The role of ATP citrate-lyase in the metabolic regulation of plasma lipids

Hypolipidaemic effects of SB-204990, a lactone prodrug of the potent ATP citrate-lyase inhibitor SB-201076

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ATP citrate (*pro-S*)-lyase (EC 4.1.3.8), a cytosolic enzyme that generates acetyl-CoA for cholesterol and fatty acid synthesis *de novo*, is a potential target for hypolipidaemic intervention. Here we describe the biological effects of the inhibition of ATP citrate-lyase on lipid metabolism in Hep G2 cells, and plasma lipids in rats and dogs, by using SB-204990, the cell-penetrant γ -lactone prodrug of the potent ATP citrate-lyase inhibitor SB-201076 ($K_i = 1 \mu\text{M}$). Consistent with an important role of ATP citrate-lyase in the supply of acetyl-CoA units for lipid synthesis *de novo*, SB-204990 inhibited cholesterol synthesis and fatty acid synthesis in Hep G2 cells (dose-related inhibition of up to 91% and 82% respectively) and rats (76% and 39% respectively). SB-204990, when administered orally to rats, was absorbed into the systemic circulation; pharmacologically relevant concentrations of SB-201076 were recovered in the liver. When administered in the diet

(0.05–0.25%, w/w) for 1 week, SB-204990 caused a dose-related decrease in plasma cholesterol (by up to 46%) and triglyceride levels (by up to 80%) in rats. This hypolipidaemic effect could be explained, at least in part, by a decrease (up to 48%) in hepatic very-low-density lipoprotein (VLDL) production as measured by the accumulation of VLDL in plasma after injection of Triton WR-1339. SB-204990 (25 mg/kg per day) also decreased plasma cholesterol levels (by up to 23%) and triglyceride levels (by up to 38%) in the dog, preferentially decreasing low-density lipoprotein compared with high-density lipoprotein cholesterol levels. Overall these results are consistent with the concept that ATP citrate-lyase is an important enzyme in controlling substrate supply for lipid synthesis *de novo* and a potential enzyme target for hypolipidaemic intervention.

INTRODUCTION

ATP citrate (*pro-S*)-lyase (EC 4.1.3.8) is a cytosolic enzyme that catalyses the cleavage of citrate into acetyl-CoA and oxaloacetate [1–3]. In non-ruminating mammals this enzyme is abundantly expressed in lipogenic tissues, such as the liver and adipose tissue [4], where it has an important role in supplying acetyl-CoA for

both cholesterol synthesis and lipogenesis. Previous studies with (–)-hydroxycitrate, a potent inhibitor of ATP citrate-lyase ($K_i = 0.15 \mu\text{M}$) [1], have demonstrated that inhibition of this enzyme leads to a decrease in the synthesis of both cholesterol and fatty acids [5–9] and an increase in low-density lipoprotein (LDL) receptor activity [10]. Furthermore a decrease in plasma cholesterol and plasma triglyceride levels was found in rats treated with (–)-hydroxycitrate, suggesting a potential utility of ATP citrate-lyase inhibitors as hypolipidaemic agents [9]. However, hydroxycitrate is not well absorbed, which hampers its use as a test compound to investigate the effects of ATP citrate-lyase inhibition *in vivo*.

We have recently identified a series of potent inhibitors of ATP citrate-lyase, one of which, SB-201076 (Figure 1), has a K_i of $1 \mu\text{M}$ for the human enzyme. Here we report the effects of this compound and its prodrug lactone, SB-204990 (Figure 1), on cholesterol and fatty acid synthesis in Hep G2 cells, as well as the hypolipidaemic properties of SB-204990 in rats and dogs.

MATERIALS AND METHODS

Reagents

Dulbecco's minimal essential medium (DMEM) containing Hepes buffer was purchased from Flow Laboratories (Irvine, Scotland, U.K.). Foetal bovine serum, glutamine and sodium bicarbonate were obtained from Gibco Life Technologies (Paisley, Scotland, U.K.). $^3\text{H}_2\text{O}$ (3700 MBq/ml) was obtained

SB-201076

SB-204990

Figure 1 Structure of SB-201076 and its γ -lactone SB-204990

Abbreviations used: DMEM, Dulbecco's minimal essential medium; HDL, high-density lipoprotein; LDL, low-density lipoprotein; RO-22-0654, 4-amino-5-ethyl-3-thiophenecarboxylic acid methyl ester hydrochloride; VLDL, very-low-density lipoprotein.

