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RP 64477: A Potent Inhibitor of Acyl-coenzyme A

CHOLESTEROL O-ACYLTRANSFERASE WITH LOW SYSTEMIC BIOAVAILABILITY

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ABSTRACT. RP 64477 (N-butyl-3-(p-decyloxybenzamido)-4-(methylthio)benzamide) has been shown to be a potent inhibitor of the cholesterol esterifying enzyme Acyl-coenzyme A:cholesterol O-acyltransferase (EC 2.3.1.26; ACAT) in intestinal, hepatic, adrenal, and arterial tissue preparations obtained from a range of animal species. Drug concentrations producing 50% inhibition of enzyme activity (IC_{50} values) ranged from 14–283 nM. Inhibition by RP 64477 in a rabbit intestinal enzyme preparation was shown to be non-competitive with respect to the substrate oleoyl-CoA. In whole cell assays using human intestinal (CaCo-2), hepatic (HepG2) and monocytic (THP-1) cell lines, RP 64477 inhibited ACAT activity with IC_{50}s of 113, 503, and 180 nM, respectively. RP 64477 (0.03% w/w by diet) reduced significantly cholesterol absorption in cholesterol/cholic acid-fed rats from 94 ± 8% to 65 ± 4%. In cholesterol-fed rabbits, cholesterol absorption was reduced from 72 ± 5% to 50 ± 5% and 44 ± 5% at dose levels of 10 and 30 mg kg^{-1} b.i.d., respectively. Plasma cholesterol levels were reduced dose-dependently in both cholesterol/cholic-acid-fed rats and cholesterol-fed rabbits. Neither cholesterol absorption nor plasma cholesterol levels were reduced significantly in animals maintained on standard laboratory diets. Pharmacokinetic studies indicated that RP 64477 was very poorly absorbed following oral administration to rats. Plasma levels of drug were <2 ng mL^{-1} following a dose of 2000 mg kg^{-1} p.o.. When radiolabelled RP 64477 was administered orally, limited absorption was indicated by the overwhelming elimination of radioactivity in the faeces (96.4% of administered material) coupled with low renal clearance (0.6% of dose) and biliary excretion (0.05% of dose). In conclusion, this work shows that RP 64477 is a potent inhibitor of ACAT obtained from a range of animal species and man. Inhibition of cholesterol absorption and hypocholesterolaemic activity has been demonstrated in rats and rabbits maintained on diets supplemented with cholesterol. Pharmacokinetic studies indicate low systemic exposure to RP 64477 as a result of limited absorption of this drug. BIOCHEM PHARMACOL 51;4:413–421, 1996.

KEY WORDS. ACAT; cholesterol; absorption; hypocholesterolaemic; bioavailability; RP 64477

Hypercholesterolaemia is firmly established as a primary risk factor for the development of coronary heart disease in man. Furthermore, lowering of plasma LDL† cholesterol levels by both dietary and pharmacological manipulation has been shown to result in reduced cardiovascular morbidity and mortality [1–3]. Bile acid sequestrants are often used as first-line therapy for moderate hypercholesterolaemia because of their low potential for systemic toxicity and, also, in combination with inhibitors of cholesterol biosynthesis when administration of such agents alone fails to bring plasma LDL cholesterol levels into the normal range. Bile acid sequestrants have the disadvantages of requiring multigram dose regimens, being extremely unpalatable, and of interfering with the absorption of vitamins and other drugs [4].

An alternative approach to eliciting hypocholesterolaemic responses *via* an intestinal site of drug action, is to inhibit the absorption of cholesterol of both dietary and biliary origin. Cholesterol absorption efficiency has been reported to regulate plasma cholesterol levels in man [5] and reports of clinical studies with plant sterols, which are known inhibitors of this process, have shown marked hypocholesterolaemic responses [6]. Like bile acid sequesterants, plant sterols also have to be given in large amounts and are associated with a high incidence of gastrointestinal disturbances. Development of a drug that inhibits a specific molecular mechanism which is an integral part of cholesterol absorption has been an area of interest to the pharmaceutical industry for a number of years. In this regard, much attention has focused on developing inhibitors of the cellular cholesterol esterifying enzyme ACAT (EC 2.3.1.26). The bulk of free cholesterol absorbed from the gas-

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† *Abbreviations:* ACAT, acyl-coenzyme A: cholesterol O-acyltransferase; LDL, low density lipoprotein; DMSO, dimethyl sulphoxide; PCEH, pancreatic cholesterol ester hydrolase; LCAT, lecithin: cholesterol acyltransferase.

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trointestinal tract appears in esterified form in lymph chylomicrons [7]. ACAT has been proposed as the principal mediator of this esterification step [8] which, with the use of enzyme inhibitors, has been shown to be rate-limiting for cholesterol absorption under certain experimental conditions [9–13]. This paper describes pharmacological activities of RP 64477 [N-butyl-3-(p-decyloxybenzamido)-4-(methylthio)benzamide, Fig. 1] *in vitro* and in rats and rabbits *in vivo*. Pharmacokinetic properties of RP 64477 in rats are also reported.

MATERIALS AND METHODS

Materials

RP 64477 and the internal standard for hplc, RP 68574 [N-butyl-3-(p-dodecyloxybenzamido)-4-(methylthio) benzamide)] were synthesised by the Discovery Chemistry Department, Dagenham Research Centre, Rhône-Poulenc Rorer, Ltd. [^{14}C] RP 64477 labelled in the carbonyl position, of specific activity 1.11 mCi mmol^{-1} and radiochemical purity 97.9% was synthesized by the Radiochemistry Section, Dagenham Research Centre, Rhône-Poulenc Rorer, Ltd.; $CaCo_2$, HepG2, and THP-1 cells were purchased from the ECCAC, Porton Down, U.K. Plasma cholesterol concentrations were determined enzymatically using standard assay kits (Boehringer Mannheim, Lewis, U.K.). Native and acetylated human LDLs were purchased from Organon Teknika (Rockville, MD).

