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EFFICACY, TISSUE DISTRIBUTION AND BILIARY EXCRETION OF METHYL (3*R**,5*S**)-(E)-3,5-DIHYDROXY-9,9-DIPHENYL-6,8-NONADIENOATE (CP-83101), A HEPATOSELECTIVE INHIBITOR OF HMG-CoA REDUCTASE ACTIVITY IN THE RAT

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Abstract—Methyl (3*R**,5*S**)-(E)-3,5-dihydroxy-9,9-diphenyl-6,8-nonadienoate, CP-83101, was identified as a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity, inhibiting enzyme activity *in vitro* with an IC_{50} of 8.5 ± 0.7 μM and a K_i with respect to HMG-CoA of 2.6 μM. CP-83101 also inhibited rat hepatic sterol biosynthesis by 39 ± 7% at a dose of 100 mg/kg. [^{3}H]CP-83101, administered orally to rats, exhibited peak plasma levels at approximately 1 hr that declined thereafter with an apparent half-time of 2–3 hr. Peak tissue levels also occurred 1 hr following oral administration of [^{3}H]CP-83101. The decline in radioactivity in the liver, however, was considerably slower than that noted in blood, whereas the half-life in non-hepatic tissues was approximately 1 hr. Liver/blood ratios of 14, and liver/lens ratios of greater than 3000, following oral administration of [^{3}H]CP-83101, were similar to those previously reported for other HMG-CoA reductase inhibitors, suggesting a high degree of tissue selectivity. In addition, liver/adrenal and liver/ovary ratios were approximately 1000 at all time points examined between 30 min and 24 hr following oral [^{3}H]CP-83101 administration, indicating a high specificity for hepatic versus other steroidogenic tissues. Evaluation of intravenous versus oral administration of the water-soluble, free acid, sodium salt of [^{3}H]CP-83101 in bile duct canulated rats indicated that approximately 20% of orally administered CP-83101 is absorbed from the gastrointestinal tract, and that absorbed CP-83101 is cleared rapidly from the plasma via the liver and from the liver via the bile. In addition, several lines of evidence suggest that CP-83101 may undergo enterohepatic recirculation. Agents of this synthetic series may thus possess advantages over other HMG-CoA reductase inhibitors with respect to tissue kinetics and specificity.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors inhibit the rate-determining step in cholesterol and polyisoprenoid biosynthesis [1], and are effective in reducing plasma cholesterol levels in humans [2, 3] due to an increase in hepatic receptor-mediated low density lipoprotein (LDL) cholesterol removal from the circulation [4, 5]. Increased LDL cholesterol internalization results from the coordinate derepression of the synthesis of both HMG-CoA reductase and the LDL receptor [6, 7], as a compensatory response presumably due to a reduction in intracellular oxysterol levels [8, 9] that occurs following inhibition of hepatic cholesterolgenesis [10–12].

Although the results of hepatic HMG-CoA reductase inhibition on plasma cholesterol concentrations are dramatic [2, 3] and may be beneficial to patients with elevated plasma cholesterol levels [13, 14], the consequences of reduction in sterol and polyisoprenoid synthesis in extrahepatic tissues are generally regarded as undesirable [3, 15–20]. The potential for adverse consequences of inhibition of cholesterolgenesis in nonhepatic tissues is a concern especially in tissues, such as the adrenals, ovaries and testis, for which nascent cholesterol synthesis is required for steroidogenesis [21], and in tissues such as the ocular lens that satisfy much, if not all of their cholesterol requirements through nascent synthesis [22, 23]. Thus, the ability of an HMG-CoA reductase inhibitor to be selectively delivered to the liver would reduce dramatically the potential for adverse effects due to systemic inhibition of cholesterolgenesis.

In this study, we have examined the efficacy, tissue kinetics and specificity, and absorption and biliary excretion profile for a representative member of a synthetic class of HMG-CoA reductase inhibitors that shows a high degree of hepatoselectivity. Although the HMG-CoA reductase inhibitor used in these studies is somewhat less potent than those isolated as fungal metabolites [5, 24, 25], its hepatoselectivity, particularly with regard to the ocular lens and nonhepatic steroidogenic tissues, suggests that other, more potent compounds of this class may exhibit improved hepatoselectivity.

MATERIALS AND METHODS

Chemicals and solutions. [^{14}C]HMG-CoA, [^{3}H]cholesterol, and [^{3}H]mevalonate were purchased from New England Nuclear (Boston, MA).

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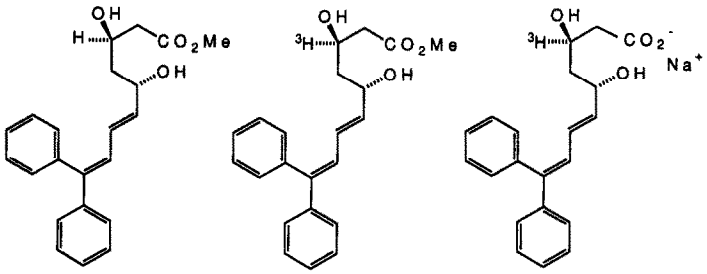

Fig. 1. Structures of CP-83101, methyl (3*R**,5*S**)-(E)-3,5-dihydroxy-9,9-diphenyl-6,8-nonadienoate (left), [^{3}H]CP-83101, methyl (*R**,5*S**)-(E)-[3-^{3}H]-3,5-dihydroxy-9,9-diphenyl-6,8-nonadienoate (center), and [^{3}H]CP-83101 sodium salt, sodium (3*R**,5*S**)-(E)-[3-^{3}H]-3,5-dihydroxy-9,9- diphenyl-6,8-nonadienoate (right).

[^{14}C]Acetate was purchased from Amersham (Arlington Heights, IL). All other chemicals were purchased from previously listed sources [26–28]. Mevinolin was a gift from Dr. Alfred W. Alberts (Merck Sharp & Dohme Research Laboratories, Rahway, NJ). Pravastatin (eptastatin) was a gift from Dr. S. J. Lucania (Squibb Institute for Medical Research, Princeton, NJ). TEDK buffer consisted of 50 mM Tris (pH = 7.5), 1 mM EDTA, 5 mM dithiothreitol, and 50 mM KCl.