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from Amersham International (Little Chalfont, Bucks., U.K.). ATP assay kits were supplied by LKB Pharmacia (Milton Keynes, Bucks., U.K.). Halothane anaesthetic was from Rhone-Merieux (Harlow, Essex, U.K.). CB-300 capillary tubes were from Sarstedt (Leicester, Leics., U.K.). Triton WR-1339 (Tyloxapol) was from Sigma Chemical Co. (Poole, Dorset, U.K.). CHOD-Iodide cholesterol test kits and GPO-PAP triglyceride test kits were purchased from Merck Ltd. (Lutterworth, Leics., U.K.). Ames cholesterol test kits were from Miles Laboratories (Slough, Berks., U.K.). SB-201076 and SB-204990 were synthesized by the department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, and [^3H]SB-204990 was synthesized by the department of Synthetic and Isotope Chemistry, SmithKline Beecham Pharmaceuticals.

Preparation of SB-204990 and hydroxycitrate free acid

(–)-Hydroxycitrate, isolated from the fruit rind of *Garcinia cambogia* [11,12], was prepared as a free acid solution (100 mM) by hydrolysing the lactone in 3.3 equiv. of NaOH, followed by neutralization to pH 7.4. SB-204990 (supplied as the free acid) was dissolved in water with the addition of approx. 1 equiv. of sodium bicarbonate to bring the pH to 7.4.

ATP citrate-lyase inhibition studies

Rat liver ATP citrate-lyase and recombinant human ATP citrate-lyase were purified as described previously [13,14]. The yield of rat ATP citrate-lyase was increased by isolating the enzyme from the livers of male rats refed with a high-carbohydrate diet for 3 days after the withdrawal of food for 24 h [15]. ATP citrate-lyase activity was determined by the malate dehydrogenase-catalysed reduction of oxaloacetate by NADH. NADH consumption was measured by monitoring the reaction at 340 nm over 20 min. Assays were run at the K_m for citrate (100 μM) and saturating ATP (250 μM) and CoA (200 μM), as described previously [12].

Cholesterol and fatty acid synthesis studies in Hep G2 cells

Hep G2 cells were cultured in DMEM containing Hepes (20 mM), sodium bicarbonate (0.85 g/l), glutamine (2 mM) and 10% (v/v) foetal bovine serum. For experiments, cells were seeded into 24-well cell culture dishes. When the cells were 80–90% confluent they were washed twice in culture medium without added foetal bovine serum and incubated overnight in this medium. The cells were then incubated for a further 2.5 h after the addition of vehicle or compound. In some experiments the vehicle or compound was added immediately before the overnight incubation. The rates of cholesterol and fatty acid synthesis were determined by the addition of $^3\text{H}_2\text{O}$ (1.25 mCi per well) for the final 90 min of the incubation, basically as described previously [10]. Minor modifications to the lipid extraction and separation methods were as follows. Saponification of the cells was performed in 4 ml of 2 M NaOH followed by the addition of 3.5 ml of 1 M NaOH and 1 ml of 9.75 M NaCl. The organic-phase extracts of the cholesterol and fatty acid fractions were washed with NaCl solutions (1.5 M) of pH 12 and 2 respectively. Silica gel TLC of the cholesterol fractions was performed in chloroform containing 1% (v/v) methanol. All other details remained the same. The effect of compounds on cell viability was determined in parallel incubations by measuring cellular ATP levels with an ATP assay kit (LKB Pharmacia) based on the firefly luciferase method. Cellular ATP was extracted into a solution containing 0.5% trichloroacetic acid and 2 mM EDTA immediately after the cells had been rapidly washed with 1 ml of PBS per well.

Studies of cholesterol and fatty acid synthesis in the rat

Male Sprague–Dawley rats were fed on a normal powdered diet (RM1, containing 8%, 71% and 21% energy as fat, carbohydrate and protein respectively; Special Diet Services, Witham, Essex, U.K.) and maintained on a reverse light cycle for 2 weeks before the study. After the intravenous injection of SB-204990 or vehicle (rat serum), as described in the legend to Table 1 (below), the synthesis of cholesterol and fatty acids was measured over 65 min after an intraperitoneal injection of $^3\text{H}_2\text{O}$ (1 mCi per 100 g rat; 4 mCi/ml in 0.9% saline). The animals were then anaesthetized with a controlled flow of ether into an induction chamber and terminal blood and liver samples taken. The amount of ^3H incorporated into the cholesterol and fatty acids of 1 g of liver was determined essentially as described by Gibbons et al. [16] and Stansbie et al. [17]. The specific radioactivity of the water pool in each animal was determined by measuring the ^3H content of aliquots of serum isolated from 1 ml of blood. The rate of cholesterol and fatty acid synthesis was calculated by assuming that 23 g-atoms of ^3H are incorporated/mol of cholesterol [18] and 14 g-atoms of ^3H /mol of fatty acids [19].