Animals and Diets

Male Sprague-Dawley rats weighing approximately 200 g were obtained from Charles River U.K. Ltd. (Margate, U.K.). Rats were fed standard rat diet (ERD, SDS, Witham, U.K.) in powdered or pelleted formulation as indicated. Cholesterol-fed rats received powdered diet supplemented with 0.5% w/w cholesterol and 0.25% w/w cholic acid. Male Golden Syrian hamsters weighing 90–110 g were obtained from Bantin and Kingman Ltd. (Hull, U.K.) and received powdered diet supplemented with cholesterol and cholic acid as indicated for rats. Male New Zealand White and Froxfield Heritable Hyperlipidaemic rabbits (2–3 kg bodyweight) were obtained from Froxfield Rabbits Ltd. (Petersfield, U.K.). Rabbits received Labsure R14 diet supplemented with 0.5% w/w cholesterol where indicated. Male marmosets were obtained from a colony bred in-house at the Dagenham Research Centre and were fed CPDX diet (SDS) supplemented with 12% w/w lard and 0.75% w/w cholesterol. Male Yucatan minipigs were supplied by Charles River U.K. Ltd. and fed Sow and Weaner diet (SDS) containing 10% w/w beef suet, 2% w/w cholesterol and 1% w/w cholic acid.

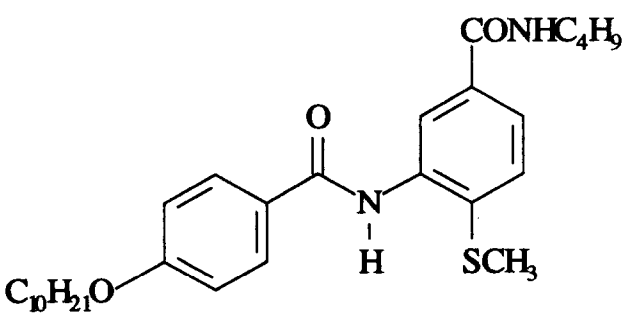

FIG. 1. Chemical structure of RP 64477, N-butyl-3-(p-decyloxybenzamido)-4-(methylthio) benzamide.

Tissue Preparation and ACAT Assays

Microsomes were prepared from liver, arterial, and adrenal tissue obtained from rats, rabbits, hamsters, pigs, and marmosets using standard methodology [14]. Enterocyte homogenates were prepared from cells isolated from the jejunum of cholesterol-fed rats, rabbits, and marmosets using methodology based on that described by Weisser [15]. Animals were maintained on cholesterol-containing diets for the time periods indicated in Table 1 prior to obtaining tissue.

ACAT activity was measured according to the method of Lichenstein and Brecher [14] using [^{14}C] oleoyl-CoA and endogenous cholesterol as substrates. Approximately 60 μg of protein was added to 0.1 M sodium phosphate buffer, pH 7.4, containing bovine serum albumin (fatty acid-free) and dithiothreitol to produce a total volume of 170 μL. RP 64477, dissolved in 1 μL of DMSO, was added and, after a 5-min preincubation at 37°C, the reaction was started by the addition of 30 μL of assay buffer containing 18 nmol of [^{14}C] oleoyl-CoA (8.3 nCi nmol^{-1}). Final concentrations in the assay medium were 90 μM oleoyl-CoA, 90 μM albumin and 2 mM dithiothreitol. The reaction was stopped after 5 minutes by the addition of 4 mL of dichloromethane: methanol (2:1 v/v) containing 10 μg mL^{-1} cold cholesterol oleate. Tritiated cholesterol oleate in dichloromethane was added to determine recovery. Cholesterol ester extraction was carried out using methodology originally described by Folch *et al.* [16]. Choles-

TABLE 1. Inhibitory potencies of RP 64477 in animal tissue preparations and human cell lines

Species	No. of days on cholesterol diet	Tissue/Cell	IC_{50} (nM)
Rat	5	Liver	194 (1)
		Intestine	89 (5)
Rabbit	35	Liver	20 (6)
		Intestine	32 (6)
		Adrenal	21 (2)
		Artery	14 (6)
Rabbit (FHHL)	0	Intestine	60 (1)
		Artery	6 (1)
Marmoset	14	Liver	136 (3)
		Intestine	76 (3)
		Adrenal	57 (4)
Pig	36	Liver	283 (3)
Hamster	7	Liver	42 (2)
Human	NA	CaCo-2	113 (4)
		HepG2	503 (4)
		THP-1	180 (4)

Preparation of tissues and experimental procedures were carried out as described in the methods section. IC_{50} values were calculated from the line of best fit for inhibitory activity of RP 64477 at 5 concentrations (4 replicates at each concentration). Values are the mean of a number of separate experiments (indicated in brackets).

terol oleate was quantified by liquid scintillation counting following separation by TLC. Enzyme activity was calculated as pmol cholesterol oleate formed per mg protein per min. ACAT inhibitory activity was determined in quadruplicate at 5 concentrations. Fifty per cent inhibition (IC_{50}) values were calculated from the line of best fit for all data points.