*Synthesis of CP-*83101 *and* [^{3}H]*CP-*83101. Methyl (3*R**,5*S**)-(E)-3,5-dihydroxy-9,9-diphenyl-6,8-nonadienoate, CP-83101 (Fig. 1, left structure), was prepared from β-phenylcinnamaldehyde as previously described by Wint and McCarthy [29]. Methyl (3*R**,5*S**)-(E)-[3-^{3}H]-3,5-dihydroxy-9,9-diphenyl-6,8-nonadienoate, [^{3}H]CP-83101 (Fig. 1, center structure), was prepared as described for CP-83101 synthesis [29] except that tritium-labeled sodium borohydride was used in place of unlabeled sodium borohydride in the reduction of methyl (E)-5-hydroxy-9,9-diphenyl-3-oxo-6,8-nonadienoate to methyl (*R**,5*S**)-(E)-3,5-dihydroxy-9,9-diphenyl-6,8-nonadienoate.

Preparation of the water-soluble, free acid, sodium salt of [^{3}H]*CP-*83101. To render [^{3}H]CP-83101 water-soluble to permit intravenous administration, [^{3}H]CP-83101 was dissolved in 0.5 mL of EtOH, mixed with an equal molar ratio of NaOH, and incubated for 90 min at 37° in a final volume of 2.0 mL water (final EtOH concentration = 20%). The resulting sodium salt of the free acid of [^{3}H]CP-83101, sodium (3*R**,5*S**)-(E)-[3-^{3}H]-3,5-dihydroxy-9,9-diphenyl-6,8-nonadienoate, is shown in Fig. 1 (right structure).

Measurement of HMG-CoA reductase activity. HMG-CoA reductase activity was measured essentially as described by Harwood *et al.* [26] with the following modifications. A catalytically active, proteolytically modified fragment of HMG-CoA reductase, solubilized from the microsomal membrane and partially purified as described by Rogers *et al.* [30], was used as the source of enzyme activity. Immediately prior to assay, a portion of this preparation was thawed and diluted with TEDK buffer such that a 48 μL portion of the diluted enzyme solution contained enzyme sufficient to convert 40–60 pmol of HMG-CoA to mevalonate per min of incubation at 37°. A 48-μL volume of the diluted solubilized enzyme solution was then mixed with 2 μL of either dimethyl sulfoxide (DMSO, control incubations) or DMSO containing various amounts of CP-83101, and 25 μL of a solution containing sufficient amounts of the following substrates to give final concentrations of 200 μM $NADP^+$, 1.7 mM glucose-6-phosphate, 0.2 units glucose-6-phosphate dehydrogenase, 66.7 μM [^{14}C]HMG-CoA (sp. act. 10 cpm/pmol), 15,000–20,000 cpm [^{3}H]mevalonate (0.6 to 1.2 Ci/mmol) as an internal standard, and 60 mM EDTA to prevent conversion of mevalonate to phosphomevalonate during incubation. Following incubation at 37° for 30 min, 10 μL of 6 M HCl was added to each tube to terminate the enzymatic reaction and to convert the newly formed mevalonate into mevalonolactone. The mevalonolactone was then separated from unreacted substrate by silica gel thin-layer chromatography. Following development in toluene:acetone (1:1), the region of the chromatogram corresponding to $R_f = 0.4$ to 1.0 was removed, immersed in liquid scintillation fluid, and counted, using a dual-channel $^3H/^{14}C$ program. HMG-CoA reductase activity is expressed as picomoles of mevalonate formed from HMG-CoA per minute of incubation at 37° per milligram of microsomal protein. Mevinolin was used as a positive standard for HMG-CoA reductase inhibition. At the substrate concentrations employed in this assay, mevinolin (K_i = 0.64 nM; [25]) exhibited an IC_{50} for HMG-CoA reductase inhibition, based on eight consecutive independent experiments, of 11.3 ± 2.0 nM (data not shown). DMSO had no inhibitory effect on the solubilized enzyme preparation at concentrations less than 10% (v/v) but exhibited an IC_{50} for inhibition of the solubilized enzyme of 21% (v/v; data not shown). The final concentration of DMSO in the assay was 2.7% (v/v).

Michaelis constants (K_m) for HMG-CoA and inhibition constants (K_i) for CP-83101 were determined at the above-mentioned concentrations of NADPH(H) and various concentrations of HMG-CoA between 1 and 38 μM by omitting [^{14}C]HMG-CoA from the substrate–cofactor solution and adding it directly to the assay mixture at the desired final concentration.

Measurement of in vivo *cholesterolgenesis from* [^{14}C]*acetate.* Male rats, receiving food and water *ad lib.* and weighing approximately 200 g, were administered an oral bolus of water containing 0.25%

carboxymethyl cellulose and various concentrations of an HMG-CoA reductase inhibitor 60 min prior to the diurnal peak for hepatic HMG-CoA reductase activity. One hour following administration, animals received an interperitoneal injection of 2.5 μL [^{14}C]acetate (16 mM; 30 μCi/mL) per g of body weight. An hour after administration of the radiolabeled acetate, animals were anesthetized with pentobarbitol, killed by decapitation, and two 1-g liver pieces were removed. Tissue samples were saponified for 120 min at 60° in 2.4 mL of 2.5 M KOH. Following saponification, 5 mL of 80% ethanol and 0.5 mL of a [^{3}H]cholesterol internal recovery standard (36 nCi/mL) were added and the solution was mixed gently. Ten milliliters of petroleum ether was then added, and the mixture was first shaken vigorously for 2 min and then centrifuged at 2000 *g* in a bench-top Sorvall for 10 min. The resultant upper (petroleum ether) layer was removed and two 3-mL aliquots were mixed with liquid scintillation fluid and assessed for radioactivity, using a dual-channel ^{3}H/^{14}C program. Rates of cholesterolgenesis estimated by this method are expressed in terms of disintegrations per minute of [^{14}C]acetate incorporated into total nonsaponifiable lipids per hour per gram of hepatic tissue. Total radioactivity obtained by this method, which lacks the traditional digitonin precipitation step, was somewhat higher than that noted from the same liver following digitonin precipitation (data not shown). However, inhibition of cholesterolgenesis from acetate by pravastatin using this method was log-linear for concentrations ranging between 0.5 and 50 mg/kg, with 85% inhibition occurring at the maximum dosage examined (data not shown). Thus, the radioactivity assessed by this method is primarily, if not totally, the result of HMG-CoA reductase-catalyzed acetate conversion to mevalonate and subsequently to nonsaponifiable sterols.