Oral bioavailability study with [^3H]SB-204990 in the rat

A single oral dose of [^3H]SB-204990 (150 $\mu\text{mol/kg}$ body weight with a specific radioactivity of 0.7 $\mu\text{Ci}/\mu\text{mol}$) was administered by gavage to male Sprague–Dawley rats (200–250 g body weight). The rats were subsequently anaesthetized with gaseous Halothane anaesthesia 2, 4 or 6 h after being dosed, and terminal EDTA blood samples were taken from the descending aorta. Liver and kidney samples were also taken and stored on ice before the extraction of radioligand. Samples of liver and kidney were homogenized in 2 vol. of water per gram of tissue with a Polytron homogenizer. Plasma was isolated from the blood samples by centrifugation at 1500 g for 15 min. Aliquots (400 μl) of plasma or liver homogenate were extracted with 800 μl of acetonitrile. These extracts were then separated by HPLC (UV, 220 nm detection) with a Waters C_{18} $\mu\text{Bondapak}$ column and a mobile phase composed of acetonitrile/0.1% (v/v) trifluoroacetic acid (11:9, v/v). The amount of radiolabel in fractions matching the elution profile of SB-204990 and SB-201076 was measured by liquid-scintillation spectrometry; recovery of radiolabel from the HPLC column was 89%, 87% and 100% for the plasma, liver and kidney extracts respectively, of which the majority (48–70%) was accounted for by SB-204990 and SB-201076. The concentrations of SB-204990 and SB-201076 in the plasma and tissue samples were calculated from the specific radioactivity of the [^3H]SB-204990 injected. The identities of the SB-201076 and SB-204990 peaks in plasma extracts from rats treated with SB-204990 were established by MS in a separate study.

Hypolipidaemic studies in the rat

Male Sprague–Dawley rats were fed with a normal powdered diet (RM1, details as above) and maintained on a reverse light cycle for 2 weeks before the study. The rats were then weighed and allocated to treatment groups: control RM1 diet, or RM1 diet containing SB-204990 at various concentrations in the diet. The body weights of the rats at the beginning of these studies were approximately 180 g (range 166–193 g). The rats were maintained on one of these diets for 1 week and body weight and food consumption were recorded daily.

At 1 week after starting treatment, the rats were anaesthetized with gaseous Halothane anaesthesia, and a blood sample (0.3 ml) was taken from a tail vein with a CB-300 capillary tube (EDTA) for the measurement of total plasma cholesterol and triglyceride

concentrations. Rates of very-low-density lipoprotein (VLDL) synthesis were determined by measuring the increase in plasma VLDL triglyceride concentration after the intravenous administration of the non-ionic detergent Triton WR-1339 [20]. Triton WR-1339 [20% (w/v) solution in 0.9% saline; 200 μ l per 100 g rat] was administered via a tail vein and the animal was allowed to recover from the anaesthesia. The rats were subsequently re-anaesthetized, as above, and a terminal EDTA blood sample was taken from the descending aorta 90 min after the injection of Triton WR-1339. This procedure was performed in the middle of the dark cycle. Terminal liver samples were also taken for quantification of hepatic SB-204990 content. Extracts of liver samples were prepared and analysed by HPLC as described for the bioavailability study. The concentration of SB-204990 in the liver was determined by measuring the peak areas of HPLC fractions matching the elution profile of SB-204990 and conversion to nmol/g on the basis of peak areas for [3 H]SB-204990 measured in hepatic extracts from previous experiments.

Plasma was isolated from the blood samples by centrifugation at 1500 *g* for 15 min. Chylomicrons were isolated from post-Triton plasma essentially as described by Khan et al. [21]: plasma (0.25 ml) was placed into a Beckman (13 mm $\times$ 64 mm, 355645) tube and over-layered with 3.25 ml of salt solution [*d* 1.006; 0.9% saline/0.1% EDTA/0.02% azide (pH 7.2–7.4)] and centrifuged at 8000 *g* and 12 $^{\circ}$ C for 35 min (acceleration 3, deceleration 3) in a Beckman 50.4 rotor in a Beckman L8-M ultracentrifuge. The chylomicrons were then isolated from the top layer and stored frozen at -20° C, along with the aliquots of the pre-Triton and post-Triton plasma samples until assayed. Cholesterol and triglyceride concentrations in both plasma and centrifugation fractions were measured enzymically, with the Merck CHOD-iodide test kit for cholesterol and the Merck GPO-PAP test kit for triglyceride.

