Cholesterol in tyloxapol (1:75 w/w ratio) was incubated with bovine adrenal cortex mitochondria (1 mg/mL), bovine adrenal cytosol (1 mg/mL), bovine serum albumen (0.25 mg/mL), potassium phosphate, pH 7.4 (20 mM), calcium chloride (0.5 mM), magnesium chloride (4 mM), EDTA (0.5 mM), glucose-6-phosphate dehydrogenase (2 units/mL), glucose-6-phosphate (5 mL), and NADPH (1.0 mM) for 1 h. The reactions were quenched with 2 mL of methanol and the products applied to 3-mL C-18 Bond Elut extraction columns previously activated with methanol (3 mL) and water (2 × 3 mL). The amount of [^{14}C]isocaproic acid formed was determined from the radioactivity in the aqueous eluent.

CYP19—Aromatase. The microsomal incubations contained potassium phosphate buffer (pH 7.4, 5 mM), dithiothreitol (0.3 mM), microsomal protein from human placenta (0.1 mg), [1β,2β-^{3}H$_2$]androstenedione, and NADPH (0.01 M). Following incubation for 10 min, the reactions were terminated with 25 μL of 40% trichloroacetic acid. The activity was determined from the radioactivity not retained on activated 1-mL C-18 Bond-Elut extraction columns as described for the DHL assay. The apparent K_m for product formation was 9 nM, and the V_{max} was approximately 200 pmol/min/mg of protein.

CYP17—Progesterone 17α/20-Lyase. Reaction mixtures with microsomes from the testes of neonatal pigs contained protein (0.025 mg/mL), NADPH (1 mM), magnesium chloride (3 mM), potassium phosphate buffer, pH 7.25 (100 mM), and 17α-hydroxyprogesterone. All reactions were terminated after 10 min by addition of 6 mL of methylene chloride followed immediately by 1 nmol of internal standard (11β-hydroxytestosterone in 50 μL of methanol). The residues from the organic phase were dissolved in 200 μL of methanol, sonicated, and analyzed by HPLC. Separation of substrate (17α-hydroxyprogesterone), product (androstenedione), and internal standard was achieved with a Jones chromatography 5-μm, 25-cm ODS column. The column was eluted isocratically with a 42/20/38 ratio of methanol/acetonitrile/water. The internal standard, detected at 254 nm, eluted with a retention time of 6.6 min, androstenedione at 10.7 min, and 17α-hydroxyprogesterone at 12.9 min. The apparent K_m was observed to be 4 μM and V_{max} 2.1 nmol/min/mg.

Progesterone Hydroxylase—CYP21 and CYP3A4. The progesterone assay was conducted as previously reported (Swinney, 1990). Briefly, mixtures containing either bovine adrenal microsomes (0.1 mg/mL) for determining activity associated with CYP21 or microsomes from human liver (1 mg/mL) for determining activity associated with CYP3A4, NADPH (1 mM), magnesium chloride (3 mM), potassium phosphate buffer, pH 7.4 for human liver and pH 7.25 for bovine adrenal (50 mM), and progesterone were incubated for 10 min. All reactions were terminated by addition of 6 mL of methylene chloride followed immediately by 1 nmol of internal standard (11β-hydroxytestosterone in 50 μL of methanol). The residues from the organic phase were dissolved in 200 μL of methanol, sonicated, and analyzed by HPLC. Separation was achieved with a Jones chromatography 5-μm, 25-cm ODS column at a flow rate of 2 mL/min. The column was eluted with tetrahydrofuran/methanol/acetonitrile/water under the following conditions: 3 min isocratic at 4/5/6/85; 25 min with a 0.5 convex gradient to 8/13/11/68; 10 min with linear gradient to 11/17/14.5/57.5; and a 5-min ramp to 4/3/3/90. Products detected at 254 nm had the following retention times: internal standard, 18 min; 16α-hydroxyprogesterone, 21 min; 6β-hydroxyprogesterone, 31 min; 21-hydroxyprogesterone, 35 min; and progesterone, 45 min. The kinetic constants associated with human hepatic 6β-hydroxyprogesterone formation were V_{max}, 714 pmol/min/mg, and K_m, 171 μM, and those with human hepatic 16α-hydroxyprogesterone formation were V_{max}, 125 pmol/min/mg, and K_m, 132 μM.

CYP11B1—11β-Hydroxylase. Incubations with bovine adrenal mitochondria containing protein (0.05 mg/mL), NADPH (1 mM), magnesium chloride (3 mM), potassium phosphate buffer, pH 7.4 (100 mM), and deoxycorticosterone were agitated for 10 min. Mitochondria were sonicated 5 min on ice before addition to the incubation mixture. The workup and analysis of the reaction mixture were the same as those for progesterone hydroxylations. The retention time for the product, corticosterone, was 22 min, and the kinetic constants associated with its formation were V_{max}, 2 nmol/min/mg, and K_m, 0.4 μM.

CYP1A2. The 3-demethylation of caffeine associated with CYP1A2 was determined in reaction mixtures (200-μL final volume) containing hepatic microsomes from human (0.4 mg), potassium phosphate buffer (50 mM, pH 8.0), magnesium chloride (2 mM), potassium chloride (0.23%), glucose-6-phosphate (5 mM), NADPH (10 mM), glucose-6-phosphate dehydrogenase (2 units/mL), and [^{14}C]caffeine, and they were incubated for 120 min at 37 °C. The reactions were stopped by the addition of 200 μL of pH 3.0 buffer containing 80 mM phosphoric acid and 5 mM heptanesulfonic acid. Following centrifugation, the supernatants were injected directly into the HPLC system and eluted isocratically with 80 mM phosphoric acid, 5 mM heptane sulfonic acid, pH 3.0, and 1% tetrahydrofuran at a flow rate of 1.5 mL/min. Radiolabeled product, paraxanthine, eluted at 9.2 min and caffeine at 18.5 min. The apparent K_m associated with the 3-demethylation was 184 μM and the V_{max} 4.6 pmol/min/mg of protein.

CYP2D6. The 4-hydroxylation of debrisoquine associated with CYP2D6 was determined in reaction mixtures (200-μL final volume) containing hepatic microsomes from human (0.5 mg), potassium phosphate buffer (50 mM, pH 8.0), magnesium chloride (3 mM), EDTA (0.5 mM), dithiothreitol (0.5 mM), debrisoquine, and NADPH (10 mM). The samples were incubated for 30 min and the reactions stopped by the addition of methanol (500 μL). The eluates from activated C-18 Bond Elut were evaporated, reconstituted in HPLC buffer, injected on to the HPLC system, and eluted isocratically with 13% acetonitrile in 80 mM phosphoric acid and 5 mM heptanesulfonic acid, pH 3.0, at a flow rate of 2 mL/min. 4-Hydroxydebrisoquine (retention time 5.5 min) was detected at 210 nm and quantitated with external standards. The apparent K_m was observed to be 82 μM and the V_{max} 57 pmol/min/mg of protein.

CYP4A1. Lauric acid 12-hydroxylation was measured as previously reported (Swinney et al., 1991). Briefly, reactions contained microsomal protein (0.1 mg) from clofibrate-treated rats (400 mg/kg in corn oil, ip, once daily for 3 days), potassium phosphate buffer (50 mM, pH 7.4), magnesium chloride (3 mM), EDTA (0.5 mM), NADPH (1 mM), and [^{14}C]lauric acid. The 10-min incubations were terminated and samples prepared as for the progesterone assay (without internal standard). Radiochemical detection following HPLC separation on a 5-μm, 25-cm C-18 column eluted at 1 mL/min with 1% acetic acid and acetonitrile (38% acetonitrile for 14 min, increased to 90% over the next 8 min and then held constant) showed 12-hydroxylauric acid eluting at 15 min and lauric acid at 31 min. The apparent K_m for the reaction was 4.2 μM and the V_{max} 7.6 nmol/min/mg of protein.