Measurement of [^{3}H]*CP*-83101 *tissue distribution.* Female rats receiving food and water *ad lib.*, and weighing approximately 170 g, were administered by oral gavage a 1.3-mL bolus of [^{3}H]CP-83101 (sp. act. = 2–5 dpm/ng CP-83101) in water containing 8% ethanol and 0.25% carboxymethyl cellulose. Blood and tissue samples were removed and assessed for radioactivity at various times following administration. For determination of peak plasma levels, blood samples of 25 μL were obtained by repetitive tail vein slicing and combusted in a Packard (model 306) tissue oxidizer. Following combustion, the [^{3}H]water/scintillation fluid mixture recovered from the oxidizer was assessed for radioactivity and was used as a measure of plasma [^{3}H]CP-83101 levels. For tissue distribution analysis, blood, the steroidogenic organs (liver, adrenals, ovaries), lens and muscle (as an indicator of whole body distribution) were obtained at sacrifice and subjected to tissue oxidation as described above. In addition, for absorption/gastrointestinal transit studies, a 1-cm segment of the small intestine, 3 cm from the stomach-intestine interface, was also obtained and subjected to tissue oxidation. For the lenses, ovaries and adrenals, organ pairs were used to estimate total tissue radioactivity. For the blood, a 200-μL sample taken from the anterior vena cava was assessed for radioactivity, whereas for the other tissues, samples weighing approximately 400 mg were assessed for radioactivity. Radioactivity contained in the various tissues is expressed in terms of either dpm per whole tissue or dpm per mg tissue.

Measurement of the rate of biliary secretion of [^{3}H]*CP*-83101. Male rats receiving food and water *ad lib.*, and weighing approximately 350 g, were anesthetized and their bile ducts canulated. Bile was then collected for 10 min as an estimate of biliary flow rate and to assure that biliary flow was maintained. A 20 mg/kg bolus of the water-soluble, free-acid, sodium salt of [^{3}H]CP-83101 (sp. act. 5–10 dpm/ng CP-83101) was then administered either orally (as a 1.2-mL bolus via gavage or direct stomach injection) or intravenously (as a 0.4-mL bolus via femoral vein injection), and bile samples were collected in 10-min increments over the next 2 hr. Following sample volume measurement, 100-μL portions of each sample were assessed for radioactivity. Radioactivity contained in the biliary secretions is expressed in terms of dpm per fraction or total cumulative dpm secreted following drug administration.

RESULTS

In vitro *inhibition of HMG-CoA reductase activity by CP*-83101. CP-83101 inhibited HMG-CoA reductase activity with an IC_{50} of 8.5 ± 0.7 μM in seven independent analyses. HMG-CoA reductase inhibition was log-linear between 0.1 and 100 μM (Fig. 2), and was competitive with respect to the substrate HMG-CoA (Fig. 3). The K_i value obtained for inhibition of enzyme activity by CP-83101 was 2.6 μM with respect to HMG-CoA (Fig. 3, inset).

A 40:60 mixture of diastereomers of CP-83101 (IC_{50} = 20.8 ± 2.2 μM) exhibited only approximately half the potency of the diastereomerically pure, 3*R**,5*S** form of CP-83101 (IC_{50} = 8.5 ± 0.7 μM), suggesting that the 3*R**,5*R** isomer possesses little, if any, inhibitory activity. Base hydrolysis of the methyl ester of CP-83101 to yield the free acid, sodium salt (IC_{50} = 4.0 μM) resulted in a substantial increase in inhibitory potency, indicating that the free acid is the active moiety.

In vivo *inhibition of rat hepatic cholesterolgenesis by CP*-83101. As shown in Table 1, CP-83101 also inhibited rat hepatic cholesterolgenesis *in vivo*. Two hours following a single, oral 100 mg/kg dose of CP-83101, hepatic cholesterolgenesis from acetate was inhibited by approximately 39 ± 7% in four independent experiments.

Plasma and tissue kinetics of [^{3}H]*CP*-83101. As shown in Figs. 4 and 5, peak plasma levels were reached between 1 and 2 hr following oral administration of [^{3}H]CP-83101. A reproducible multiplicity of sub-peaks, occurring at approximately 60-min intervals throughout the peak of plasma radioactivity, was also noted (Fig. 4), suggesting the possibility of enterohepatic recirculation (see below). Radioactivity disappeared from the plasma with an apparent half-time of approximately 2–3 hr (Figs. 4 and 5). Peak hepatic levels also occurred approximately 1 hr following oral administration (Fig. 5). The decline in radioactivity in the liver, however, was considerably slower than that observed in blood

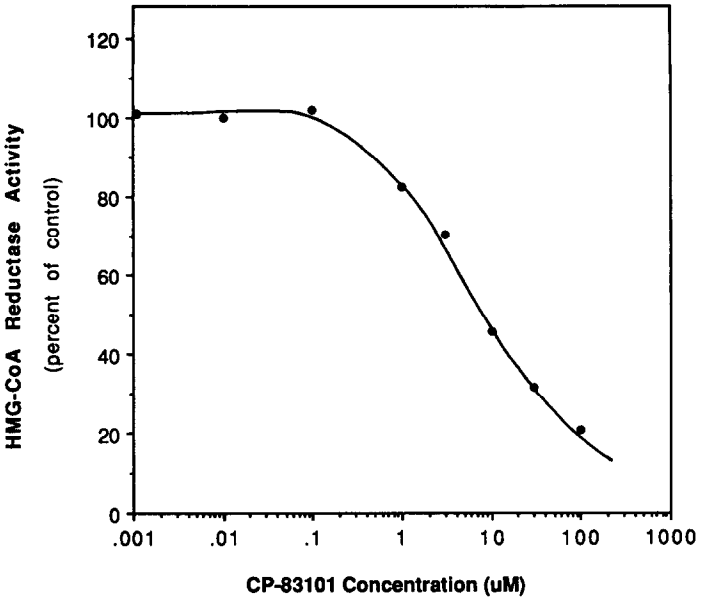

Fig. 2. Concentration-dependent inhibition of HMG-CoA reductase activity by CP-83101. Protease-solubilized, partially purified rat liver HMG-CoA reductase (2.0 μg protein; 27.1 nmol/min/mg) was incubated for 30 min at 37° in a final volume of 75 μL of TEDK buffer containing 200 μM $NADP^+$, 1.7 mM glucose-6-phosphate, 0.2 units glucose-6-phosphate dehydrogenase, 66.7 μM [^{14}C]HMG-CoA (sp. act. 10 cpm/pmol), 15,000–20,000 cpm [^{3}H]mevalonate (0.6 to 1.2 Ci/mmol), 60 mM EDTA, 2.7% DMSO and the indicated concentrations of CP-83101. Following incubation, mevalonate synthesis was assessed and HMG-CoA reductase activity was quantitated as described in Materials and Methods. HMG-CoA reductase activity in control incubations averaged 56.9 ± 1.3 pmol/min.