ACAT Assays in Human Cell Lines

ACAT activity was determined in CaCo-2 cells using methodology similar to that described by Field *et al.* [17]. Cells cultured in 6-well plates were preincubated for 2 hr in 2 mL of Medium 199 supplemented with 10 mM Hepes, pH 7.4, and cholesterol-rich micelles (giving a final concentration of 150 μM cholesterol, 10 mM sodium taurocholate, 300 μM monoolein, and 50 μM phosphatidylcholine) in the presence or absence of RP 64477 that had been initially prepared in neat DMSO. The final concentration of DMSO in the culture medium (for all cell assays) was 0.2% v/v. Preincubation medium was then replaced with the same medium containing 50 μM [^{14}C] oleic acid (6.1 μCi μmol^{-1}) complexed with 17 μM bovine serum albumin (fatty acid-free) and cells incubated for a further 2 hr. RP 64477 or vehicle was present during both incubations. ACAT activity in HepG2 cells was determined using a procedure similar to that described by Havekes *et al.* [18]. Cells cultured in 6-well plates were incubated for 22 hr in 1 mL Dulbecco's Modified Eagles Medium supplemented with 1% bovine serum albumin (fatty acid-free) and 100 μg mL^{-1} of human LDL. ACAT activity was measured over the final 2 hr of the incubation by the addition of [^{14}C] oleic acid/albumin complex (to give a final concentration of 100 μM [^{14}C] oleic acid with a specific activity of 0.5 μCi μmol^{-1} and 164 μM albumin). RP 64477 was present throughout the 22-hr incubation.

ACAT activity was measured in THP-1 cells using a modification of the method of Via *et al.* [19]. Cells cultured in 6-well plates were first differentiated by incubation for 72 hr in RPMI 1640 medium supplemented with 10% foetal calf serum, 50 μM 2-mercaptoethanol and 80 nM phorbol 12,13 myristate. Cells were then incubated for 24 hr in fresh medium supplemented with 100 μg mL^{-1} of acetylated human LDL, and ACAT activity measured over the final 5 hr of the incubation by the addition of [^{14}C] oleic acid/albumin complex (to give a final concentration of 200 μM [^{14}C] oleic acid with a specific activity of 5 μCi μmol^{-1} and 100 μM albumin). RP 64477 was present for the final 6 hr of the incubation period. In all experiments, immediately following incubation cells were washed twice with phosphate buffered saline, left to dry overnight at 4°C, then dissolved in 0.5 mL of 0.5% SDS. Cholesterol esters were extracted using 20 volumes of chloroform: methanol (2:1 v/v) and ACAT activity and inhibitory potency of RP 64477 calculated as described for microsomal assays.

Assays of PCEH and LCAT Activity

Activity of RP 64477 against PCEH, and LCAT-mediated cholesterol esterification was measured by methods described previously [20, 21] using rat pancreas (PCEH) or plasma (LCAT) as the enzyme source.

Hypocholesterolaemic Activity of RP 64477 in Rats and Rabbits

Hypocholesterolaemic activity of RP 64477 was investigated by administering the drug (0.001%–0.03% w/w by diet) to rats maintained for 3 days on powdered laboratory diet supplemented with cholesterol/cholic acid. Animals were then killed by asphyxiation in carbon dioxide, and terminal blood samples taken by cardiac puncture into a heparinised syringe for preparation of plasma. Plasma cholesterol concentrations were determined enzymatically using standard assay kits. Hypocholesterolaemic activity of RP 64477 in rabbits was investigated by administering RP 64477 at doses of 1, 3, 10, and 30 mg kg^{-1} b.i.d. for 7 days to animals receiving standard laboratory diet supplemented with cholesterol. Drug suspensions were prepared in aqueous 1% w/v sodium carboxymethylcellulose/0.5% w/v Tween 80 and administered in a dose volume of 1 mL kg^{-1} by means of a rubber stomach tube. Blood samples were obtained from the central ear artery on days 0 (predosing), 4, and 7 of the study. Preparation of plasma and determination of plasma cholesterol concentrations were carried out as described for rats.

[^{14}C] Cholesterol Absorption in Rats and Rabbits

Cholesterol absorption was measured in rats and rabbits using dual isotope methodology originally described by Zilversmit [22]. Briefly, rats were fed standard diet or cholesterol/cholic acid-containing diet with or without RP 64477 at a level of 0.03% for 4 days. After 2 days of dosing, animals received an intravenous injection into the tail vein of 5 μCi of [^{3}H] cholesterol (46 μCi nmol^{-1}) as a colloidal suspension in 400 μL of 0.9% w/v sodium chloride/7.5% v/v ethanol followed by a gavage dose of 5 μCi of [^{14}C] cholesterol (50 nCi nmol^{-1}) as a colloidal suspension in 1 mL of distilled water containing 7.5% v/v ethanol. Animals were killed by asphyxiation in carbon dioxide 48 hr after isotope administration and terminal blood samples obtained by cardiac puncture. Plasma was prepared as described previously and radiolabelled sterol extracted from 250 μL samples following saponification. Percentage absorption of orally administered [^{14}C] cholesterol was determined using the equation:

$$\text{Absorption (\%)} = \frac{[^{14}\text{C}]\ \text{plasma dpm}/[^{14}\text{C}]\ \text{oral dose dpm} \times 100}{[^{3}\text{H}]\ \text{plasma dpm}/[^{3}\text{H}]\ \text{i.v. dose dpm}}$$

Rabbits were fed the cholesterol-containing diet for 9 days with or without concomitant administration of RP 64477 (10 and 30 mg kg^{-1} b.i.d.). On day 6 of drug administration, animals received oral [^{14}C] cholesterol and i.v. [^{3}H] cholesterol (*via* an ear vein) prepared as described for rats. Blood samples were obtained 72 hr after isotope administration and processed as described for rats.

Pharmacokinetics of Parent Compound

RP 64477, prepared as a 44.3% w/v suspension in aqueous 1% w/v sodium carboxymethylcellulose/0.5% w/v Tween 80, was administered by stomach intubation to groups of rats (N = 4) at a dose level of 2000 mg kg^{-1}. One group was sacrificed, predose, and the remainder at 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 hr postdose. Blood samples were obtained by cardiac puncture and plasma prepared and stored at −20°C until analysed for drug content.