CYP2C9. The incubations contained potassium phosphate buffer (100 mM, pH 7.4), magnesium chloride (6 mM), EDTA (0.05 mM), glucose-6-phosphate (10 mM), NADPH (1 mM), and 0.4 unit of glucose-6-phosphate dehydrogenase, tolbutamide, and hepatic microsomes from human (1 mg/mL) in a final volume of 200 μL. After 120 min, the reactions were stopped by the addition of an equal volume of 80 mM phosphoric acid and 5 mM heptanesulfonic acid, pH 3.0, and 1 nmol of the internal standard, chlorpropamide, was added. The samples were injected directly on to the HPLC system following the separation of protein by centrifugation and eluted isocratically with 20% acetonitrile in 80 mM phosphoric acid and 5 mM heptanesulfonic acid, pH 3.0. The flow rate was maintained at 1 mL/min and monitored at a wavelength of 230 nm. Retention times for 4-hydroxybutamide, chlorpropamide, and tolbutamide were 7.0, 23.0, and 36.3 min, respectively. The apparent K_m was observed to be 246 μM, and the V_{max} was 126 pmol/min/mg of protein.

General Methods. Protein concentration was determined by the method of Lowery et al. (1951). Cytochrome P450 determinations were by the method of Omura and Sato (1964) in potassium phosphate (10 mM), DTT (0.1 mM), EDTA (0.1 mM), glycerol (20%), emulgen 911 (0.2%), and sodium cholate (0.5%).

Determination of Kinetic Constants. Tight-binding inhibition kinetics were evaluated with the linear equations described by Henderson (1972). The apparent K_i values associated with inhibition of the various cytochromes P450 were determined by Dixon analysis and/or Lineweaver–Burk analysis or from an extrapolation of IC_{50} data by Dixon analysis (cholesterol 7α-hydroxylase, CYP7). The need for the extrapolation was due to the fact that substrate concentrations (cholesterol) could not be adequately varied as a result of the high endogenous concentrations. For a Dixon plot, $1/v$ versus [I] gives an x-intercept of $-K_i([S]/K_m + 1)$. Via a substrate concentration determined from total microsomal cholesterol concentration and a K_m value determined from partially purified cholesterol 7α-hydroxylase (36 μM), the apparent K_i values were determined. Analysis of data only in the log linear portion of the IC_{50} determinations was used to calculate apparent K_i values by the method of Dixon (10–90% control activity). All K_i values associated with LDM activity were determined from a plot of K/V (determined from a $1/v$ versus $1/[S]$ plot) versus $[I](V_o/K_o)$. The r^2 values of the line (four to seven points) were always greater than 0.96. Standard deviations were calculated from multiple ($n \geq 3$) determinations.

In Vivo Assays. Hepatic dihydrolanosterol accumulation and cholesterol depletion were determined in male Syrian hamsters (LAK:LVG(SYR)VAF+, 90–110 g, Charles River Labs, MA). Groups of five to seven animals were administered the dihydrochloride salt of RS-21607 dissolved in water or water (vehicle) orally by gavage. At 90 min prior to sampling, the hamsters received an ip dose of approximately 10 μCi of [^{14}C]mevalonate in 0.5 mL of isotonic saline. At sampling time, livers were removed, rinsed in saline, blotted, weighed, minced, and immersed in 40 mL of acetone containing 0.5 μCi of [32-^{3}H$_3$]dihydrolanosterol and 0.5 μCi of [^{3}H]-cholesterol. The samples were homogenized with a Brinkman polytron and frozen overnight. The precipitated protein was then removed by filtration and the acetone evaporated under a stream of nitrogen. The residue was reconstituted in a 50/50 mixture of isopropyl alcohol/acetonitrile containing 0.02% BHT, and the sterols analyzed were separated and quantitated by HPLC. Dihydrolanosterol was separated from lanosterol and cholesterol with the HPLC analysis used for determining lanosterol demethylase activity. Cholesterol was separated from the methyl sterols on a Beckman 5-μm Ultrasphere silica column (4.6 cm × 25 cm) with a 97/3 ratio of hexane to isopropyl alcohol isocratically at 1 mL/min. For both analyses, the flow rate of the scintillation cocktail through the radioflow detector was 3 mL/min. The values are presented as dpms of [^{14}C]dihydrolanosterol or -cholesterol formed from [^{14}C]-mevalonic acid administered 90 min prior to sampling, normalized to the dpm's of the internal standards, [^{3}H]-dihydrolanosterol or -cholesterol, [^{14}C]mevalonic acid added, and liver weight.

The effect of RS-21607 upon plasma steroid concentrations was determined in male Sprague–Dawley rats (Crl:CD-BR-VAF+, 200 g, Charles River Labs, MA). Groups of seven animals were administered the dihydrocholoride salt of RS-21607 dissolved in water or water (vehicle) orally by gavage. At 60 min prior to sampling, the hamsters received an im dose of 0.5 μg of LHRH and 25 μg of ACTH in isotonic saline. To avoid stress, the animals were also sedated with an ip dose of nembutal (33 mg/kg). At the appropriate sampling time, the animals were anesthetized with halothane and blood was collected by cardiac puncture into heparinized tubes. Plasma testosterone, progesterone, and corticosterone concentrations were determined using radioimmunoassay.

Chemistry. [24,25-$^3H_2$]Dihydrolanosterol. [24,25-^{3}H$_2$]-DHL was synthesized as described previously with minor modifications (Nicolas et al., 1978). A 10-mL side-arm septum flask containing a stirring magnet and 5% Pt/carbon (5 mg) was connected to a high-vacuum line and evacuated. A solution of lanosterol (4.25 mg, 0.01 mmol) in ethyl acetate was injected into the flask. The mixture was degassed and then frozen in liquid nitrogen. Tritium gas (10 Ci, 58 Ci/mmol carrier-free) was transferred into the reaction flask via a Toepler pump, and the reaction mixture was allowed to stir overnight at ambient temperature. Unused tritium gas and most of the solvent were transferred into a liquid-nitrogen-cooled waste bulb connected to the vacuum line. After removal of the flask from the line, the residue was dissolved in ethyl acetate (5 mL) and filtered through a Gelman 0.45-μm nylon filter. To remove any labile radioactivity, the filtrate was concentrated three times from ethanol/ethyl acetate and then dissolved in ethyl acetate (10 mL) to give 350 μCi at a purity of 60%. Purification by flash chromatography (10- × 120-mm column; 90:10 hexanes:ethyl acetate) gave 201.4 mCi at >99% radiochemical purity.

(2R,4S)-trans-2-[2-(4-Chlorophenyl)ethyl]-2-methyl-4-[[(4-aminophenyl)thio]methyl]-1,3-dioxolane, Hydrogen Oxalate Salt (trans-2R,4S-DI). A solution of *p*-toluenesulfonic acid monohydrate (570 mg, 3.0 mmol) in toluene (10 mL) was dried by refluxing through a bed of 4-Å sieves in a Dean-Stark side-arm apparatus for 1.5 h. The solution was cooled, and a separate solution of 4-(4-chlorophenyl)-2-butanone (274 mg, 1.5 mmol), *S*-solketal tosylate (640 mg, 2.25 mmol), and *n*-butanol (0.275 mL, 3 mmol) in toluene (3 mL) was added. The mixture was allowed to reflux through a bed of 4-Å molecular sieves for 5 h and then cooled and poured into aqueous sodium bicarbonate. Extraction of the aqueous mixture with ethyl acetate followed by removal of the solvent by evaporation under reduced pressure gave the crude product as a yellow oil (1.25 g). Separation of the mixture by flash chromatography (45- × 240-mm silica gel, 75:25 hexanes/ethyl acetate) afforded pure less polar isomer as an oil (154 mg, 25.0% yield) and impure more polar isomer. Rechromatography of the more polar isomer (32- × 240-mm silica

gel, 65:35 hexanes/diethyl ether) gave pure material as an oil (164 mg, 29.9% yield). Nuclear Overhauser effect (NOE) difference experiments with ^{1}H NMR spectra of the isomers demonstrated that the less polar isomer had 2*R*,4*S*-*trans*-stereochemistry (wherein the 2-methyl group and the 4-tosylate side chain are *cis*), while the more polar isomer possessed 2*S*,4*S*-*cis*-stereochemistry. Thus, irradiation of the 2-methyl protons of the more polar *cis*-isomer resulted in enhancement of the resonance of the 4-proton, whereas no similar effect was noted with the less polar *trans*-isomer.