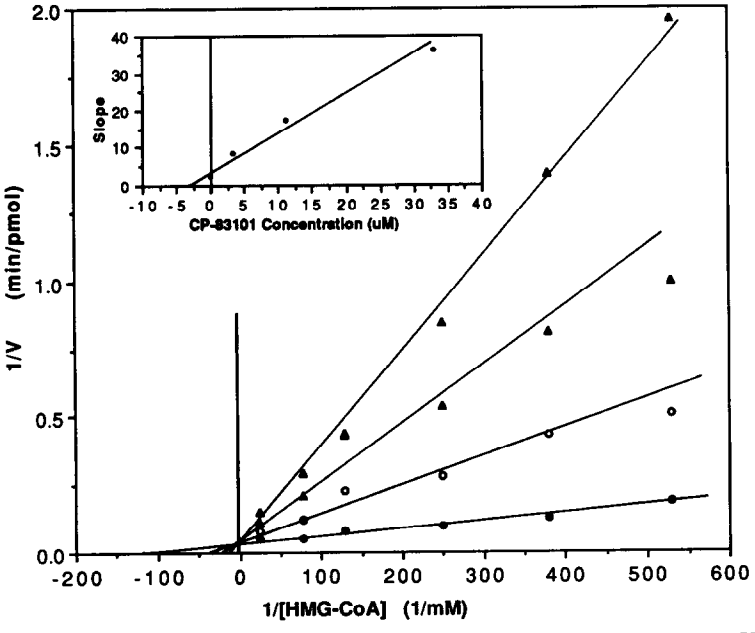

Fig. 3. Competitive inhibition of HMG-CoA reductase activity by CP-83101 with respect to HMG-CoA. Partially purified rat liver HMG-CoA reductase (2.0 μg protein; 27.1 pmol/min/mg) was incubated for 30 min at 37° in a final volume of 75 μL of TEDK buffer containing either 0 μM (●), 3.3 μM (○), 11 μM (▲) or 33 μM (△) CP-83101, 2.7% DMSO, the indicated concentrations of [^{14}C]HMG-CoA, and the remaining HMG-CoA reductase cofactors as described in Materials and Methods. Following incubation, mevalonate synthesis was assessed, and HMG-CoA reductase activity was quantitated as described in Materials and Methods. Data are the averages of duplicate measurements of HMG-CoA reductase activity and are expressed as reciprocal velocity as a function of reciprocal HMG-CoA concentration. Inset: secondary plot of the slope as a function of CP-83101 concentration.

Table 1. *In vivo* inhibition of hepatic cholesterolgenesis by CP-83101

Expt.	Cholesterol synthesis (dpm/hr/g liver) Control	CP-83101 (100 mg/kg)	Inhibition of cholesterolgenesis (%)
1	4700	3370	28
2	3350	2560	24
3	3670	2170	41
4	6080	3330	45

Male rats, weighing 200 g, received food and water *ad lib.* and were administered a 6.7 mL/kg body weight oral bolus of water containing 0.25% carboxymethyl cellulose and 15 mg/mL CP-83101 (final dose = 100 mg/kg), 60 min prior to the diurnal peak for hepatic HMG-CoA reductase activity. One hour later, animals received an interperitoneal injection of 2.5 μL [^{14}C]acetate (16 mM; 30 μCi/mL) per g of body weight. An hour after administration of the radiolabeled acetate, animals were killed, and two 1-g liver samples were obtained. Tissue samples were processed and rates of [^{14}C]acetate incorporation into neutral sterols were determined as described in Materials and Methods.

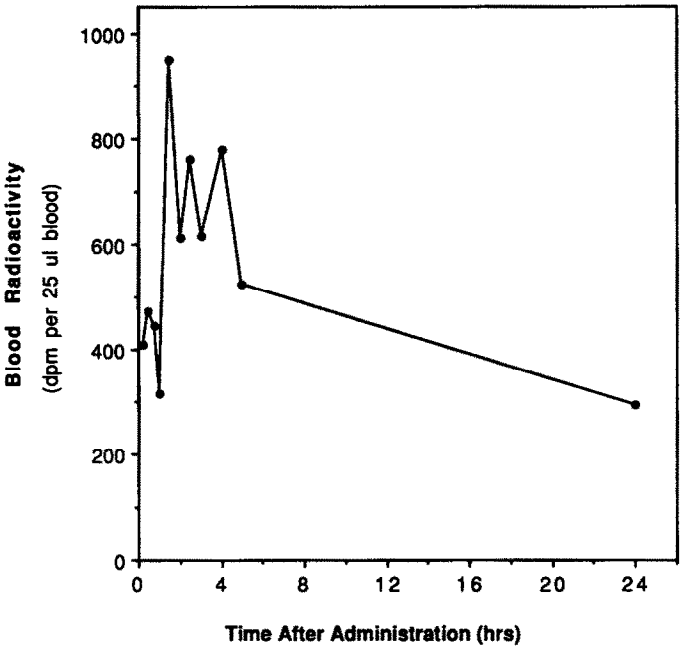

Fig. 4. Plasma kinetics of [^{3}H]CP-83101 following oral administration. [^{3}H]CP-83101, 4.7 mg, was mixed with 100 mg of unlabeled CP-83101 and dissolved in 1.1 mL of ethanol. To this solution was added 5.0 mL of a solution of 0.25% carboxymethyl cellulose in water. A 1.5 mL volume of the resulting suspension of [^{3}H]CP-83101 (85,950 dpm/μL; sp. act. = 5.02 dpm/ng CP-83101) was administered orally by gavage to each of four 170-g female rats (dosage = 152 mg/kg; 129 × 10^6 dpm/animal). Blood samples of 25 μL were obtained by tail vein slicing at the indicated times after [^{3}H]CP-83101 administration, and were combusted in a model 306 Packard Tissue Oxidizer. The [^{3}H]water/scintillation fluid mixture recovered from the oxidizer following combustion was quantitated in a liquid scintillation counter and was used as a measure of plasma [^{3}H]CP-83101. Shown are the average plasma tritium levels at the indicated times after dosing.