Rates of VLDL synthesis were calculated as [(post-Triton triglyceride – pre-Triton triglyceride) – chylomicron triglyceride]/time between Triton injection and post-Triton blood sample, i.e. in mg of triglyceride/h per dl of plasma. Only 5% of the post-Triton plasma triglyceride concentrations were composed of chylomicron triglyceride.

Hypolipidaemic studies in the dog

Male beagle dogs were housed two to a pen and maintained on SDS-A SQC diet (containing 21%, 48% and 31% energy as fat, carbohydrate and protein respectively; Special Diet Services), fed daily at 09:30h, with free access to water. Any food remaining in the pen at 16:00h was removed and daily food consumption was determined. Blood samples (2 ml maximum) were obtained from the cephalic vein, 30 min before and 6.5 h after the daily meal on two occasions per week. These blood samples were placed into heparin and EDTA plasma tubes and plasma was isolated by centrifugation at 1500 *g* for 10 min. Heparinized plasma samples were analysed for cholesterol and triglyceride concentrations with the Ames and Merck GPO-PAP test kits respectively. Both plasma cholesterol and plasma triglyceride levels were stable before the study. EDTA plasma samples were used for the isolation of VLDL, LDL and high-density lipoprotein (HDL) fractions. SB-204990 was administered once daily (09:15h) orally in gelatin capsules at 10 mg/kg per day for 7 days, immediately followed by 25 mg/kg per day for 15 days. Food intake and body weight of the dogs, monitored before, during and after the studies, were unaffected by treatment. VLDL, LDL and HDL lipoprotein fractions were isolated from the EDTA plasma samples as described previously [22].

All procedures involving animals in this paper were subject to both internal review and UK Home Office regulations.

RESULTS

ATP citrate-lyase enzyme inhibition studies

The ability of SB-201076 to inhibit ATP citrate-lyase activity was determined as described previously [12] with purified rat liver [13] or recombinant human ATP citrate-lyase [14]. SB-201076 was found to be equally potent in inhibiting either the rat ($K_i = 1 \pm 0.05 \mu\text{M}$) or the human ($K_i = 1 \pm 0.1 \mu\text{M}$) ATP citrate-lyase enzyme (means $\pm$ S.E.M.). Inhibition of ATP citrate-lyase by SB-201076 was predominantly competitive against citrate with a small but significant uncompetitive component. SB-204990, the γ -lactone prodrug of SB-201076, was found to be inactive.

The effect of ATP citrate-lyase inhibition on cholesterol and fatty acid synthesis

SB-204990 caused a dose-dependent decrease in the rates of cholesterol and fatty acid synthesis in Hep G2 cells, measured by the incorporation of ^3H into the sterol and fatty acid fractions of saponifiable cell lipids during a 2.5 h incubation (Figure 2). At 30 μM SB-204990, decreases in the rates of cholesterol and fatty acid synthesis were 91% and 82% respectively. Furthermore the inhibition of cholesterol and fatty acid synthesis by SB-204990 was maintained for at least 16 h (results not shown). In contrast, SB-201076 did not inhibit cholesterol or fatty acid synthesis in Hep G2 cells when presented to the cells at concentrations up to 1 mM (results not shown). For comparison, similar studies were performed with a known inhibitor of ATP citrate-lyase, (–)

Figure 2 Effect of SB-204990 and (–)-hydroxycitrate on the rate of synthesis of cholesterol and fatty acids in Hep G2 cells

Hep G2 cells were incubated in DMEM for 2.5 h in the presence of SB-204990 or (–)-hydroxycitrate at the concentrations indicated in the figure. The rates of cholesterol synthesis (●, SB-204990; ○, (–)-hydroxycitrate) and fatty acid synthesis (■, SB-204990; □, (–)-hydroxycitrate) were measured by using $^3\text{H}_2\text{O}$ as described in the Materials and methods section. Rates of cholesterol synthesis were 2.4 and 3.5 pmol/min per well and fatty acid synthesis 17.2 and 18.7 pmol/min per well for the (–)-hydroxycitrate and SB-204990 experiments respectively. The results are expressed as means $\pm$ S.D. for four to five replicates in representative experiments. Significant changes from control were determined by Student's *t* test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Table 1 Effect of SB-204990 on hepatic synthesis of cholesterol and fatty acids in rats

Rates of hepatic synthesis of sterols and fatty acids were measured in male rats, fed with a standard RM1 diet, by measuring the incorporation of ^3H label into hepatic sterol and fatty acid fractions, 65 min after an intraperitoneal injection of $^3\text{H}_2\text{O}$ as described in the Materials and methods section. SB-204990 or vehicle was administered by intravenous injection at the doses shown, 245 and 65 min before the injection of $^3\text{H}_2\text{O}$. Results are means $\pm$ S.D. ($n = 8$). Significant changes were determined by two-way (treatment, day) analysis of variance, followed by pairwise *t*-tests of each treatment group against the control. * $P \leq 0.05$; *** $P \leq 0.001$.