Less polar isomer (2*R*,4*S*)-*trans*-2-[2-(4-chlorophenyl)-ethyl]-2-methyl-4-[[(*p*-toluenesulfonyl)oxy]methyl]-1,3-dioxolane was an oil: $[\alpha]^{20}_D = -3.2°$ (c = 0.4, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (s, 3H, 2-CH_3), 1.86 (m, 2H, CH_2), 2.46 (s, 3H, $ArCH_3$), 2.62 (m, 2H, $ArCH_2$), 3.79 (dd, 1H, J = 6.1, 8.7 Hz) and 4.07 (dd, 1H, J = 6.3, 8.7 Hz) (CH_2O), 4.02 (m, 2H, CH_2OS), 4.27 (m, 1H, CHO), 7.09 (d, 2H, J = 8.4 Hz, H-2, H-6 of C_6H_4Cl), 7.23 (d, 2H, J = 8.4 Hz, H-3, H-5 of C_6H_4Cl), 7.36 (d, 2H, J = 8.0 Hz, H-3, H-5 of C_6H_4S), 7.81 (d, 2H, J = 8.4 Hz, H-2, H-6 of C_6H_4S); MS m/e 410 (M^+).

More polar isomer (2*S*,4*S*)-*cis*-2-[2-(4-chlorophenyl)ethyl]-2-methyl-4-[[(*p*-toluenesulfonyl)oxy]methyl]-1,3-dioxolane was also an oil: $[\alpha]^{20}_D = -1.8°$ (c = 0.4, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.31 (s, 3H, 2-CH_3), 1.88 (m, 2H, CH_2), 2.40 (s, 3H, $ArCH_3$), 2.58 (m, 2H, $ArCH_2$), 3.56 (dd, 1H, J = 5.6, 8.6 Hz) and 4.08 (dd, 1H, J = 6.7, 8.7 Hz) (CH_2O), 4.04 (m, 2H, CH_2OS), 4.35 (m, 1H, CHO), 7.06 (d, 2H, J = 8.5 Hz, H-2, H-6 of C_6H_4Cl), 7.22 (d, 2H, J = 8.4 Hz, H-3, H-5 of C_6H_4Cl), 7.31 (d, 2H, J = 8.0 Hz, H-3, H-5 of C_6H_4S), 7.78 (d, 2H, J = 8.3 Hz, H-2, H-6 of C_6H_4S); MS m/e 410 (M^+).

To a solution of the (2*R*,4*S*)-*cis*-tosylate (95 mg, 0.23 mmol) prepared as above and 4-aminobenzenethiol (95 mg, 0.76 mmol) in acetone (5 mL) was added solid potassium carbonate (125 mg, 0.9 mmol), and the mixture was heated under reflux for 4 h. The mixture was cooled, and an additional quantity of thiol (80 mg, 0.64 mmol) was added and the mixture refluxed another 4 h. The mixture was cooled, poured into water, and extracted into ethyl acetate. The extracts were washed with H_2O, dried (Na_2SO_4), and evaporated under reduced pressure to give a residue which was purified by flash chromatography (22- × 215-mm silica gel, 75:25 hexanes/ethyl acetate), affording the pure title compound as an oil (70 mg, 83.3% yield). The hydrogen oxalate salt was formed by treating an ethereal solution of the compound with ethereal anhydrous oxalic acid. Recrystallization of the salt from ethyl acetate–hexane afforded an off-white solid: mp 130.5–131.3 °C; $[\alpha]^{20}_D = -2.0°$ (c = 0.3, $CHCl_3$); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.31 (s, 3H, 2-CH_3), 1.79 (m, 2H, CH_2), 2.57 (m, 2H, $ArCH_2$), 2.81 (dd, 1H, J = 7.5, 13.3 Hz) and 2.96 (dd, 1H, J = 5.2, 13.4 Hz) (CH_2S), 3.58 (m, 1H) and 4.00 (m, 1H) (CH_2O), 4.07 (m, 1H, CHO), 6.55 (d, 2H, J = 8.5 Hz, H-3, H-5 of C_6H_4N), 7.14 (d, 2H, J = 8.5 Hz, H-2, H-6 of C_6H_4N), 7.20 (d, 2H, J = 8.5 Hz, H-2, H-6 of C_6H_5Cl), 7.28 (d, 2H, J = 8.4 Hz, H-3, H-5 of C_6H_5Cl); MS m/e 363 (M^+). Anal. Calcd for $C_{21}H_{24}ClNO_6S$: C, 55.56; H, 5.33; N, 3.09. Found: C, 55.70; H, 5.16; N, 3.18.

(2S,4S)-cis-2-[2-(4-Chlorophenyl)ethyl]-2-methyl-4-[[(4-aminophenyl)thio]methyl]-1,3-dioxolane, Hydrogen Oxalate Salt (cis-2S,4S-DI). This compound was prepared from the (2*S*,4*S*)-*cis*-tosylate as described for the *trans*-isomer. Recrystallization from ethyl acetate–hexane gave a pale yellow solid: mp 129.2–131.8 °C; $[\alpha]^{20}_D = -3.4°$ (c = 0.3, $CHCl_3$); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.25 (s, 3H, 2-CH_3), 1.85 (m, 2H, CH_2), 2.61 (m, 2H, $ArCH_2$), 2.82 (dd, 1H, J = 7.4, 13.3 Hz) and 2.95 (dd, 1H, J = 5.2, 13.3 Hz) (CH_2S), 3.56 (dd, 1H, J = 6.5, 8.0 Hz) and 4.03 (dd, 1H, J = 6.2, 8.2 Hz) (CH_2O), 4.14 (m, 1H, CHO), 6.55 (d, 2H, J = 8.5 Hz, H-3, H-5 of C_6H_4N), 7.14 (d, 2H, J = 8.5 Hz, H-2, H-6 of C_6H_4N), 7.21 (d, 2H, J = 8.5 Hz, H-2, H-6 of C_6H_5Cl), 7.30 (d, 2H, J = 8.5 Hz, H-3, H-5 of C_6H_5Cl); MS m/e 363 (M^+). Anal. Calcd for $C_{21}H_{24}ClNO_6S$: C, 55.56; H, 5.33; N, 3.09. Found: C, 55.85; H, 5.41; N, 3.10.

3β-Hydroxy-24,25-dihydrolanost-8-en-32-al. Prepared from the previously reported 3β-hydroxy-32-(benzyloxy)-24,25-dihydrolanost-8-ene (Takano et al., 1991) by modification of the procedures reported by the same authors. Thus, to an ice-cooled solution of the 32-benzyl ether (125 mg, 0.23 mmol) and collidine (0.062 mL, 0.47 mmol) in methylene chloride (6 mL) was added *tert*-butyltrimethylsilyl (TBDMS) triflate (0.065 mL, 0.27 mmol). The ice bath was removed, and the mixture was stirred for 2 h, after which it was poured into a 1 *N* aqueous solution of $NaHSO_4$. Extraction with a 2:1 hexane/methylene chloride mixture followed by evaporation of solvents under reduced pressure gave the crude 3β-TBDMS ether. To this material in absolute ethanol (10 mL) was added 10% palladium on carbon catalyst (25 mg), and the mixture was stirred under a hydrogen atmosphere for 16 h. After filtration through a pad of Celite, evaporation of solvent under reduced pressure gave a residue which was then recrystallized from methanol–methylene chloride affording pure 3β-[(*tert*-butyltrimethylsilyl)oxy]-32-hydroxy-24,25-dihydrolanost-8-ene, mp 157–160 °C (103 mg, 78.8% yield for two steps).