(Fig. 5). For the adrenals, ovaries and lenses, peak tissue levels also occurred 1 hr following administration (Fig. 5). However, the half-life in these tissues was less than 1 hr (Fig. 5).

Tissue distribution specificity of [^{3}H]*CP-83101.* At all time points examined following oral administration of [^{3}H]CP-83101, the majority of the absorbed radioactivity was present in the liver, with the remainder (approximately 10%) present in the blood (Fig. 5). On a per tissue basis, the amount of radioactivity present in the adrenals and ovaries was more than 1000-fold less than the radioactivity contained in the liver (Table 2, Fig. 5). The amount of radioactivity present in the lens was the lowest of

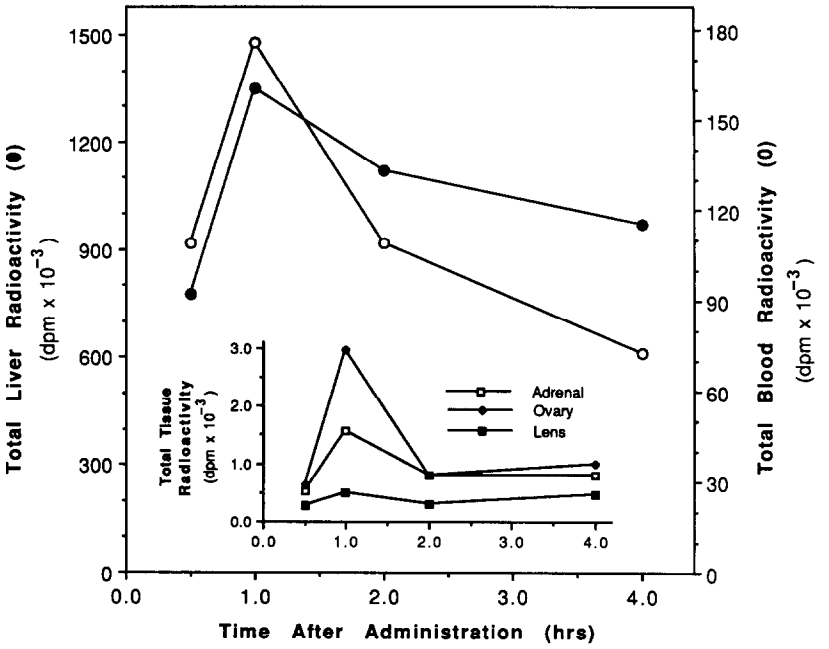

Fig. 5. Kinetics of [^{3}H]CP-83101 in blood, liver and extrahepatic tissues following oral administration. [^{3}H]CP-83101, 4.23 mg, was mixed with 100 mg of unlabeled CP-83101 and dissolved in 0.5 mL of ethanol. To this solution was added 6.0 mL of a solution containing 0.25% carboxymethyl cellulose in water. Following a brief sonication to obtain an even suspension, a 1.3 mL volume of the resulting suspension of [^{3}H]CP-83101 (40,870 dpm/μL; sp. act. = 2.55 dpm/ng CP-83101) was administered orally by gavage to each of four 170-g female rats (dosage = 123 mg/kg; 53.1 × 10^6 dpm/animal). Animals were anesthetized with an injection of pentobarbital at the indicated times after dosing, and a 200-μL blood sample was removed from the anterior vena cava. Two 400-mg samples of liver tissue were also removed. For the lens, ovaries and adrenals, both organs were removed and weighed. Blood, liver, and extrahepatic tissue samples were subjected to tissue oxidation as described in Materials and Methods. Tissue radioactivity was calculated based on tissue weights and blood volumes and is expressed in terms of dpm per whole tissue. Shown is the total radioactivity present in the liver (●), blood (○) and extrahepatic tissues (inset) as a function of time after [^{3}H]CP-83101 administration.

Table 2. Ratio of hepatic to nonhepatic tissue levels of [^{3}H]CP-83101 as a function of time after oral administration

	[^{3}H]dpm in liver/[^{3}H]dpm in extrahepatic tissue				
	Time after oral administration of CP83101				
Nonhepatic tissue	30 min	1 hr	2 hr	4 hr	24 hr
Blood	7.3	8.2	10.2	13.2	14.0
Ovary	1260	485	1430	968	1460
Adrenal	1530	931	1430	1220	1280
Lens	2800	2900	3870	2040	3320

[^{3}H]CP-83101, 4.23 mg, was mixed with 100 mg of unlabeled CP-83101 and dissolved in 0.5 mL of ethanol. To this solution was added 6.0 mL of 0.25% carboxymethyl cellulose in water. Following brief sonication to obtain an even suspension, a 1.3 mL volume of the resulting suspension of [^{3}H]CP-83101 (40,870 dpm/μL; sp. act. = 2.55 dpm/ng CP-83101) was administered orally by gavage to each of four 170-g female rates (dosage = 123 mg/kg; 53.1 × 10^6 dpm/animal). Animals were anesthetized with an interperitoneal injection of pentobarbital at the indicated times after dosing, and a 200-μL blood sample was removed from the anterior vena cava. At sacrifice, the adrenals, ovaries and ocular lenses were removed and weighed. Two 400-g pieces of liver tissue were also removed. All tissues were subjected to tissue oxidation as described in Materials and Methods. Tissue radioactivity was calculated based on tissue weights and blood volumes. Shown are the ratios of total hepatic radioactivity to total tissue radioactivity for the indicated tissues.