Plasma levels of RP 64477 were determined following liquid-liquid extraction using a reverse phase HPLC procedure with UV detection. Prior to extraction, 300 ng of internal standard contained in 30 µL of acetonitrile was added to 1-mL aliquots of plasma and vortex mixed. Acetonitrile (500 µL) was then added, the samples mixed again, and n-hexane (10 mL/tube) added. The tubes were then mechanically shaken for 15 min before being centrifuged at 2000 × g for 10 min. The organic phase was then separated, evaporated to dryness, and the residue resuspended in acetonitrile (200 µL). Aliquots (150 µL) were injected onto the analytical column (Spherisorb ODS 2, 150 × 4.6 mm, 5 µm), which was maintained at 40°C. Chromatography was effected with a mobile phase that consisted of acetonitrile: aqueous 2 mM KH_2PO_4 (4:1 v/v) pumped at a flow rate of 1.5 mL min^{-1}. The eluent was monitored at 270 nm. The assay had a lower limit of accurate quantification of 10 ng mL^{-1} when 1 mL of plasma was used, and a limit of detection of 2 ng mL^{-1}. The method was linear over the range 10–500 ng mL^{-1}.

ADME of Radiolabelled Product

[^{14}C] RP 64477, formulated as a 2% w/v suspension in 1% w/v sodium carboxymethylcellulose/0.5% w/v Tween 80, was administered by stomach intubation to 4 groups of rats (N = 4) at a dose level of 100 mg kg^{-1}. Animals were transferred to glass metabolism cages (Jencons Ltd., Hemel Hempstead, U.K.) for the separate collection of urine and faeces (over solid CO_2). Excreta was collected up to the time of sacrifice for the 1, 6, and 24-hr groups and at 24-hr intervals for 7 days for the 168-hr group. In addition, for the 168-hr group, expired air was collected at 24-hr intervals up to the time of sacrifice. Tissues (see Table 4) were rapidly excised, pooled for each group, and the fresh weights determined. Gastrointestinal sections were initially flushed through with saline to remove the contents. Thereafter, each section was cut open, washed successively 3 times with fresh saline and, finally, dabbed dry with Whatman filter paper. All samples plus residual carcass and skin plus fur were stored at −20°C until required for analysis of radioactivity content.

[^{14}C] RP 64477 (100 mg kg^{-1} p.o.) was also administered to rats that had been implanted with a bile-duct cannula. Drug administration was carried out immediately following recovery from anesthesia. Animals were housed individually in glass metabolism cages, permitted to roam freely, and had access to Vamine nutrient solution (Kabivitrum Ltd, Uxbridge, U.K.). Bile was collected predose and for the following periods postdose: 0–3, 3–6, 6–12, and 12–24 hr. Urine and faeces (0–24-hr collections) were collected separately over solid CO_2. Animals were sacrificed 24 hr postdose, blood was removed by cardiac puncture and the gastrointestinal tract *in toto* and the liver excised. These samples plus the residual carcass, skin plus fur, and plasma were stored at −20°C until required for analysis of radioactivity content.

Radioactivity determinations were made using aliquots of urine, plasma, bile, and apparatus washes added directly to scintillant. Tissues were solubilised by incubation with Lumasolve (Lumac b.v., Holland) for 3 days at 37°C prior to counting. Aliquots of blood were solubilised with Lumasolve/isopropyl alcohol (1:2 v/v) under the above conditions and then bleached with H_2O_2. Skin plus fur were digested for 14 days at 37°C with 2 M KOH in industrial methylated spirit.

Statistical Analysis

Statistical comparisons were made using one- or two-way analysis of variance (ANOVAR) with Student's *t* tests to determine significant differences ($P < 0.05$) or by using Student's *t* tests for unpaired data as indicated.

RESULTS

Inhibitory Activity of RP 64477 in vitro

Inhibitory potencies of RP 64477 *in vitro* in tissue preparations obtained from a range of species and in human cell cultures are shown in Table 1. For animal tissues, IC_{50} values in the range 6–283 nM were recorded, with no obvious species/tissue differences apparent. Potent inhibitory activity was also recorded in human cell lines of hepatic (HepG2), intestinal (CaCo-2), and monocytic (THP-1) origin. IC_{50}s of 503, 113, and 180 nM, respectively, were recorded using the assay protocols described. The nature of this inhibitory activity was investigated further using rabbit enterocyte homogenates preincubated with RP 64477 (40 nM) or vehicle, and assayed in the presence of varying concentrations of [^{14}C] oleoyl-CoA. The increase in incorporation of substrate with respect to varying concentration demonstrated typical saturation kinetics (Fig. 2). A reciprocal plot of the data (inset, Fig. 2) suggests that RP 64477 is a noncompetitive inhibitor of ACAT with respect to oleoyl-CoA. No inhibitory activity was recorded against rat PCEH or LCAT at test concentrations up to 200 µM and 20 µM, respectively.

Hypocholesterolaemic Activity of RP 64477 in Rats and Rabbits

Rats fed cholesterol/cholic acid-supplemented diets exhibited plasma cholesterol levels approximately 3-fold higher than those recorded in animals receiving the basal diet (5.9 ± 0.5 *versus* 2.1 ± 0.1 mM for cholesterol diet-fed and basal diet-fed, respectively). Administration of RP 64477 (0.01% and 0.03% w/w by diet) reduced significantly plasma cholesterol levels in cholesterol/cholic acid-fed rats by 29% and 61%, respectively (Fig. 3). Food consumption was not affected by dietary incor-