To an ice-cooled solution of the above 32-alcohol (84 mg, 0.15 mmol) in acetone (10 mL) was added Jones reagent (0.1 mL, 0.21 mmol of chromic acid in acetone), and the mixture was stirred for 1 h. The mixture was then poured into water and extracted with ethyl acetate, after which concentration of the extracts under reduced pressure gave a residue consisting of crude 3β-TBDMS ether 32-aldehyde. This material was dissolved in THF (10 mL), and 2 *N* aqueous H_2SO_4 (8 mL) was then added, after which the mixture was heated at 40 °C for 28 h. The mixture was poured into water and extracted into ethyl acetate, the extracts then being concentrated under reduced pressure. The residue was further purified by flash chromatography (175- × 16-mm silica gel, 88:12 hexanes/acetone), which afforded the title compound (40 mg, 60.1% yield). Recrystallization from acetone–hexane provided an analytical sample, mp 178–181 °C [lit. mp 177–179 °C, Takano et al. (1991); 177–179 °C, Trzaskos et al., (1987)]. All other physical data agreed with that reported by Trzaskos et al. (1987).

[7β-^{3}H]-7α-Hydroxycholesterol. A freshly prepared solution of [^{3}H]sodium borohydride (100 mCi, 58.1 Ci/mmol, 0.0017 mmol) in aqueous 0.01 N sodium hydroxide (0.1 mL) was added to 7-ketocholesterol (3β-hydroxycholest-5-en-7-one) (3.1 mg, 0.0077 mmol) in a small screw-cap vial. Ethanol (0.3 mL) was added, and the reaction mixture was allowed to stir at room temperature. After 2 days, acetone (0.3 mL) was added to quench the reaction followed by 10% aqueous hydrogen chloride (0.002 mL), and the solvent was removed by evaporation under a nitrogen stream. The residue was dissolved in ethyl acetate (10 mL) to determine the amount of activity (104 mCi). Radio-TLC (silica gel, 50:50 hexane/isopropyl acetate, three passes) showed a 1:7 mixture of [7β-^{3}H]-7α-hydroxycholesterol and [7α-^{3}H]-7β-hydroxycholesterol. The solution was concentrated, taken up in toluene (1.0 mL), and purified three times by flash chromatography (10- × 120-mm column; 80:20 hexanes/ethyl acetate) to give

Table 1: Kinetic Constants Associated with Microsomal Metabolism of Dihydrolanosterol[a]

species	substrate	$K_{m,app}$ (μM)	V_{max} (pmol/min/mg)
human ♂	[32-$^3H_3$]DHL	10.3 ± 4.7	21.5 ± 10.2
human ♀	[32-$^3H_3$]DHL	10.3	46.5
hamster ♂	[32-$^3H_3$]DHL	34.2 ± 5.1	233 ± 28
rat ♂	[24,25-$^3H_2$]DHL	181 ± 123	1553 ± 925
rat ♂	[32-$^3H_3$]DHL	79.0 ± 25.2	766 ± 311
purified rat	[32-$^3H_3$]DHL	36.7 ± 8.4	3.41 ± 1.05 min^{-1}

[a] Rate constants were determined from Lineweaver–Burk analysis of product formation at substrate concentrations ranging from 5 to 50 μM (higher substrate concentrations had very limited solubility). When [32-$^3H_3$]dihydrolanosterol was used as substrate, the rate of product formation was determined from the amount of tritium soluble in water. The amount of 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol formed from [24,25-$^3H_2$]dihydrolanosterol in the presence of AY-9944 was determined by HPLC.

2.78 mCi of [7β-^{3}H]-7α-hydroxycholesterol at >99% radiochemical purity.

RESULTS

Lanosterol Demethylase Activity. Lanosterol 14α-demethylase activity was determined utilizing either [24,25-$^3H_2$]-DHL or [32-$^3H_3$]DHL as substrate. The rates of reaction following incubation with [32-$^3H_3$]DHL were associated with the rate of tritium release into the aqueous media. The rates following incubation with [24,25-$^3H_2$]DHL were determined using HPLC with a radioflow detector as described by Trzaskos and co-workers (1984). Further metabolism of the demethylation product, 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (8,14-diene), was blocked by the addition of AY-9944, a Δ^{14}-reductase inhibitor. The formation of the products by both methods was comparable and linear with time and protein.

The kinetic constants associated with microsomal lanosterol demethylation as monitored by the tritium release assay are shown in Table 1. The apparent K_m associated with the human was lower than those of rat and hamster (10.3 ± 4.7, 79.0 ± 25.2, and 34.2 ± 5.1 μM, respectively). The rat enzyme was purified to apparent homogenity as determined by SDS–polyacrylamide gel electrophoresis. The specific activities of two separate preparations were 2.35 and 5.00 nmol of P450/mg of protein. The reason for the low specific activity is unknown. The activity in a system reconstituted with cytochrome P450 reductase was unaffected by cytochrome b_5 (0.5–1.0 nmol) or dilauroylphosphatidylcholine (1–20 μg/mL). The apparent K_m for the partially pure enzyme was 36.7 ± 8.4 μM, and the V_{max} was 3.41 ± 1.05 min^{-1} (Table 1); accordingly, the second-order rate constant for the reaction, V/K, is approximately 1550 M^{-1} s^{-1}.

Inhibition of Lanosterol Demethylase. The inhibition of LDM purified from rat by RS-21607 showed that under the conditions employed (5 and 10 nM LDM), RS-21607 acted kinetically as tight-binding inhibitor with an apparent K_i of 840 pM (Figure 3). The increase in slope with an increase in substrate concentration in the $I_t/1 - (v_i/v_o)$ versus v_o/v_i plot (Figure 3A) is consistent with a competitive inhibition mechanism (Henderson, 1972).

RS-21607 and its three stereoisomers were also observed to be competitive inhibitors of microsomal LDM. The apparent K_i values for the four stereoisomers of RS-21607 associated with the inhibition of LDM in microsomes from cholestyramine-treated rats are shown in Table 2. The *cis*-2*S*,4*S*-compound (RS-21607, apparent K_i = 2.5 ± 1.5 nM) was approximately 50-fold more effective than its *trans*-2*R*,4*S*-diastereomer. Both the 2*S*-enantiomers were over an order of magnitude more effective than the corresponding 2*R*-enantiomers, and, consistent with related antifungal agents, the *cis*-diastereomers were more effective than the *trans*-diastereomers. Ketoconazole, a *cis*-dioxolane racemic mixture,