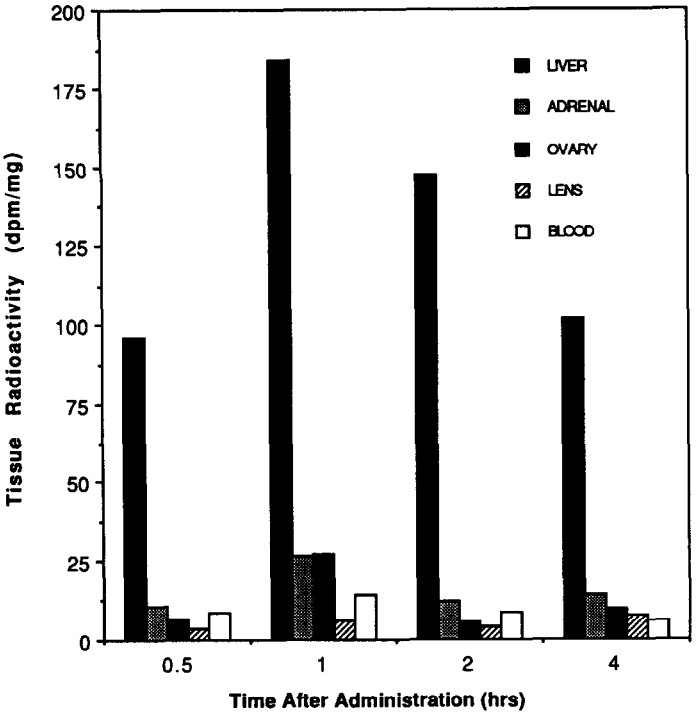

Fig. 6. Tissue weight distribution specificity of [^{3}H]CP-83101 following oral administration. The experimental conditions are as described in the legend of Fig. 5. Radioactivity obtained following tissue oxidation is expressed as dpm per unit tissue weight. Shown is a comparison of radioactivity per mg tissue for the indicated tissue as a function of time after [^{3}H]CP-83101 administration.

any tissue examined (Fig. 5), and was approximately 3000-fold less than that found in the liver at all time points studied (Table 2, Fig. 5). On a per mg tissue basis, the levels of radioactivity present in the nonhepatic tissues was also very small compared to that present in the liver (Fig. 6). Similar tissue distribution and hepatospecificity were obtained at all time points examined between 30 min and 24 hr (Table 2), suggesting that little release of label into the aqueous pool occurred within the time-frame of the experiment.

Although the blood contained the highest levels of radioactivity of any tissue other than the liver (Fig. 5), the radioactivity present in the blood was also substantially less than that found in the liver (Figs. 5 and 6, Table 2). At all time points examined following oral [^{3}H]CP-83101 administration, the radioactivity present in the blood was approximately 10-fold less than that recovered in the liver (Figs. 5 and 6, Table 2). The amount of radioactivity present in the blood relative to that present in the liver decreased as a function of time after oral dose administration (Table 2, Fig. 7), indicative of continued removal of the compound by the liver.

*Rapid biliary excretion of CP-*83101. Following administration, [^{3}H]CP-83101 was excreted rapidly via the bile. As shown in Figs. 8 and 9, appearance of radioactivity in the bile was noted within 10 min following intravenous administration of [^{3}H]CP-83101 and peak levels of biliary radioactivity were noted within 20 min following intravenous administration (Fig. 9). Approximately 12, 30, and 40% of the intravenously administered dose of [^{3}H]CP-83101 was excreted via the bile within 30 min, 1 hr, and 2 hr respectively (Fig. 8). In addition, less than 15 and 2% of an intravenous dose of [^{3}H]CP-83101 was found in the liver and blood, respectively, 30 min following administration (Table 3). Similarly, rapid appearance of radioactivity in the bile was also noted following oral administration of [^{3}H]CP-83101 (Fig. 8). The delayed appearance relative to that noted for intravenous administration (Fig. 8) was due to the time required for gastrointestinal transit and absorption following oral administration. As a result, peak levels of biliary radioactivity occurred 50 min following oral administration (Fig. 9). For both routes of administration, similar liver to blood distributions were noted at all time points examined (Table 3), suggesting similar tissue kinetics regardless of the route of administration. Taken together, these results are indicative of rapid absorption, rapid hepatic clearance, and rapid biliary excretion of CP-83101.

The extent to which this agent is excreted via the bile suggests that this may be a major route for its metabolism. In addition, the extent to which and the rapidity with which this agent is excreted via the bile suggest that enterohepatic recirculation of CP-83101 may occur. Indeed, the broad peak of plasma radioactivity (Fig. 4), the reproducible multiplicity of subpeaks of plasma radioactivity occurring at approximately 60-min intervals (Fig. 4), and the sustained hepatic levels (Fig. 5) even in the presence of rapid biliary secretion (Fig. 9) support this suggestion.

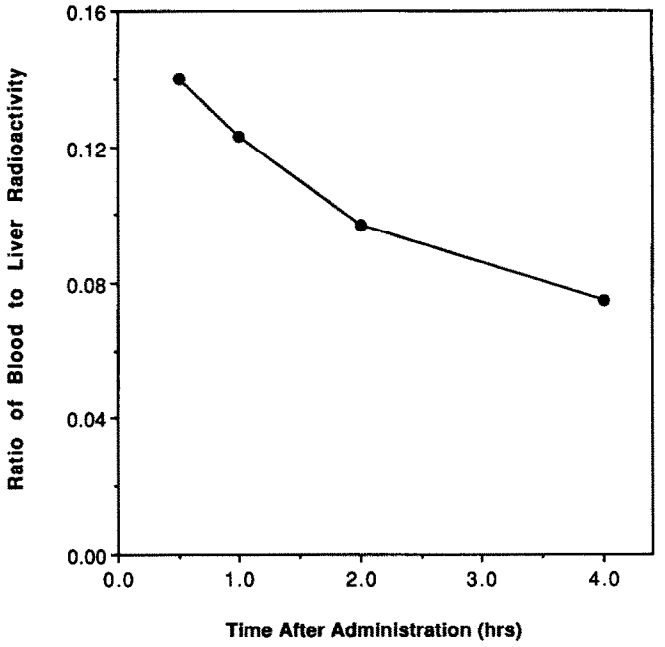

Fig. 7. Ratio of total blood to total hepatic radioactivity as a function of time after oral [^{3}H]CP-83101 administration. The experimental conditions are as described in Table 2.

Furthermore, in contrast to the 1-hr peak tissue levels of radioactivity noted in liver (Figs. 5 and 10), blood (Fig. 5) and other tissues examined (Fig. 5), the peak of radioactivity noted in the small intestine, 3 cm from the stomach/intestinal interface, occurred 2 hr after oral dosage administration (Fig. 10), at a time when a majority of absorbed [^{3}H]CP-83101 had been returned to the intestine via the bile.