Treatment	Cholesterol synthesis ($\mu\text{mol/h per g}$)	Change (%)	Fatty acid synthesis ($\mu\text{mol/h per g}$)	Change (%)
Control	0.335 ± 0.052	—	0.29 ± 0.11	—
SB-204990 ($2 \times 150 \mu\text{mol/kg}$)	$0.081 \pm 0.020^{***}$	—76	$0.18 \pm 0.03^*$	—39

Figure 3 Concentrations of SB-204990 and SB-201076 in liver and plasma after oral administration of a single dose of [^3H]SB-204990 to rats

Male rats (200–250 g body weight) received a single dose of [^3H]SB-204990 ($150 \mu\text{mol/kg}$ body weight) by gavage. Animals were killed 2, 4 and 6 h after being dosed and the amounts of SB-204990 and SB-201076 in plasma (A) and liver (B) were determined by HPLC. Results are means $\pm$ S.D. for extracts from three animals.

hydroxycitrate. Although (–)-hydroxycitrate is a potent inhibitor of ATP citrate-lyase ($K_i = 0.15 \mu\text{M}$), it was much less potent than SB-204990 at inhibiting lipid synthesis *de novo*, presumably owing to its restricted cell permeability (Figure 2). None of the concentrations of inhibitors used were toxic to the cells as judged by cellular ATP levels, although SB-204990 ($30 \mu\text{M}$) caused a small significant decrease (6%) in cellular ATP levels after 2.5 but not 16 h (results not shown).

The ability of SB 204990 to inhibit hepatic sterol and fatty acid synthesis was subsequently tested in rats (Table 1). Intravenous administration of SB-204990 (twice, each $150 \mu\text{mol/kg}$) significantly inhibited both hepatic cholesterol and fatty acid synthesis (by 76% and 39% respectively). No significant change in hepatic

ATP, ADP, AMP or energy charge were determined in a parallel experiment, indicating that the decrease in hepatic lipid synthesis was not due to a toxic effect (results not shown).

Oral bioavailability of SB-204990 and SB-201076

To investigate whether SB-204990 acts as a prodrug to deliver SB-201076 to its site of action *in vivo*, its absorption and uptake by the liver were studied in rats. An HPLC assay was developed to quantify radiolabelled SB-204990 and SB-201076 in plasma and tissue extracts. After oral administration of a single dose of [^3H]SB-204990 ($150 \mu\text{mol/kg}$ body weight) to rats, the concentration of both compounds in the plasma and liver was determined 2, 4 and 6 h after dosing (Figure 3). At 4 h the quantities of radiolabel measured in the liver, plasma and kidney were 21%, 3.5% and 1% respectively of the administered dose. Both SB-204990 and SB-201076 were detected in plasma, liver and kidney. The concentration of SB-204990 detected in the plasma decreased from 30 nmol/ml after 2 h to 9 nmol/ml after 6 h. Maximal concentrations of SB-201076 (41 nmol/ml) were detected in the plasma after 4 h. Hepatic concentrations of SB-204990 and SB-201076 were higher, reaching a maximum of 173 and 87 nmol/g respectively, 4 h after dosing. The concentrations of SB-204990 and SB-201076 in the kidney at 4 h, 80 and 45 nmol/g respectively were lower than those detected in the liver at the same time point. At the doses employed, the concentrations of SB-201076 found in the liver are high in relation to its K_i for ATP citrate-lyase ($1 \mu\text{M}$) and would be sufficient to inhibit this enzyme effectively.