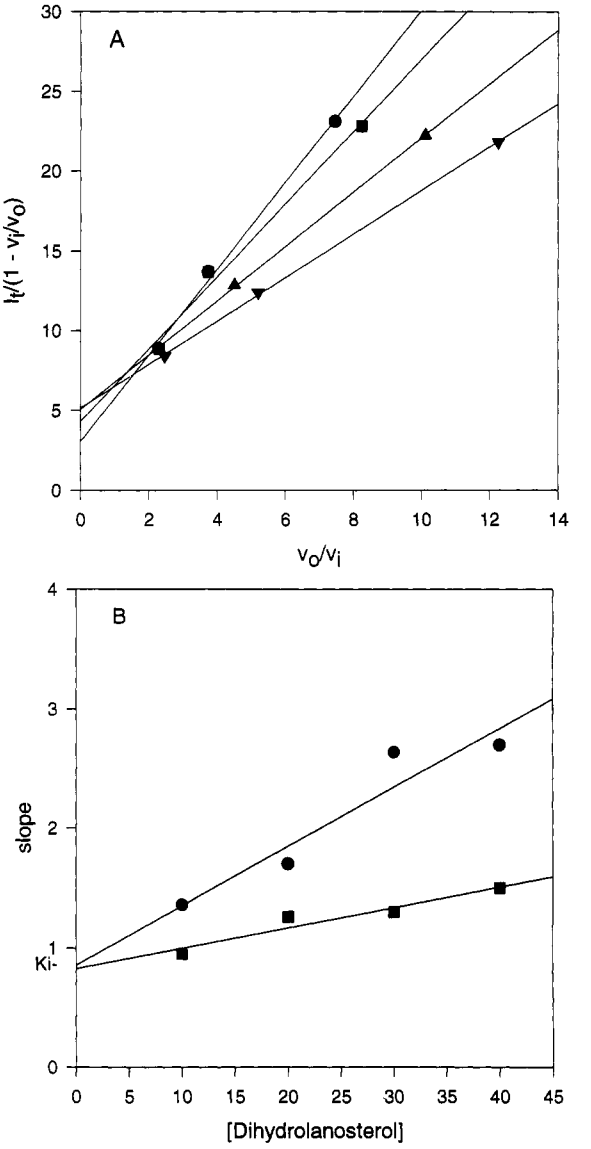

FIGURE 3: Tight-binding inhibition of purified rat lanosterol demethylase by RS-21607. (A) Relationship of dose–response curves to changes in inhibitor concentration with 10 nM LDM analyzed by the method of Henderson (1971): (●) 40 μM DHL, (■) 30 μM DHL, (▲) 20 μM DHL, and (▼) 10 μM DHL. (B) Replot of slopes of (A): (●) 10 nM LDM and (■) 5 nM LDM. The data from the dose–response curves with 5 nM LDM were similar to those shown with 10 nM LDM and also intercepted the y-axis at approximately 5. I_t is total concentration of inhibitor; v_i is velocity in the presence of inhibitor; and v_o is velocity without inhibitor.

Table 2: Inhibition of Rat Microsomal Lanosterol Demethylase

compound	K_i (nM)
cis-2*S*,4*S* (RS-21607)	2.5 ± 1.5
cis-2*R*,4*R*	37 ± 11
trans-2*S*,4*R*	11.0
trans-2*R*,4*S*	117
cis-2*S*,4*S* + 2*R*,4*R*	11.5 ± 8.5
ketoconazole	64, 67
trans-2*R*,4*S*-DI[a]	64800
cis-2*S*,4*S*-DI[a]	10400

[a] DI is the abbreviation for desimidazole.

Table 3: Inhibition of Lanosterol Demethylase in Hepatic Microsomes from Human, Rat, and Hamster[a]

	human K_i (nM)		rat K_i	hamster K_i
	male	female	(nM)	(nM)
cis-2*S*,4*S* (RS-21607)	0.85, 0.35	0.82, 1.17	2.5 ± 1.5	1.4
cis-2*R*,4*R*	17.7		37 ± 11	40.4
cis-2*S*,4*S* + cis-2*R*,4*R*	1.24		6.3 ± 4	2.5
ketoconazole	63.5		64, 67	24.5

[a] Data from Lineweaver–Burk analysis of the rate of tritium release from [$^3H_3$]dihydrolanosterol.

Table 4: Selective Inhibition of Cytochrome P450 Activity[a]

	inhibitor	
cytochrome P450	RS-21607	keto-conazole
Sterol Hydroxylases		
lanosterol 14α-demethylase (human)	0.80 ± 0.34	66
cholesterol 7α-hydroxylase (CYP7)	1625 ± 784	334
cholesterol 27-hydroxylase (CYP27)	71%[b]	32%[b]
27-hydroxycholesterol 7α-hydroxylase	66%[b]	45%[b]
cholesterol side-chain cleavage (CYP11A1)	18400 ± 800	1400 ± 0
Steroid Hydroxylases		
aromatase (CYP19)	7.6 ± 1.3	103%[b]
progesterone 17α,20-lyase (CYP17)	446, 448	38, 29
corticoid 11β-hydroxylase (CYP11B1)	27, 42	81
progesterone 21-hydroxylase (CYP21)	51%[b]	92%[b]
Xenobiotic Hydroxylases		
hepatic progesterone hydroxylases		
6β-hydroxylase (human CYP3A)	33.0 ± 10.2	140
16α-hydroxylase (human)	35.6 ± 12.4	80
lauric acid 12-hydroxylase (rat CYP4A)	NI	NI
debrisoquine (human CYP2D6)	17500 ± 9300	NI
caffeine (human CYP1A2)	3100 ± 1500	100000
tolbutamide (human CYP2C9)	7170	27330

[a] Data are expressed as apparent K_i in nM. NI, no inhibition at 100 μM. [b] Percent control at inhibitor concentration of 5 μM.

was approximately 6-fold less effective than the racemic *cis*-RS-21607.

RS-21607 was at least as potent in hepatic microsomes from male and female human and male hamster as in microsomes from male rat (Table 3) with an apparent K_i value at least an order of magnitude lower than that of the *cis*-2*R*,4*R*-enantiomer. This implies that the intrinsic affinity of these compounds for LDM is independent of the enzyme source and suggests that the nature of the active site is conserved across these species.

Cytochrome P450 in Vitro Selectivity. The high affinity of imidazoles for cytochromes P450 can result in undesired interactions with nontarget cytochromes P450. RS-21607 was selected for further study as a potential cholesterol-lowering agent, based on selective inhibition of LDM relative to the other sterol hydroxylases, steroid hydroxylases, or xenobiotic hydroxylases (Tables 4 and 5). RS-21607 was a minimum of 10-fold more active against LDM than the other cytochromes P450 investigated (Table 5).

The importance of resolving racemic mixtures is emphasized by comparing the activities of RS-21607 and its enantiomer *cis*-2*R*,4*R*. RS-21607 is a much better LDM inhibitor than the *cis*-2*R*,4*R*-enantiomer; however, the *cis*-2*R*,4*R*-enantiomer was a considerably more effective inhibitor of CYP7, CYP17, and CYP11B1, the enzymes responsible for degradation of cholesterol and synthesis of androgens and corticoids, respectively. The apparent K_i values associated with inhibition of LDM, CYP7, CYP17, and CYP11B1 by the *cis*-2*R*,4*R*-enantiomer were 37, 109, 55, and 16 nM, respectively. Accordingly, the difference in K_i values between LDM and CYP7, CYP17, and CYP11B1 was only 2.9, 1.5, and 0.4, respectively, as compared to 559, 2031, and 44 for RS-21607 (Table 5). Strong interactions with these other cytochromes P450 would have been expected if the activity of the two enantiomers had not been separated by preparation of the individual enantiomers. Ketoconazole, a *cis*-dioxolane racemic mixture, is relatively nonselective for the cytochromes P450 (Tables 4 and 5). We have previously prepared the individual enantiomers of ketoconazole and also found differential activities in the separate enantiomers (Rotstein, 1992). These data show RS-21607 to be a selective inhibitor of LDM in vitro, with a much better selectivity profile than ketoconazole.