Absorption kinetics of CP-83101. The existence of rapid and extensive biliary excretion of CP-83101, together with the probability for enterohepatic recirculation, complicates an accurate determination of the degree to which CP-83101 is absorbed from the intestinal lumen. For example, following oral administration of [^{3}H]CP-83101, approximately 3% of the administered dose was present in the liver and blood at a time corresponding to peak tissue levels (Fig. 5). However, due to the rapidity with which CP-83101 is excreted via the bile (see above), this representation is an underestimate of the degree to which CP-83101 is absorbed.

A reliable estimate of the degree of intestinal absorption of CP-83101 can be made based on a comparison of the levels of [^{3}H]CP-83101 (administered as the free acid sodium salt) following oral versus intravenous administration. However, due to differences in the time required to reach peak CP-83101 levels following oral and intravenous administration (Fig. 9), estimation of the degree of intestinal absorption requires comparison of the cumulative biliary excretion of CP-83101 following the two routes of administration. Based on the area under the curves for biliary excretion of the free acid sodium salt of [^{3}H]CP-83101 (Figs. 8 and 9), it is estimated that CP-83101 is absorbed from the gastrointestinal tract to the extent of approximately 20%.

DISCUSSION

Direct inhibition of hepatic HMG-CoA reductase activity, as an effective method for reducing plasma cholesterol levels, has been well documented in experimental animals [4, 5, 24, 25, 31] and in humans [2, 3, 5], and thus represents a useful therapeutic mechanism for combating atherosclerosis. A variety of competitive [4, 5, 24, 25, 27, 32–34] and noncompetitive [31] inhibitors of hepatic HMG-CoA reductase have been identified. Of these, the most notable are the fungal metabolites, compactin [24] and mevinolin [4, 25], their derivatives, pravastatin [5] and simvastatin [32], and unrelated synthetic agents SRI-62320 [33] and BMY-22089 [34], that inhibit enzyme activity directly through competition with the substrate HMG-CoA.

While very effective in inhibiting hepatic HMG-CoA reductase activity, potent inhibitors of enzyme activity, such as these, also possess the potential, in extrahepatic tissues, to inhibit enzyme activity, and hence cholesterol and polyisoprenoid biosynthesis. These tissues contribute relatively little to plasma cholesterol modulation [35] but are either self-reliant on endogenous cholesterolgenesis for polyisoprenoid and cholesterol metabolites (e.g. the ocular lens) or play an important role in intermediary metabolism through the formation of a variety of polyisoprenoid and sterol-related compounds (e.g. the adrenals, ovaries and testes). Since inhibition of polyisoprenoid and sterol synthesis in these tissues may lead to undesirable consequences [3, 15–20], inhibitors of HMG-CoA reductase activity that are selectively targeted to the liver would exhibit a dramatic reduction in the potential for inducing these adverse effects of systemic inhibition of sterol synthesis.

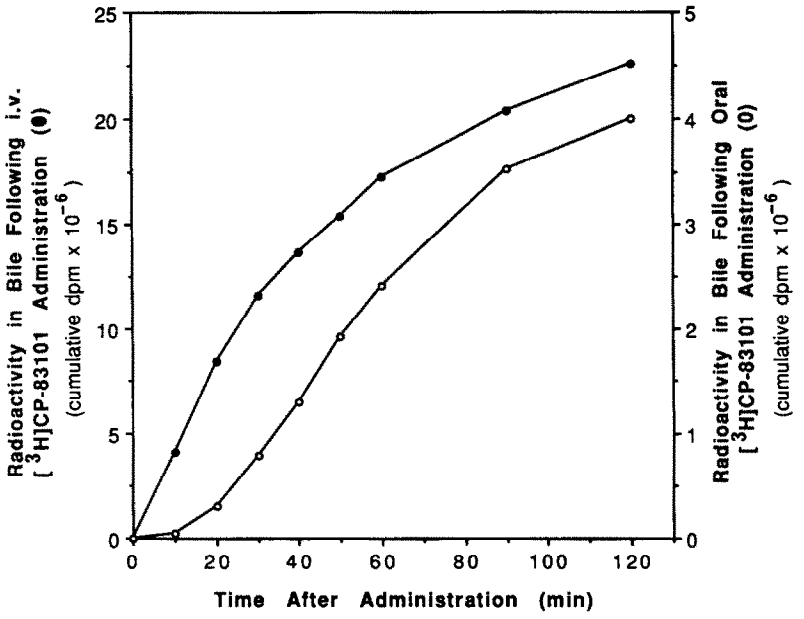

Fig. 8. Cumulative appearance of radioactivity in the bile following oral and intravenous administration of the free acid, sodium salt of [^{3}H]CP-83101. To render CP-83101 water-soluble to permit intravenous administration, 5.25 mg [^{3}H]CP-83101 was dissolved in 0.5 mL EtOH, mixed with 51.6 mg of unlabeled CP-83101 (total CP-83101 = 162 μmol) and hydrolyzed to the water-soluble, free acid, sodium salt by incubation for 90 min at 37° in a final volume of 2.0 mL water containing 80 μL of DMSO and 162 μmol of NaOH. Following dilution with water to a final volume of 3.25 mL, the resulting solution of the free acid, sodium salt of [^{3}H]CP-83101 (122,800 dpm/μL; sp. act. = 7.03 dpm/ng [^{3}H]CP-83101, sodium salt) was administered to animals at a dose of 20 mg/kg (49.2 × 10^6 dpm/animal). Male rats receiving food and water *ad lib.*, and weighing approximately 350 g, were anesthetized, and their bile ducts were canulated. Bile was then collected for 10 min as an estimate of biliary flow rate and to assure that biliary flow was maintained. For animals intravenously administered the free acid, sodium salt of CP-83101, 0.4 mL of the 17.5 mg/mL solution described above was injected into the femoral vein of the anesthetized animals. For animals orally administered the free acid, sodium salt of CP-83101, the solution used for intravenous administration was diluted 1:3 by addition of 2 vol. of water, and 1.2 mL of the resulting solution was administered into the stomach by direct injection. Bile samples were collected in 10-min increments over the next 2 hr. Animals were maintained under anesthesia during the course of the study by dropwise administration of pentobarbitol to the peritoneal cavity when necessary. Following bile collection and sample volume measurement, 100-μL portions of each sample were mixed with scintillation fluid and assessed for radioactivity. Radioactivity contained in the biliary secretions is expressed in terms of total cumulative dpm secreted up to the indicated times following drug administration. Shown is the cumulative radioactivity measured in the bile following intravenous (●) or oral (○) administration of the free acid, sodium salt of [^{3}H]CP-83101 as a function of time after administration.