Hypocholesterolaemic and hypolipidaemic effects of ATP citrate-lyase inhibition in the rat and dog

The hypocholesterolaemic and hypotriglyceridaemic effects of SB-204990 were subsequently determined in rats after its administration in the diet at various dose levels for 1 week (Table 2). Hepatic concentrations of SB-204990 ($60\text{--}103 \text{ nmol/g}$) were dose-related and again at such a level that effective inhibition of ATP citrate-lyase by SB-201076 would be expected (compare with the results above). Mass measurements of SB-201076 (in contrast with the radiolabelled assay used above) could not be accomplished owing to co-eluting material in HPLC. SB-204990 caused a significant and dose-related decrease in plasma cholesterol and triglyceride levels (by up to 46% and 80% respectively). The effects on plasma triglyceride levels were greater than on plasma cholesterol levels. To investigate the mechanism of the hypolipidaemic action of SB-204990, rates of VLDL synthesis were determined by measuring the increase in plasma triglyceride levels after the intravenous injection of Triton WR-1339 [20]. The VLDL synthesis rate recorded for the controls, 195 mg/h per dl , was similar to those previously published [23,24]. SB-204990 significantly decreased the rate of VLDL

Table 2 Effect of SB-204990 on VLDL synthesis and plasma lipid levels in rats

Male rats were fed with a standard RM1 diet supplemented with SB-204990 at the indicated dose levels for 1 week. Food intake and body weights were recorded daily and compound intakes per day were calculated. The effects of SB-204990 on plasma cholesterol levels, plasma triglyceride levels and VLDL synthesis were determined as described in the Materials and methods section. Results are means $\pm$ S.D. ($n = 6$). Significant changes were determined by two-way (treatment, day) analysis of variance, followed by pairwise t tests of each treatment group against the control. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Treatment	Compound intake ($\mu\text{mol/day per kg}$)	Food intake (g/week)	SB-204990 in liver (nmol/g)	Plasma lipids		
				Cholesterol (mg/dl)	Triglyceride (mg/dl)	VLDL synthesis (mg/h per dl)
Control	0	173 $\pm$ 15	—	78 $\pm$ 11	105 $\pm$ 27	195 $\pm$ 36
SB-204990 (0.05%, w/w)	156 $\pm$ 13	177 $\pm$ 22	60 $\pm$ 13	70 $\pm$ 13	52 $\pm$ 14***	121 $\pm$ 37**
SB-204990 (0.125%, w/w)	382 $\pm$ 27	172 $\pm$ 6	90 $\pm$ 13	68 $\pm$ 9	35 $\pm$ 11***	101 $\pm$ 39***
SB-204990 (0.25%, w/w)	729 $\pm$ 74	160 $\pm$ 6	103 $\pm$ 22	42 $\pm$ 12***	21 $\pm$ 6***	116 $\pm$ 35**

Figure 4 Hypocholesterolaemic and hypotriglyceridaemic effects of SB-204990 in the dog

Male beagle dogs were fed with a standard pelleted diet and received SB-204990 (10 mg/kg per day for 7 days, followed by 25 mg/kg per day for an additional 15 days) administered orally once a day (09:15h). Blood samples were taken at 09:00h. Results are means $\pm$ S.D. and expressed as a percentage of the control plasma cholesterol or triglyceride concentration immediately before dosing (day 13) for each dog ($n = 4$). Control plasma cholesterol and plasma triglyceride levels were 135 mg/dl and 29 mg/dl respectively. Data were analysed by analysis of variance, taking account of effects due to treatment, day, animal and body weight. Comparisons with the average of the final two pre-dose values were used to determine significant changes due to treatment; * $P \leq 0.05$.

Table 3 Effect of SB-204990 on plasma lipoprotein fractions in the dog

Experimental details and data analysis were as described in the legend to Figure 4. Plasma VLDL, LDL and HDL fractions were isolated from samples taken in the morning 1 and 4 days before dosing (control) and on the last day of dosing (SB-204990) as described in the Materials and methods section. The cholesterol contents of these fractions are expressed as means $\pm$ S.D. *Significant changes from control, $P \leq 0.05$.

Fraction	Cholesterol (mg/dl)		Change (%)
	Control	SB-204990	
VLDL	1.4 $\pm$ 0.1	1.1 $\pm$ 0.5	—21
LDL	9.5 $\pm$ 3.5	5.7 $\pm$ 2.1*	—40
HDL	148 $\pm$ 12	115 $\pm$ 11*	—22

synthesis at all dose levels tested [by up to 48% at the 0.125% (w/w) dose level; Table 2] although no clear dose-response relationship was evident, suggesting that maximal inhibition of VLDL synthesis was already accomplished at the 0.05% (w/w) dosing level. Food intake was not significantly affected by the treatment.

The effects of SB-204990 on plasma lipids and lipoproteins were also tested in the dog, because the plasma cholesterol levels in this animal model are particularly sensitive to the effects of cholesterol synthesis inhibitors. SB-204990, administered at 25 mg/kg for 15 days (after a 7-day starting period at a dose of 10 mg/kg), caused a significant and sustained decrease in fasting plasma cholesterol and triglyceride levels of up to 23% and 38% respectively (Figure 4). Similar decreases were seen in post-absorptive plasma samples taken in the afternoon (results not shown). Once the treatment had stopped, plasma cholesterol and triglyceride levels quickly returned to pretreatment levels.