Table 5: Relative Selectivity of Cytochrome P450 Inhibition[a]

	inhibitor	
cytochrome P450	RS-21607	ketoconazole
Sterol Hydroxylases		
lanosterol demethylase	1	1
cholesterol 7α-hydroxylase (CYP7)	2031	5
cholesterol side-chain cleavage (CYP11A1)	23000	22
Steroid Hydroxylases		
aromatase (CYP19)	10	>1560
progesterone 17α,20-lyase (CYP17)	559	0.5
corticoid 11β-hydroxylase (CYP11B1)	44	1.2
Xenobiotic Hydroxylases		
hepatic progesterone hydroxylases		
6β-hydroxylase (human CYP3A)	41	2.2
16α-hydroxylase (human)	45	1.3
lauric acid 12-hydroxylase (rat CYP4A)	>125000	>1560
debrisoquine (human CYP2D6)	21875	>1560
caffeine (human CYP1A2)	3875	1560
tolbutamide (human CYP2C9)	8963	427

[a] Data are extrapolated from Table 4.

Cytochrome P450 Activities in Vivo. The interaction of RS-21607 with LDM in vivo was investigated by measuring the accumulation of newly synthesized hepatic DHL and the depletion of newly synthesized cholesterol following oral administration of RS-21607. RS-21607 was given to hamsters as the dihydrochloride salt dissolved in water; 90 min prior to sampling, approximately 10 μCi of [^{14}C]mevalonic acid was administered intraperitoneally. At the appropriate time, livers were removed and extracted with acetone, and the sterols were separated by HPLC and their levels determined by radiochemical detection. It is important to note that this method provides information only for the 90-min period prior to sampling. Figure 4A shows that administration of RS-21607 caused a large accumulation (>10-fold) of newly synthesized DHL in somewhat of a dose-responsive fashion. At all the doses of RS-21607, DHL accumulation reached a maximum around 10 dpm of [^{14}C]DHL/dpm of [^{3}H]DHL/μCi of mevalonic acid/g of liver. At later time points, the accumulation of DHL decreases in a dose-dependent fashion, presumably as a result of drug clearance. RS-21607 caused a dose-responsive decrease in hepatic cholesterol biosynthesis with the greatest inhibition between 5 and 8 h (Figure 4B). Cholesterol biosynthesis 18 h postdose was unaffected by 3 mg/kg RS-21607, inhibited by 25% at 10 mg/kg, and inhibited by 52% and 54% by 30 mg/kg (two separate experiments). Accordingly, RS-21607 has an 18-h ED_{50} for cholesterol lowering of 30 mg/kg. *These data indicate that RS-21607 can effectively reach the target enzyme in vivo and inhibit DHL turnover and cholesterol synthesis.*

The in vitro cytochrome P450 selectivity data predicts that RS-21607 should selectively inhibit LDM and cholesterol biosynthesis with minimal effects upon other cytochrome-P450-dependent reactions. This assumption was evaluated

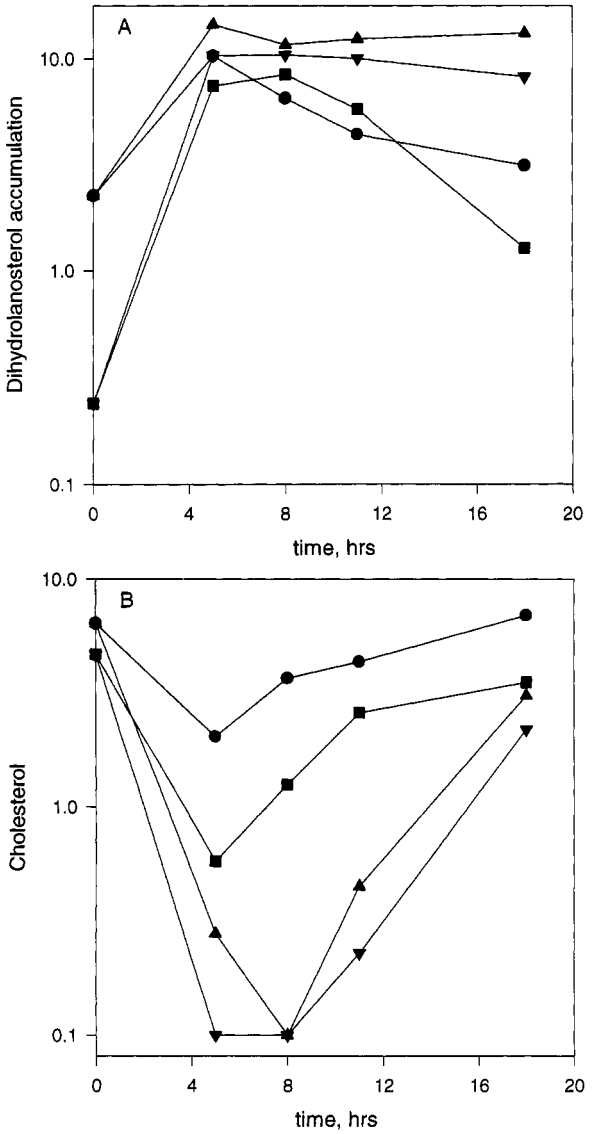

FIGURE 4: Effect of RS-21607 upon lanosterol demethylase activity in hamster. A single oral dose of RS-21607 was given to hamsters and the ability to inhibit lanosterol demethylase determined by the accumulation of hepatic dihydrolanosterol (A) and the depletion of cholesterol (B). The values are presented as dpms of [^{14}C]-dihydrolanosterol or -cholesterol formed from [^{14}C]mevalonic acid administered 90 min prior to sampling, normalized to the dpm's of the internal standards, [^{3}H]dihydrolanosterol or -cholesterol, [^{14}C]-mevalonic acid added, and liver weight, and are plotted against the time after the initial dose of drug. The data represent groups of five animals, and the standard errors are all less than 10% of the final value. Animals were dosed with 3 mg/kg (●), 10 mg/kg (■), or 30 mg/kg (▲) and (▼) (two separate experiments).

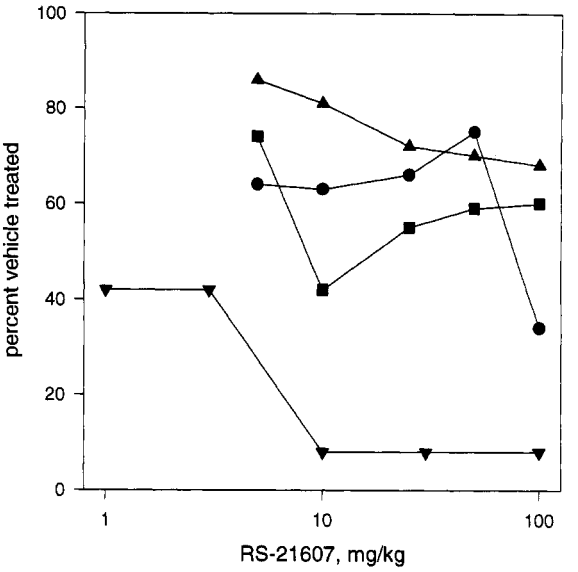

FIGURE 5: Comparison of ability of RS-21607 to inhibit hepatic cholesterol biosynthesis versus its effect upon cytochrome-P450-dependent steroid formation. Inhibition of cholesterol biosynthesis (▼) was determined in hamsters 5 h following a single dose of RS-21607. Cholesterol levels were determined from the dpm's of [^{14}C]-cholesterol formed from [^{14}C]mevalonic acid administered 90 min prior to sampling, normalized to the dpm's of the internal standards, [^{3}H]cholesterol, [^{14}C]mevalonic acid added, and liver weight. Plasma steroid concentrations were measured in LHRH/ACTH-primed male rats (N = 7) 2 h following a single oral dose of RS-21607. The steroid concentrations in untreated animals were 19.9 ng/mL for testosterone (●), 7.15 ng/mL for progesterone (■), and 964 ng/mL for corticosterone (▲). Standard errors were all less than 20% of final values.