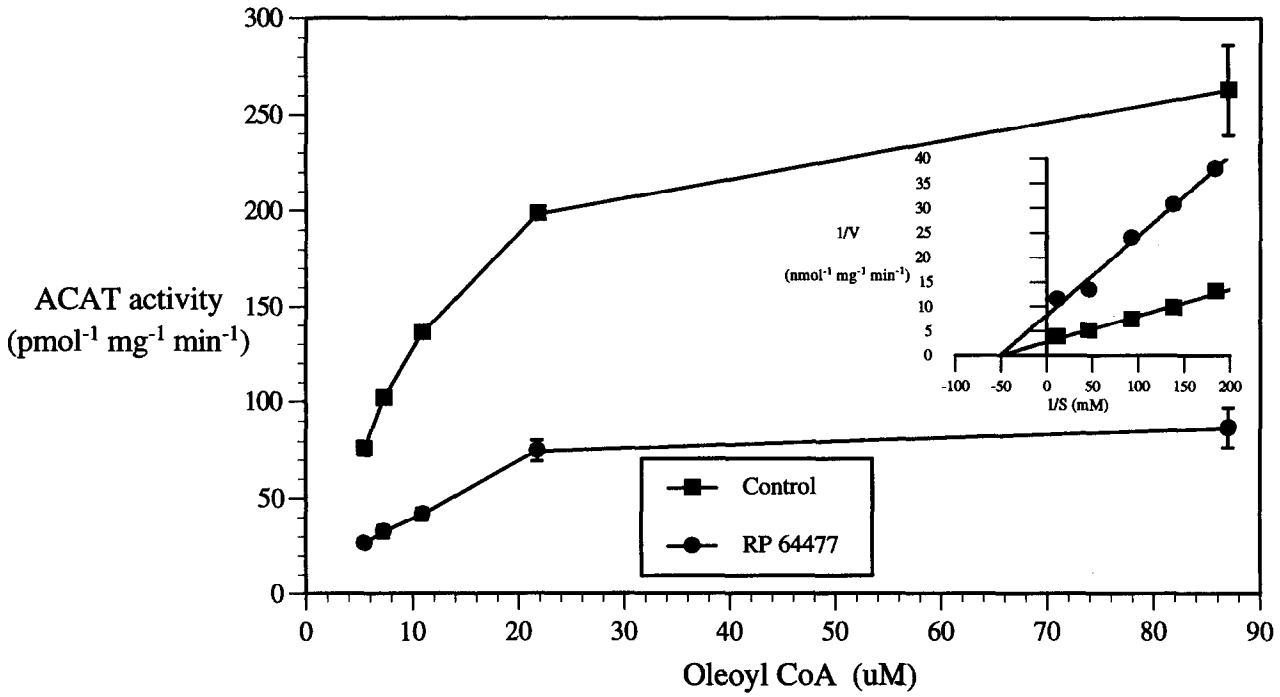

FIG. 2. ACAT activity in homogenates prepared from rabbit enterocytes measured in the presence of varying concentrations of oleoyl-CoA. Homogenates were incubated with 40 nM RP 64477 for 5 min prior to the addition of oleoyl-CoA.

poration of RP 64477. Incorporation at levels of 0.001, 0.003, 0.01, and 0.03% corresponded to daily drug intakes of 1, 3, 9, and 26 mg kg^{-1} day^{-1}, respectively.

Feeding of a cholesterol-enriched diet to rabbits resulted in an approximately 7-fold increase in plasma cholesterol levels after 4 days, rising to a 13-fold increase after 7 days. Animals receiving RP 64477 (10 and 30 mg kg^{-1} b.i.d.) over this period exhibited significantly lower plasma cholesterol levels on both days 4 and 7 when compared to values recorded from vehicle-treated animals fed the cholesterol-containing diet (Fig. 4). Compared to cholesterol-fed controls, after 7 days of dosing, plasma cholesterol levels were 35% and 53% lower in animals receiving 10 and 30 mg kg^{-1} b.i.d. doses, respectively, of RP 64477.

[^{14}C] Cholesterol Absorption in Rats and Rabbits

Results shown in Fig. 5 indicate that RP 64477 did not significantly affect cholesterol absorption when administered to normocholesterolaemic rats for 4 days at a dose level of 0.03% w/w by diet or to normocholesterolaemic rabbits for 9 days at a dose level of 30 mg kg^{-1} b.i.d. The effects of RP 64477 on [^{14}C] cholesterol absorption were also studied in animals rendered hypercholesterolaemic by cholesterol/cholic acid (rats) or cholesterol (rabbits) feeding. The percentage of [^{14}C] cholesterol absorbed appeared to be higher in animals fed cholesterol-containing diets than in animals fed standard laboratory diets (94 vs 67% in rats, $P < 0.05$ by unpaired student's t test; 72 vs 54% in rabbits, NS by unpaired student's t test). RP 64477 significantly reduced cholesterol absorption at a dose level of 0.03% w/w by diet in cholesterol/cholic acid-fed rats and at 10 and 30 mg kg^{-1} b.i.d. in rabbits. Levels of cholesterol absorption recorded in treated rats and rabbits were similar to those observed in untreated normocholesterolaemic animals of the respective species.

RP 64477 Plasma Pharmacokinetics

Following oral administration to rats of a single dose of 2000 mg kg^{-1} (the maximum dose that could be formulated for administration by this route), drug levels in plasma were generally not detectable (<2 ng mL^{-1}) and always below the limit of accurate quantification (10 ng mL^{-1}). These data were indicative of minimal systemic exposure to RP 64477.

Excretion of Total Radioactivity

Limited absorption of orally administered [^{14}C] RP 64477 (100 mg kg^{-1}) in rats was indicated by the overwhelming elimination of administered radioactivity in faeces (96.4% of the dose in 1 week, Table 2), coupled to low renal clearance (0.6% of the dose in 1 week, Table 2) and biliary excretion (0.05% of the dose in 24 hr, Table 3). In addition, nonquantifiable levels of radioactivity were determined in expired air, indicating the stability of the ^{14}C label in the molecule to metabolic degradation.