To investigate which lipoprotein fractions accounted for the hypolipidaemic effects of SB-204990, plasma samples taken from dogs before and during dosing were fractionated and VLDL, LDL and HDL cholesterol contents were measured. SB-204990 significantly decreased cholesterol levels in the LDL and HDL fractions, the effects on LDL being proportionately the greatest (Table 3).

DISCUSSION

The ATP-driven conversion of citrate and CoA into oxaloacetate and acetyl-CoA by ATP citrate-lyase provides a major source of

acetyl-CoA units within the cytosolic compartment of mammalian cells. Because acetyl-CoA is the basic precursor for the synthesis of both sterols and fatty acids, inhibition of ATP citrate-lyase should decrease both cholesterol and fatty acid synthesis [5–8]. ATP citrate-lyase therefore represents a potential target for hypolipidaemic intervention, particularly because inhibitors of either cholesterol or fatty acid synthesis have hypolipidaemic properties [9,10,25]. The importance of the cholesterol synthesis pathway in controlling plasma lipid levels has been demonstrated clearly with the statins. These compounds are potent inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, a key regulator in the mammalian cholesterol synthesis, and their hypocholesterolaemic activity results from an increase in hepatic LDL receptor numbers consequent on the inhibition of cholesterol synthesis [26]. Inhibitors of fatty acid synthesis such as 5-tetradecyloxy-2-furancarboxylic acid ('TOFA') and 4-amino-5-ethyl-3-thiophenecarboxylic acid methyl ester hydrochloride (RO-22-0654) decrease plasma triglyceride levels, at least in part, through their ability to inhibit hepatic VLDL synthesis and output [27–30]. The inhibition of ATP citrate-lyase is thus expected to result in combined hypocholesterolaemic and hypotriglyceridaemic effects through its effects on the synthesis of both cholesterol and fatty acids. Furthermore the inhibition of ATP citrate-lyase might be particularly effective in lowering plasma LDL levels, first by decreasing hepatic VLDL output (VLDL being the precursor for LDL) and secondly by increasing hepatic LDL receptor levels.

We have previously demonstrated that inhibition of cholesterol synthesis in Hep G2 cells with (–)-hydroxycitrate, a potent inhibitor of ATP citrate-lyase, results in an increase in LDL receptor activity in these cells [10]. Here we have confirmed that (–)-hydroxycitrate caused a dose-related inhibition of both cholesterol and fatty acid synthesis in Hep G2 cells, but with an IC_{50} approx. 1000-fold greater than its K_i for ATP citrate-lyase, reflecting the poor cellular access of this compound previously recognized by Sullivan et al. [9] in their studies in rat hepatocytes. Following on from our previously published work on the design and synthesis of inhibitors of ATP citrate-lyase [12], we have now identified SB-201076 as a potent inhibitor of this enzyme ($K_i = 1 \mu\text{M}$ for both rat liver ATP citrate-lyase and recombinant human ATP citrate-lyase). However, SB-201076 was unable to inhibit the synthesis of either cholesterol or fatty acids when incubated with Hep G2 cells at concentrations up to 1 mM, indicating that it has poor access to its site of action in cells. In marked contrast, SB-204990, the γ -lactone of SB-201076, strongly inhibited both cholesterol synthesis and fatty acid synthesis in Hep G2 cells (up to 91 % and 82 % respectively at 30 μM) for at least 16 h. Because SB-204990 does not inhibit ATP citrate-lyase directly, these results indicate strongly that SB-204990 is absorbed by the hepatocytes and hydrolysed to the potent ATP citrate-lyase inhibitor SB-201076 within these cells. We therefore hypothesized that SB-204990 acts as a prodrug for SB-201076. SB-204990 was also approx. 30-fold more potent than hydroxycitrate at decreasing the synthesis of cholesterol and fatty acids in Hep G2 cells, thus providing a useful tool for studying the hypolipidaemic effects of ATP citrate-lyase inhibitors *in vivo*.