by comparing the ability of RS-21607 to lower cholesterol biosynthesis in hamster to its effects upon steroid hormone levels in rat (Figure 5). These data show that RS-21607 is a much more effective inhibitor of cholesterol biosynthesis than of the biosynthesis of testosterone, corticosterone, or progesterone. These steroids are formed by the CYP17, CYP11B1, and CYP11A1, respectively. The effect of RS-21607 upon the plasma concentrations of these compounds was determined after oral administration of 5–100 mg/kg to male rats stimulated with ACTH and LHRH. Corticosterone levels decreased from 964 ± 70 ng/mL with no RS-21607 to 655 ± 54 ng/mL following a 100 mg/kg dose (32% decrease). A single 100 mg/kg dose of ketoconazole reduced corticosterone levels 86% (from 1054 ± 66 mg/mL to 147 ± 13 ng/mL). While testosterone levels were reduced only slightly after doses of 5–50 mg/kg of RS-21607 (from 19.9 ± 2.4 to 12.7 ± 1.9 and 14.9 ± 1.9 ng/mL, respectively), they dropped to 6.75 ± 1.25 ng/mL after the 100 mg/kg dose. This was a 66% decrease as compared to untreated rats. In contrast, ketoconazole decreased testosterone levels 73% following a 25 mg/kg dose and 94% following a 50 mg/kg dose. Progesterone plasma concentrations dropped from 7.15 ± 0.94 ng/mL in untreated animals to 2.99 ± 0.41 ng/mL in animals treated with 10 mg/mL RS-21607. However, this effect on progesterone was not related to dose since the plasma concentration of progesterone was 4.26 ± 0.39 ng/mL at 100 mg/kg RS-21607. These data support the conclusions of the in vitro studies.

Mechanism of Inhibition. Azoles are known to competitively inhibit cytochromes P450 in part by coordination to the heme, resulting in a type II binding spectrum, characterized by an absorption peak at 425–435 nm and an absorption minimum at 390–405 nm (Schenkman, 1981). As expected, RS-21607 interacted with the purified LDM to produce a type II binding spectrum with an absorption maximum of approximately 432 nm and a minimum of 412 nm (Figure 6). At the concentrations of enzyme needed to observe the inhibitor-induced spectrum (100–250 pmol/mL), RS-21607 appeared to bind stoichiometrically to the enzyme (data consistent with tight-binding inhibition), precluding the determination of the apparent K_d.

The relative importance of the imidazole upon binding to the rat enzyme was investigated with the desimidazole analogs of *cis*-2*S*,4*S* (RS-21607) and *trans*-2*R*,4*S*, *trans*-2*R*,4*S*-DI, and *cis*-2*S*,4*S*-DI, respectively. (The change from 2*S* to 2*R* and from *cis* to *trans* in going to the corresponding desimidazole analogs is due to a reversal of the Cahn–Ingold–Prelog priority sequence in the desimidazole compounds.) As expected, the

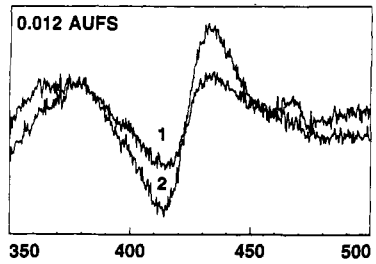

FIGURE 6: Binding spectrum of the interaction of RS-21607 with LDM purified from rat. RS-21607 was added to a sample cuvette containing LDM (200 pmol/mL) and the spectrum recorded between 350 and 500 nm with a SLM Aminco UV spectrophotometer in the split-beam mode. The reference cuvette contained only pure LDM and RS-21607 vehicle. Full-scale absorbance on the *y*-axis is 0.012. Spectra 1 and 2 were recorded following the addition of 50 and 150 pmol/mL RS-21607, respectively. Addition of more RS-21607 resulted in no increased absorbance. Background absorbance was automatically subtracted from the recorded spectra.

imidazole greatly increases the ability of these compounds to inhibit LDM (Table 2), presumably as a result of its ability to coordinate directly with the heme. In contrast to the imidazole compounds, the *cis*-2*S*,4*S*-desimidazole analog (in which the methyl group and side chain are *trans*) had a higher affinity for the enzyme than its *trans*-analog (methyl group and side chain are *cis*). Another interesting observation from these data is that the desimidazole analogs bind with a similar affinity to LDM as does the substrate DHL (assuming k_{-1} is much greater than k_2, making K_i and K_m values comparable). Together, these data confirm that the ligand attached to the imidazole in RS-21607 is a good non-sterol substitute for DHL and that coordination of the imidazole to the heme provides the major contributing factor for the inhibition of LDM by RS-21607.

The 32-oxysterol intermediates (32-alcohol and 32-aldehyde) formed in the demethylation of DHL accumulate under conditions of low metabolism, presumably as a result of competition for further oxidation with excess substrate (Trzaskos et al., 1986b). Ketoconazole was observed by these investigators to increase the accumulation of the 32-oxy intermediates. We also found that as the percent metabolism decreased, the ratio of accumulated oxylanosterol to demethylated product increased (Figure 7). The results shown in Figure 7 were obtained with 40 μM DHL incubated from 2 to 30 min. Addition of RS-21607 (5–30 nM) to the 20-min reactions caused a decreased in the percent metabolism as compared to control (11%), as expected. However, the percent of accumulated oxylanosterols in the products was greater than expected if the decrease in metabolism was simply a result of inhibition of the enzyme at only the first step of the reaction. If this had been the case, the inhibited profile should have had an identical slope to that without inhibitor. The increase in accumulation with increasing inhibitor and decreasing metabolism can only be interpreted to indicate that *RS-21607 inhibits more than one step in the demethylation reaction.* Other studies showed that the profiles for RS-21607 and ketoconazole (100–800 nM) were nearly identical (data not shown) and the primary oxysterol accumulated with both inhibitors was the 32-aldehyde. Similar results were obtained in experiments using 80 μM DHL. These data are interpreted to indicate that the relative affinities of RS-21607 and ketoconazole for the different steps in the sequential oxidation of DHL by LDM are similar and that both preferentially inhibit third oxidation relative to second, resulting in an accumulation of the 32-aldehyde. The difference between the two inhibitors is that 10–20 times more

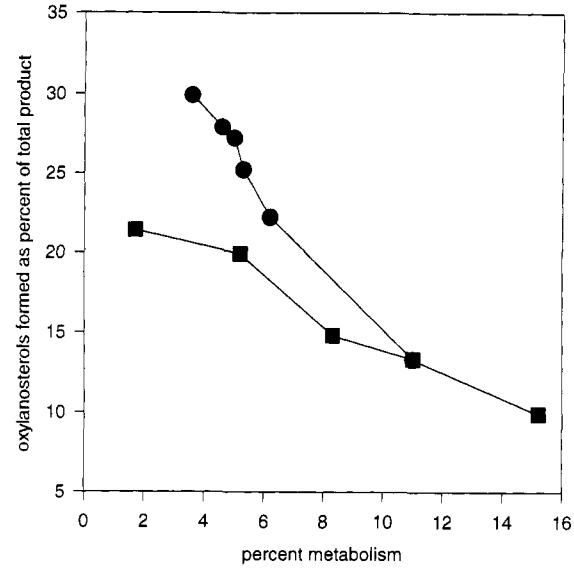

FIGURE 7: Accumulation of oxylanosterols. DHL (40 μM) was incubated with 1 mg/mL microsomal protein from cholestyramine-treated rats. The percent of 32-oxysterol formation was determined from the HPLC analysis of 32-alcohol, 32-aldehyde, and diene formation. The change in percent metabolism in the absence of RS-21607 (■) was obtained by changing the incubation time from 5 to 30 min. The data with RS-21607 (●) was obtained by changing the inhibitor concentration in 20-min incubations. RS-21607 concentration was varied from 5 to 30 nM. An example of the acutal amount of product formed per 1 mL of incubation is the 20-min incubation without inhibitor; 11% of the DHL was metabolized to 3.99 nmol of diene, 0.398 nmol of 32-alcohol, and 0.212 nmol of 32-aldehyde. Addition of 20 nM RS-21607 decreased the conversion of DHL to 4.6%; 1.328 nmol of diene, 0.172 nmol 32-alcohol, and 0.344 nmol of 32-aldehyde were formed. Similar ratios of products were observed at other concentrations of inhibitors. Individual data points represent the mean of duplicate incubations. The data presented in this figure are representative of three separate studies, two using 40 μM DHL and one using 80 μM DHL.

ketoconazole was needed to achieve the same results as those observed for RS-21607.