Tissue Distribution of Total Radioactivity

Low systemic exposure to drug-derived radioactivity, the highest level observed being 0.11 μg equivalents mL^{-1} 1 hr post-dose, was further emphasized by generally limited tissue pen-

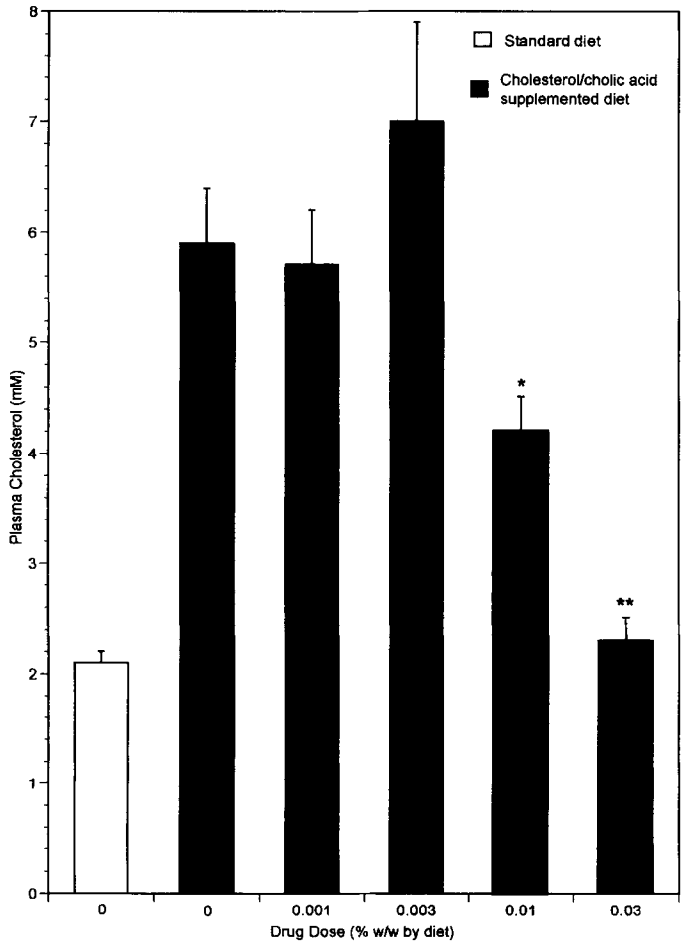

FIG. 3. Hypocholesterolaemic activity of RP 64477 in cholesterol/cholic acid-fed rats. Groups of animals (N = 8) received standard diet or diet supplemented with 0.5% w/w cholesterol/ 0.25% w/w cholic acid and RP 64477 at the doses indicated for 3 days. Statistical comparisons were made using two-way analysis of variance (ANOVAR) with Student's *t*-tests to determine significant differences. Results are expressed as mean ± SEM. ** = $P < 0.01$, *$P < 0.05$ for RP 64477-treated animals compared to cholesterol-fed controls.

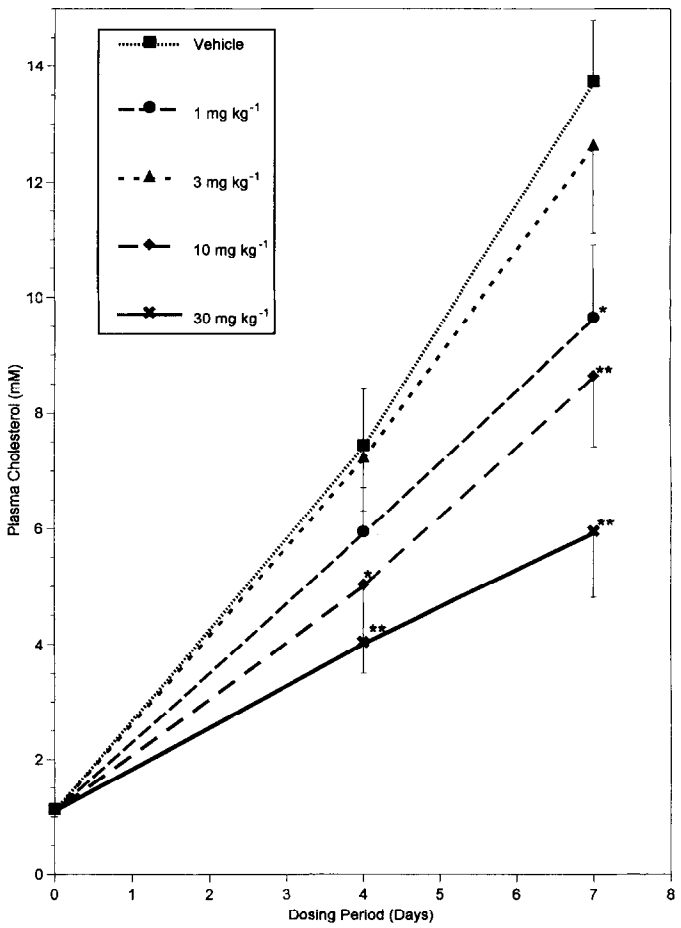

FIG. 4. Hypocholesterolaemic activity of RP 64477 in cholesterol-fed rabbits. All animals (N = 10 per group) received chow supplemented with 0.5% w/w cholesterol from day 0. Animals received vehicle or RP 64477 b.i.d. by stomach intubation at the doses indicated. Statistical comparisons were made using two-way analysis of variance (ANOVAR) with Student's *t*-tests to determine significant differences. Results are expressed as mean ± SEM.
**** = $P < 0.01$, * = $P < 0.05$ for RP 64477-treated animals compared to vehicle-treated, cholesterol-fed animals on the same day.**

etration (Table 4). Radiolabelled drug-derived material was largely confined to the gastrointestinal tract contents prior to elimination in faeces. However, low levels were determined in excretory tissues, the lung, and adipose and epididymal fat. The nature of plasma radioactivity was not investigated further owing to the low and variable levels detected. In view of the fact that RP 64477 could not be quantified in plasma following an oral dose of 2000 mg kg^{-1}, it is likely that systemic radioactivity recorded following administration of [^{14}C] RP 64477 (100 mg kg^{-1} p.o.) is attributable largely to low levels of metabolites. Radioactivity was cleared from all tissues by 168 hr postdose.