The rat was chosen as the initial animal model on which to test the hypolipidaemic properties of SB-204990 because it has been used successfully to measure the hypotriglyceridaemic activity of several hypolipidaemic agents. SB-204990, administered via the intravenous route, inhibited both hepatic cholesterol and fatty acid synthesis in the rat, confirming the potential utility of this compound in the study of the hypolipidaemic effects of ATP citrate-lyase inhibition *in vivo*. The bioavailability of SB-204990

and SB-201076 were established subsequently. When administered orally, SB-204990 was absorbed into the systemic circulation and concentrated into the liver, where substantial amounts of both SB-204990 and SB-201076 were detected. These results confirmed that SB-204990 acts as a prodrug for SB-201076 and delivers it to its site of action. In addition, sufficient concentrations of SB-201076 were achieved in the liver, relative to its K_i for ATP citrate-lyase, to effectively inhibit ATP citrate-lyase enzyme activity.

The hypolipidaemic studies with SB-204990 in the rat showed that the oral administration of SB-204990 via the diet for 1 week caused a significant decrease in the levels of both cholesterol and triglycerides in the plasma. These results are in agreement with the ability of (–)-hydroxycitrate to decrease cholesterol and triglyceride levels in the serum in rats fed with a high-carbohydrate diet and serum triglyceride levels in various hypertriglyceridaemic rat models [9], although higher doses (1.63–2.63 mmol/kg) of (–)-hydroxycitrate were used to elicit these effects. SB-204990 also significantly decreased VLDL synthesis by up to 48 %. This result, in addition to the observed decrease in plasma triglyceride levels by (–)-hydroxycitrate in Triton WR-1339-treated rats [9], is consistent with the hypothesis that the inhibition of ATP citrate-lyase results in a decrease in plasma triglyceride levels, at least in part by inhibiting VLDL secretion. The relative importance of the cholesterol and fatty acid synthesis pathways in influencing VLDL output after ATP citrate-lyase inhibition remains to be elucidated. The cholesterol synthesis pathway might well be involved, because the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor lovastatin has also been demonstrated to decrease hepatic VLDL synthesis in the rat [21,31,32]. Khan et al. [21] hypothesized that the decrease in VLDL synthesis caused by lovastatin resulted from a decrease in hepatic cholesterol esters, subsequent to a decrease in cholesterol synthesis. SB-204990 could therefore reduce VLDL synthesis in rats, at least in part, by the same mechanism as lovastatin. However, the additional effects of SB-204990 on fatty acid synthesis should also contribute to its inhibition of VLDL synthesis and hypotriglyceridaemic effects because rates of fatty acid synthesis *de novo* have been closely linked to rates of VLDL secretion, even though the proportion of newly synthesized fatty acids incorporated into VLDL is low compared with those derived from dietary fats and stored triglyceride [23,33–35]. The reason for this might be related to the expected decrease in malonyl-CoA levels, a key intermediate in the fatty acid synthesis pathway, as fatty acid synthesis is inhibited upstream at the step of ATP citrate-lyase. Because malonyl-CoA negatively regulates the entry of fatty acids into the mitochondria for β -oxidation, via the inhibition of carnitine palmitoyltransferase 1 [36], a decrease in malonyl-CoA levels would be expected to redirect fatty acids into β -oxidation rather than VLDL synthesis. Support for this hypothesis comes from the decrease in hepatic VLDL output and the activation of ketogenesis by the fatty acid synthesis inhibitor RO-22-0654 and the ability of (–)-hydroxycitrate to stimulate the oxidation of palmitate and decrease its esterification in pancreatic islets [30,37].

The hypolipidaemic effects of inhibiting ATP citrate-lyase might be caused not only by an inhibition of VLDL synthesis but also by an up-regulation of hepatic LDL receptor activity as a result of an inhibition of cholesterol synthesis. The dog has been used successfully as an animal model to test the hypocholesterolaemic activity of many cholesterol synthesis inhibitors that act by increasing LDL receptor expression [22,38–40]. Here we have demonstrated that SB-204990 (25 mg/kg per day) decreased plasma cholesterol levels and plasma triglyceride levels in the dog and that the effect was maintained throughout the treatment

period. Analysis of the plasma lipoprotein fractions in dogs treated with SB-204990 showed that SB-204990 caused a proportionately greater decrease in LDL cholesterol levels than in HDL cholesterol levels. These results are again consistent with the hypothesis that the inhibition of ATP citrate-lyase leads to a decrease in plasma lipid levels, although the exact contribution of VLDL synthesis and LDL receptor activity to the hypo-lipidaemic effect of SB-204990 in the dog remains to be determined.

Overall the results obtained here with SB-204990, and those previously published for hydroxycitrate, are consistent with the hypothesis that inhibition of ATP citrate-lyase will result in hypocholesterolaemic and hypotriglyceridaemic effects and confirms the potential of this enzyme as a therapeutic target.

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