DISCUSSION

These studies were initiated to determine if mammalian LDM could be selectively inhibited both in vitro and in vivo. Methods were established to monitor in vitro LDM inhibition and interactions with other known cytochromes P450, as well as methods to measure endpoints of specific in vivo enzyme chemistry.

The synthesis of [32-$^3H_3$]DHL made possible the development of a rapid and convenient method for evaluating lanosterol demethylase activity (DeKeczar et al., 1993). The label incorporated at the C-32 position is lost the aqueous environment during the oxidation process. This is easily separated from unreacted product and quantitated. Evaluation of LDM activity by this procedure could be complicated by kinetic isotope effects. The breaking of a C–T bond during a catalytic event should proceed much more slowly than for a C–H bond due to the lower zero-point energy; accordingly, DHL labeled with three tritiums on the C-32 methyl group might be thought to be oxidized much more slowly than unlabeled enzyme. However, the rate of demethylation from [32-$^3H_3$]DHL was very similar to that observed with [24,25-$^3H_2$]DHL which has an unlabeled C-32 methyl group (Table 1). A ^TV isotope effect of 2.03 was observed in microsomes from cholestyramine-treated rats, while no significant isotope effect associated with $^T(V/K)$ was observed (Table 1). The lack of a large isotope effect upon this reaction allows us to

reliably determine the kinetic constants associated with the inhibition of lanosterol demethylase using [32-$^3H_3$]DHL as the substrate. It also suggests that the demethylation chemistry is not rate limiting in the enzymatic reaction.[3]

RS-21607, a *cis*-2*S*,4*S*-stereoisomer, was determined to be the most effective and selective non-sterol, mammalian LDM inhibitor yet described. It was characterized against LDM purified from rat as a competitive tight-binding inhibitor with an apparent K_i of 840 pM (Figure 2). Its ability to block LDM activity was similar in microsomes from all the species studied (human, rat, and hamster) (Table 2). The ability to inhibit the enzyme appears to be largely a result of the electrostatic contribution of the imidazole binding to the heme. Removal of the imidazole from the substrate dramatically decreased the apparent K_i, data consistent with the observation of a type II binding spectrum (Figure 7). RS-21607 also had a higher affinity for LDM than the other cytochromes P450 involved in sterol, steroid, and xenobiotic oxidations. Since all cytochromes P450 have a protoporphyrin IX heme prosthetic group, an azole has the potential to bind with high affinity to all these enzymes. Therefore, the apparent selectivity of RS-21607 suggests that it interacts with the apoprotein of LDM with a much higher affinity than it interacts with the apoprotein of the other cytochromes P450. RS-21607 was also found to block the biosynthesis of cholesterol in hamsters in a dose-dependent manner with a corresponding accumulation of dihydrolanosterol. This was interpreted to indicate that RS-21607 is able to distribute to the site of action in hamsters and inhibit the target enzyme. At the same doses in rat testosterone, corticosterone, and progesterone plasma concentrations, endpoints of the enzymes involved in steroid biosynthesis were relatively unaffected. These data show RS-21607 to be an effective and selective inhibitor of LDM in vitro and in vivo.

It is well established that for many racemic compounds, one enantiomer interacts with biological systems in a manner distinct from its antipode. For example, we have previously shown that the *cis*-2*S*,4*R*-enantiomer of ketoconazole is approximately 3-fold more effective than the *cis*-2*R*,4*S*-enantiomer for inhibition of LDM (Rotstein et al., 1992). In contrast, the *cis*-2*R*,4*S*-enantiomer is an order of magnitude more effective as an inhibitor of CYP7. Therefore, the *cis*-2*S*,4*R*-enantiomer has a much more desirable profile for cholesterol lowering than its antipode. However, most studies with ketoconazole have been undertaken with the racemic mixture, i.e., a mixture of two compounds with distinctly different biological profiles. Upon investigating the profile of the four stereoisomers of RS-21607 against various cytochromes P450, we also observed the different diastereomers to have different interactions. Fortunately, the stereoisomer with the best activity for LDM, RS-21607, also had the least activity against CYP7, CYP17, and CYP11B1. Obviously, inhibition of cholesterol degradation (CYP7) should decrease the ability of the compounds to lower cholesterol in vivo. This translates to an overall maximal effect on cholesterol biosynthesis by RS-21607, with minimal effects upon cholesterol degradation and androgen and corticoid formation.

The 32-aldehyde of dihydrolanosterol has been reported to accumulate under a variety of conditions including high DHL concentration and the presence of ketoconazole (Trzaskos et al., 1986b, 1987; Saucier et al., 1987). We observed a similar accumulation with RS-21607. The accumulation of this oxysterol has been associated with a suspension of HMG-CoA reductase activity. Recently, Trzaskos and co-workers (1993) used the lanosterol analog 15α-fluorolanost-7-en-3β-ol to generate a stable sterol 32-aldehyde analog. They observed this compound to supress HMG-CoA reductase activity by reducing the translational efficiency of the reductase mRNA. They proposed that the "accumulation of the 32-aldehyde *in situ* may be the result of a designed integrated regulatory process which serves to control endogenous cholesterol biosynthesis under normal physiological fluxes of carbon flow through the sterol pathway". Accordingly, an inhibitor which increases the accumulation of the 32-aldehyde of DHL, such as RS-21607, may decrease cholesterol biosynthesis by two mechanisms, inhibition of lanosterol 14α-demethylase and feedback suppression of HMG-CoA reductase. This proposal is supported by the observation that RS-21607 suppresses the post-translational activity of HMG-CoA reductase activity in hamsters and HEP G2 cells (Dr. Pamela M. Burton, personal communication).

In this report, we have presented the methodology used to rationally evaluate an inhibitor for an enzyme of cholesterol biosynthesis. RS-21607 was characterized to be a high-affinity, tight-binding, competitive inhibitor of LDM. It inhibited the enzyme and cholesterol biosynthesis in hamster liver for at least 18 h following a 30 mg/kg oral dose. RS-21607 had minimal effects against the other cytochromes P450 investigated both in vivo and in vitro. It also caused an increase in the accumulation of the 32-aldehyde oxysterol intermediate. Therefore, as a result of its effectiveness as a LDM inhibitor and its selectivity against other cytochromes P450, RS-21607 has the potential to be a novel therapy for the treatment of hypolipidemic disorders. Preliminary results indicate that RS-21607 effectively lowers LDL cholesterol in mildly hypolipidemic patients without serious side effects (Dr. Ken Schwartz, personal communication).

[3] The intrinsic isotope effect for an enzymatic reaction can be masked by other enzymatic steps in the reaction sequence. Northrup (1977) has shown that masking of the V isotope effect is a result of the "ratio of catalysis" and represents the ratio of the rate of the catalytic step to the rate of the other forward steps contributing to the maximal velocity, whereas masking of the V/K isotope effect is a result of the "commitment to catalysis" and represents the tendency of the enzyme–substrate complex to go forward through catalysis as opposed to its tendency to break down to free enzyme and substrate. The observation of the lower V/K isotope effect with lanosterol demethylase suggests that steps preceeding the catalytic event, such as substrate binding or enzyme reduction, may be rate limiting.

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