DISCUSSION

The data presented in this report demonstrate RP 64477 to be a potent inhibitor of the enzyme ACAT in intestinal, hepatic, adrenal, and arterial tissue prepared from a number of species and also in the human hepatic, intestinal, and monocytic cell lines HepG2, CaCo-2, and THP-1. Kinetic studies performed with rabbit enterocyte homogenates indicated that RP 64477 acts noncompetitively with respect to the substrate oleoyl-CoA. No inhibitory activity against the cholesterol esterifying enzymes LCAT and PCEH could be detected.

RP 64477 inhibited cholesterol absorption and also rises in plasma cholesterol levels in both rats and rabbits fed cholesterol-containing diets. Experiments in which RP 64477 was administered to animals with pre-established dietary-induced hyperlipidaemia showed hypocholesterolaemic activity of this drug over similar dose ranges to those described in this report (data not shown). These results are in accord with the hypothesis that for cholesterol to be absorbed, an esterification step mediated by ACAT is a prerequisite. However, RP 64477 did not inhibit cholesterol absorption nor influence choles-

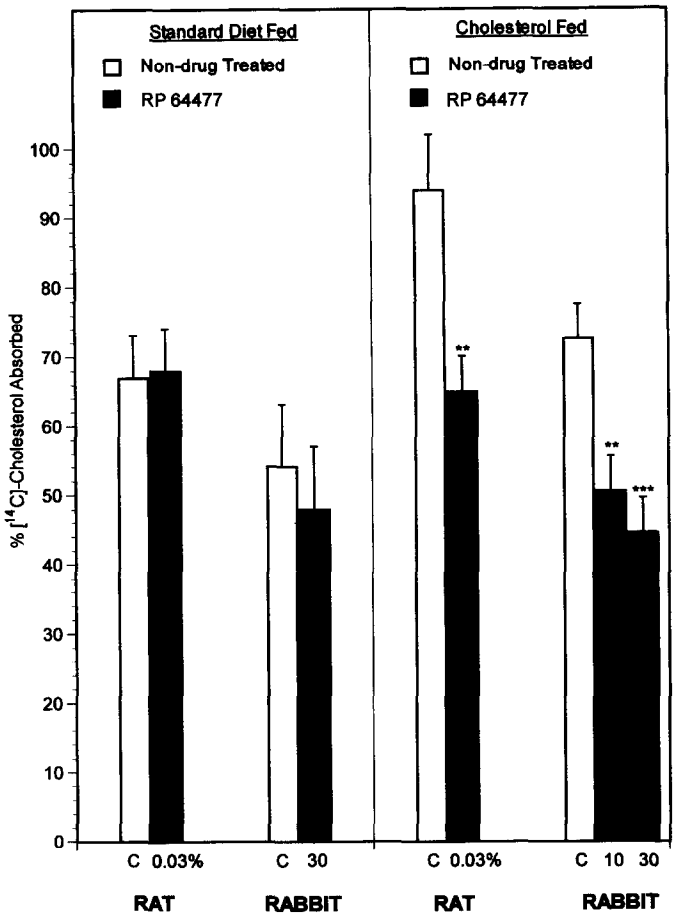

FIG. 5. The effect of RP 64477 on [^{14}C] cholesterol absorption in rats and rabbits fed standard or cholesterol-supplemented diets. RP 64477 was administered to rats by dietary incorporation (0.03% w/w) and to rabbits by stomach intubation (10 or 30 mg kg^{-1} b.i.d.). Cholesterol absorption was quantitated using the dual isotope technique as described in the Methods section. Results are expressed as mean ± SEM (N = 10 for each group except cholesterol-fed rat control group where N = 9). Statistical comparisons were made using one way analysis of variance (ANOVAR) with Student's *t*-tests to determine significant differences.
***** = $P < 0.0001$, ** = $P < 0.01$, * = $P < 0.05$ for RP 64477-treated animals compared to respective non-drug-treated controls.**

terol levels in animals maintained on standard laboratory diets. These findings are consistent with those of Heider *et al.* [12] who reported using dual isotope methodology for the measurement of cholesterol absorption, that the fatty acid amide, Sandoz compound 57-118, only inhibited cholesterol absorption and elicited hypocholesterolaemic responses in rabbits fed cholesterol-containing diets. Two major explanations can be offered for these findings. First, ACAT may only play a regulatory role in mediating cholesterol absorption in animals fed high-cholesterol diets. However, there are reports in the research literature that describe inhibitory effects of ACAT inhibitors on cholesterol absorption in noncholesterol-fed, lymph-cannulated rats [11, 13]. The second possibility is that insufficient inhibition of ACAT was achieved with the dose regimens employed in this study (and also that of Heider *et al.*)

TABLE 2. Daily excretion of radioactivity in urine and faeces of rats after a single oral dose of [^{14}C] RP 64477 (100 mg/kg^{-1})

	% Recovery	
Time	Faeces	Urine
0–24 hr	67.39	0.25
24–48 hr	22.01	0.29
48–72 hr	6.11	0.07
72–96 hr	0.73	0.01
96–120 hr	0.13	<0.01
120–144 hr	0.03	<0.01
144–168 hr	0.01	<0.01
Total 0–168 hr	96.41	0.62

Radioactivity was not quantifiable in 24-hr collections of expired air.

