Squalene synthase is a key enzyme in cholesterol biosynthesis and is rate-limiting for the pathway. Inhibition of this enzyme by squalestatin has been shown to lower serum cholesterol levels in vivo. Squalestatin is a potent inhibitor of squalene synthase and can significantly reduce serum cholesterol. The article describes the isolation of squalestatin from the fungus Phoma motteram and its development as a potential therapeutic agent for the treatment of hypercholesterolemia. The structure of squalestatin is shown, and its mechanism of action is discussed. The experimental procedures include the isolation of rat hepatocytes, the measurement of squalene synthase activity, and the determination of cholesterol biosynthesis. The results demonstrate the effectiveness of squalestatin in reducing cholesterol levels in vivo and in vitro. The potential of squalestatin as a therapeutic agent for the treatment of hypercholesterolemia is discussed.
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Measurement of ATP-Citrate Lyase Activity—A crude preparation of ATP:citrate lyase was prepared as a post-mitochondrial supernatant from rat liver homogenized in 0.25 M sucrose, 10 mM mercaptoethanol, 1 mM MgCl₂, and 20 mM Tris-HCl and centrifuged 10,000 g for 10 min. Enzyme was incubated with [14C]citrate (0.08 μCi/ml, 0.7 mM), ATP (10 mM), coenzyme A (0.7 mM), and hydroxylamine (200 mM); the last component converts one of the products ([14C] acetyl-CoA) to the corresponding hydroxamate. After incubation at 37 °C for 10 min, excess unlabeled citrate was added. The [14C] acetylhydroxamate was separated from the corresponding hydroxamate in the supernatant by precipitation of the radiolabeled acids as a complex with barium after incubation with barium hydroxide overnight at 4 °C. [14C]Acetylhydroxamate in the supernatant was measured by liquid scintillation counting. This assay gives results comparable with those described by Watson et al. (1969).

Citrate Efflux from Mitochondria—Rat liver mitochondria were prepared as described by Harper and Saggerson (1975) and checked for functional integrity prior to use by determination of their respiratory control ratio using an oxygen electrode.

Citrate efflux from mitochondria was measured using the method of Robinson et al. (1970) where intra-mitochondrial citrate is labeled by incubation with [14C]bicarbonate, and efflux of [14C]citrate stimulated by addition of extra-mitochondrial unlabeled citrate. Specific citrate-stimulated efflux was defined by that proportion of total efflux that was inhibited by 1,2,3-benzenereticarboxylic acid.

Measurement of Cholesterol Biosynthesis in Vivo—Cholesterol biosynthesis in vivo was carried out essentially as described by Tsujita et al. (1986), except that [14C]cholesterol was measured by HPLC. Squalene synthase was measured after extraction and HPLC separation as described by Watson et al. (1969). Squalane synthase activity was determined as described under "Experimental Procedures." The optimum enzyme activity was determined by intraperitoneal administration of [14C]acetate (250 μCi/kg). After 1 h, the rats were killed and the livers removed, and a sample of 0.5 g was saponified in alcoholic KOH at 80 °C for 1.5 h. [14C]Cholesterol was measured after extraction and HPLC separation as described above.

Effect on Serum Cholesterol Levels in Marmosets—Marmosets of mixed sex were fasted overnight and blood samples (500 μl) taken from the femoral vein and collected in Microtainers (BectonDickinson). Serum was obtained by centrifugation for 4 min at 10,000 g in a Beckman Microfuge. Serum cholesterol concentrations were determined using a standard assay kit (Boehringer 237574) on a Kone Progress Plus autoanalyzer. Animals were allocated to treatment groups on the basis of their fasting serum cholesterol levels such that the mean and distribution of serum cholesterol levels were similar for each group.

Effects on Apoprotein Levels—Eight marmosets were dosed orally with squalene synthase (1 mg/kg/d for 7 days). Serum samples were collected from the femoral vein prior to the animals receiving the first dose of squalene synthase, and again 24 h after the last dose. Apolipoprotein levels were measured immunoturbidimetrically using Sigma kits for human apolipoproteins B and A1 (Sigma catalog 357-A and 356-A, respectively).

RESULTS

Biological Effects in Vitro—Squalane synthase is a potent inhibitor of squalene synthase from both rat and marmoset liver, with an IC₅₀ of 12 nM (range 4–22 nM). Typical results of in vitro inhibition of the rat liver enzyme are shown in Fig. 2. Similar inhibitory activity is seen against squalene synthase present in microsomes isolated from Hep-G2 cells, a human hepatoma line, and from Candida albicans (data not shown).
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FIG. 5. Effect of squalestatin 1 on cholesterol biosynthesis in vivo in rats. Squalestatin 1 was administered intravenously to rats (n = 8 per group) and cholesterol biosynthesis in vivo was measured as described under "Experimental Procedures."

Squalene synthase 1 also inhibits the synthesis of cholesterol from [14C]acetate by isolated rat hepatocytes, with an IC50 of 39 nM (Fig. 3). The tricarboxylic acid structure of squalene 1 resembles citrate, and 1,2,3-benzenetricarboxylate has been shown to inhibit cholesterol synthesis through its effect on citrate efflux from mitochondria (Claeys and Azzi, 1989). Therefore, the effects of squalestatin 1 on the early citrate-utilizing steps in the cholesterol biosynthetic pathway were examined, i.e. citrate efflux from mitochondria and ATP:citrate lyase activity. Squalestatin 1 has no effect on ATP:citrate lyase activity at concentrations up to 100 μM and no effect on citrate efflux from mitochondria at concentrations up to 1 nM.