to influence cholesterol absorption in noncholesterol-fed animals. It has been claimed that only about 30% of ACAT activity is required to mediate cholesterol absorption in animals fed standard laboratory diets, whereas in the cholesterol-fed state more ACAT is employed in mediating absorption of a larger cholesterol load [23]. Consequently, the lower the intestinal cholesterol load, the greater the inhibition of ACAT required to attenuate cholesterol absorption. Despite the fact that inhibition of cholesterol absorption has not been demonstrated for RP 64477 nor Sandoz 57-118 in normal-fed animals, this latter hypothesis still provides perhaps the most plausible explanation for the conflicting research literature in this area. The effects of administration of higher doses of RP 64477 to normal-fed animals have not been explored. However, recent studies with the ACAT inhibitor RP 73163 [24] have shown that repeat administration of high doses of this compound results in an inhibition of cholesterol absorption in normal-fed rats (U.M. Moore and D. Riddell, unpublished data). Insufficient inhibition of intestinal ACAT may well underlie the reported failure of the ACAT inhibitor CL 277082, a trisubstituted urea, to significantly influence plasma cholesterol levels in human subjects [25].

TABLE 3. Summary of drug-derived radioactivity in postadministration bile, urine, skin, faeces, carcass, liver, gastrointestinal (GI) tract, and washes of rats after a single oral dose of [^{14}C] RP 64477 (100 mg kg^{-1})

	% of Administered dose			
Sample	Rat 1	Rat 2	Rat 3	Mean
Bile 0–3 hr	0.01	0.00	0.00	0.00
Bile 3–6 hr	0.01	0.00	0.00	0.00
Bile 6–12 hr	0.01	0.00	0.01	0.01
Bile 12–24 hr	0.04	0.04	0.04	0.04
Bile Total	0.07	0.04	0.05	0.05
Urine	0.06	0.02	0.06	0.05
Faeces	16.12	0.98	36.09	17.73
Skin	0.02	0.10	0.02	0.05
Carcass	0.29	0.31	0.18	0.26
Liver	0.00	0.01	0.01	0.01
G.I. Tract	82.66	95.85	64.52	81.01
G.I. Washes	0.03	0.15	0.02	0.07
Grand Total	99.25	97.46	100.95	99.22

TABLE 4. Distribution of radioactivity (expressed as μg drug equivalents g^{-1} tissue) in rats following single oral administration of [^{14}C] RP 64477 (100 mg kg^{-1})

Tissue	Time in hours			
	1 hr	6 hr	24 hr	168 hr
Plasma	0.11	–	0.07	–
Adrenal	–	–	–	–
Bladder	0.46	0.39	7.12	–
Blood	–	–	–	–
Eye	–	–	–	–
Bone marrow	–	–	–	–
Pituitary	–	–	–	–
Thyroid	–	–	–	–
Adipose	–	–	0.18	–
Brain	–	–	–	–
Epididymal fat	–	–	0.26	–
Heart	–	–	–	–
Kidney	–	–	0.29	–
Liver	0.42	–	0.45	–
Lung	0.76	–	–	–
Muscle	–	–	–	–
Pancreas	–	–	–	–
Spleen	–	–	–	–
Testes	–	–	–	–
Thymus	–	–	–	–
Stomach wall	28.29	4.28	3.48	–
Duodenum wall	2.78	1.89	0.70	–
Jejunum wall	1.63	1.43	0.62	–
Ileum wall	17.72	9.76	1.27	–
Caecum wall	–	26.93	29.93	–
Colon wall	–	3.33	8.41	–
Carcass	0.24	0.86	7.33	–

– = Radioactivity was not quantifiable in these samples.

The pharmacokinetic data described here indicate that RP 64477 is very poorly absorbed from the gastrointestinal tract with extremely low levels of drug-derived products appearing systemically following oral administration. The data further show only low levels of compound and/or metabolites gaining access to intestinal cells following oral administration (1.63 and 1.43 μg drug equivalents g^{-1} of jejunal tissue at 1 and 6 hr, respectively, after an oral dose of 100 mg kg^{-1}). Drug levels of this order, if representative of parent compound would, however, be sufficient to elicit ACAT inhibition. In contrast to these *in vivo* observations, good penetration of CaCo-2 cells was apparent *in vitro* as evidenced by the comparable IC_{50}s generated for RP 64477 in homogenate and whole cell systems. Good cell penetration *in vitro* is most likely explained by the preparation and presentation of the drug in solvent.

Low systemic availability minimises the potential for toxicity occurring through systemic interactions, which has allowed the use of relatively high doses of this drug in clinical studies. Despite this, RP 64477 failed to influence cholesterol levels in human subjects [26]. DUP 128, an ACAT inhibitor that is similarly only poorly available systemically, has recently been shown to be only weakly active in reducing plasma cholesterol levels in human subjects at large multigram doses [27].

In conclusion, RP 64477 is a potent inhibitor of the enzyme ACAT obtained from a number of tissues and from a range of species, including man. RP 64477 has low systemic bioavailability but is able to inhibit intestinal cholesterol absorption and elicit hypocholesterolaemic responses in cholesterol-fed rats and rabbits. In animals fed standard laboratory diets, comparable dose regimens do not influence cholesterol absorption nor plasma lipids. Despite the failure of RP 64477 to demonstrate significant hypocholesterolaemic activity in man, intestinal ACAT inhibition remains a valid target mechanism for inhibition of cholesterol absorption and hypocholesterolaemic drug therapy. For molecules acting only on intestinal ACAT to be effective clinically, however, a high level of potency and long duration of action in the intestinal cell will be required. In this respect, animal studies carried out in the noncholesterol-fed rather than cholesterol-fed state are more likely to be predictive of efficacy in man. If the issue of toxicity can be overcome for systemically available ACAT inhibitors, greater hypocholesteroleamic responses may be achievable *via* the additional property of inhibition of hepatic ACAT.

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