When isoprenoid intermediates were measured in isolated hepatocytes, [3H]FPP could be readily detected in these cells after 30 min incubation, and the level decreased slowly over the next hour; other isoprenoid intermediates could not be detected. In the presence of 50 or 500 nM squalestatin 1, the amount of radiolabel in FPP increased approximately 6-fold at all time points, as would be expected from inhibition of squalene synthase (Fig. 4). The reason for the decrease in labeling of FPP over 90 min is under further investigation; in control cells, labeling of cholesterol from [3H]mevalonate increases linearly over this time. Similar experiments in which radiolabeled squalene in cell extracts was measured by HPLC showed that squalene labeling (which is low but measurable in control cells) was not detectable in the presence of squalestatin 1 (data not shown).

Effects on Serum Cholesterol Levels in Vivo—Inhibitors of cholesterol biosynthesis which act at hydroxymethylglutaryl-CoA reductase are reported to have no effect on the serum cholesterol levels in rodents, although other mammalian species such as rabbits or primates respond to these agents (Tsujita et al., 1986). Adult marmosets have been shown to possess a serum lipoprotein profile similar to that of man (Crook et al., 1990), and we have shown that this species is sensitive to inhibitors of hydroxymethylglutaryl-CoA reductase. Fig. 6a shows the effect of squalestatin 1 on serum cholesterol in adult marmosets; a significant effect is apparent at an oral dose of 10 mg/kg/day, and cholesterol lowering of up to 75% can be achieved at an oral dose of 100 mg/kg/day. The cholesterol lowering is apparent within 24 h (data not shown) and can be maintained for at least 8 weeks on prolonged dosing with no attenuation of the response (Fig. 6b).

Table I shows the effects of squalestatin 1 on apolipoprotein levels in marmosets. Table I shows the effects of squalestatin 1 on apolipoprotein levels in marmosets. Eight animals were allocated to each group and dosed orally by intragastric intubation with squalestatin 1 dissolved in water. a, animals were dosed for 1 week with 1–100 mg/kg/day, and serum cholesterol levels measured as above at the end of the dosing period. Animals were dosed for 8 weeks with 10 mg/kg/day, and serum cholesterol levels determined every 7 days. All results are expressed as the mean of the change in serum cholesterol levels from pre-dose values (± S.E.).

| Serum parameter | Change in level |
|-----------------|----------------|
| Cholesterol     | %              |
| (4.6 ± 0.5 mM)  | 51 ± 4         |
| Apolipoprotein B| 45 ± 9         |
| (0.38 ± 0.05 mg/ml) |             |
| Apolipoprotein A1| 0 ± 4         |
| (0.90 ± 0.06 mg/ml) |           |

DISCUSSION

Many studies on the pathway of sterol synthesis have used inhibitors of various steps, including hydroxymethylglutaryl-CoA reductase, hydroxymethylglutaryl-CoA synthase, squalene epoxidase, and lanosterol 14α-demethylase. With the discovery of squalestatin 1, a potent inhibitor of squalene synthase, we have been able to study inhibition of the first committed step of cholesterol biosynthesis, after the branchpoint in the pathway. The compound does not inhibit hydroxymethylglutaryl-CoA reductase, another important enzyme in

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controlling cholesterol biosynthesis, at concentrations up to 5 μM in vitro (data not shown). Additional evidence for its selectivity is provided by its lack of effect on the mitochondrial tricarboxylate transporter and its poor activity against ATP-citrate lyase.

We have also shown squalestatin 1 to be a potent inhibitor of cholesterol synthesis in freshly isolated hepatocytes (IC_{50} = 39 nM). Similar IC_{50} values are obtained if the experiment is performed using hepatocytes that have been allowed to adhere to tissue culture dishes overnight, showing that the effect of squalestatin 1 is not related to the trauma of the collagenase-perfusion isolation method (data not shown). The observation in hepatocytes that the labeling of the precursor (FPP) increases while that of the product (squalene) decreases provides strong evidence that squalene synthase is the site of action of squalestatin 1 and further underlines the selectivity of action of the compound. In addition, the flux through the cholesterol biosynthetic pathway is readily inhibited by squalestatin 1, both in isolated hepatocytes and in rat liver in vivo; this suggests that squalene synthase activity in hepatic cells is similar to the rate of flux through the pathway, consistent with its proposed role as a rate-determining step in the pathway (Brown and Goldstein, 1980; Bruenger and Rilling, 1986).

Marmosets have been shown to have a lipoprotein metabolism similar to that of man (Crook et al., 1990), and therefore we believe that this primate is a good model in which to study the effects of cholesterol lowering drugs. Our observations with squalestatin 1 in marmosets provide the first demonstration that a potent inhibitor of squalene synthase can affect serum cholesterol in vivo. It is important that only the apo-B-containing lipoproteins are affected. As squalene synthase is the first committed step on the cholesterol biosynthetic pathway, a therapeutic agent acting by inhibiting this step should have minimal effects on non-steroidal products of the pathway from mevalonate. Perturbations in the levels of ubiquinone have been implicated in some of the side effects of inhibitors of hydroxymethylglutaryl-CoA reductase (Folkers et al., 1990). Additional studies to determine the mechanism of inhibition at a molecular level and possible clinical utility of this class of compounds are under way.

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