In this report, we have described the identification of a potent competitive inhibitor of hepatic HMG-CoA reductase activity, CP-83101, that exhibits a greater than 1000-fold selectivity for the liver when compared with other steroidogenic tissues (e.g. adrenals and ovaries) and a greater than 3000-fold hepatoselectivity when compared to tissues that rely on endogenous cholesterolgenesis for polyisoprenoid and sterol-related compounds (e.g. the ocular lens). CP-83101 is absorbed rapidly from the gastrointestinal tract to the extent of approximately 20%, is removed rapidly from the plasma by the liver, presumably first pass, and then is returned rapidly and extensively to the intestinal lumen via the bile. In addition, several lines of evidence suggest that CP-83101 may be subject to enterohepatic recirculation, thus increasing its degree of hepatoselectivity.

The rate of hepatic uptake of CP-83101, as evidenced by the rate at which CP-83101 appears in the bile, may be responsible, in part, for the degree of hepatoselectivity exhibited for this agent. In addition, the ability of CP-83101 to undergo enterohepatic recirculation may augment this selectivity by permitting sustained levels of CP-83101 to be present in the liver relative to other non-hepatic tissues. Indeed, the dramatic differences in tissue half-life noted between the liver and extrahepatic tissues support this suggestion.

With regard to the other HMG-CoA reductase inhibitors mentioned above, CP-83101 appears to be approximately 3-fold more hepatoselective than compactin with respect to liver to blood ratios, based on similar radioactive tracer methodologies [36]. However, based on a comparison of the radioactive tracer methodologies reported here and compound/metabolite mass determinations reported for other inhibitors [5, 37], CP-83101, at a dose of 152 mg/kg, appears to be somewhat less hepatoselective, with

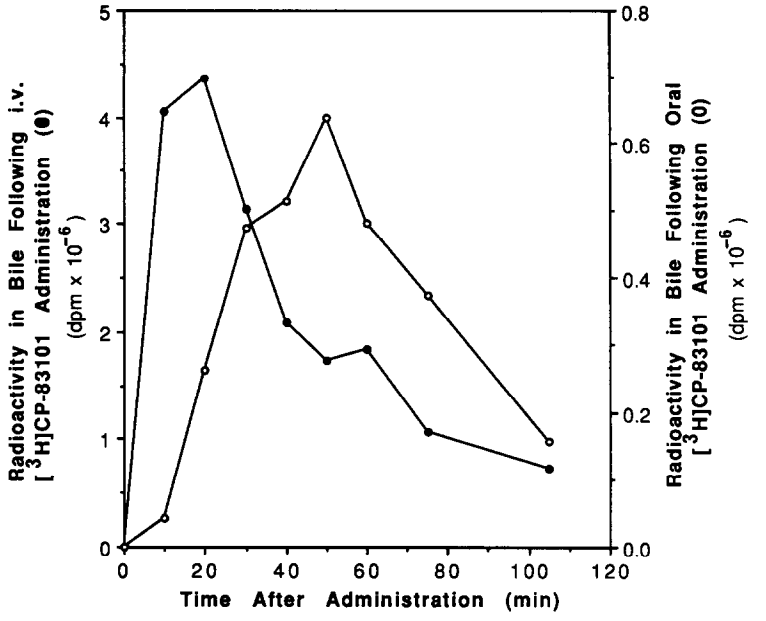

Fig. 9. Radioactivity measured in the bile following intravenous or oral administration of the free acid, sodium salt of [^{3}H]CP-83101 as a function of time after administration. The experimental conditions are as described in the legend of Fig. 8. Radioactivity contained in the biliary secretions is expressed in terms of dpm per biliary fraction. Thus, the radioactivity shown for the 10-min point represents the radioactivity excreted in the bile during the first 10-min collection period. For each animal, the biliary flow rate remained relatively constant through the course of the experiment, indicating that CP-83101 did not alter biliary flow. Key: (●) intravenous and (○) oral administration.

Table 3. Comparison of liver and plasma levels of radioactivity following oral and intravenous administration of the free acid, sodium salt of [^{3}H]CP-83101

Time after administration (min)	Oral administration		Intravenous administration	
	Radioactivity in blood (dpm × 10^{-3})	Radioactivity in liver (dpm × 10^{-3})	Radioactivity in blood (dpm × 10^{-3})	Radioactivity in liver (dpm × 10^{-3})
30	32.7	223	456	5020
60	61.8	244	971	3930
120	18.8	105	396	1280

To render CP-83101 water-soluble to permit intravenous administration, 4.54 mg [^{3}H]CP-83101 was mixed with 52.3 mg of unlabeled CP-83101 (total CP-83101 = 162 μmol) and hydrolyzed to the water-soluble, free acid, sodium salt by incubation for 90 min at 37° in a final volume of 5.6 mL water containing 162 μmol of NaOH. The resulting solution of the free acid, sodium salt of [^{3}H]CP-83101 (89,220 dpm/μL; sp. act. = 8.79 dpm/ng [^{3}H]CP-83101, sodium salt) was administered to animals at a dose of 20 mg/kg (35.7 × 10^6 dpm/animal) as follows. For animals intravenously administered the free acid, sodium salt of CP-83101, 0.4 mL of a 10 mg/mL solution was injected into the femoral vein following light anesthetization with halothane. For animals orally administered the free acid, sodium salt of CP-83101, the same solution used for intravenous administration was diluted 1:3 by addition of 2 vol. of water, and 1.2 mL of the resulting solution was administered by gavage. Animals receiving hydrolyzed CP-83101 orally were also treated briefly with halothane. Tissue radioactivity was calculated based on liver weights and blood volumes and is expressed in terms of dpm per whole tissue.

respect to the blood and steroidogenic tissues, than 20 mg/kg doses of either mevinolin [37], pravastatin [5], or simvastatin [37]. BMY-22089, a synthetic HMG-CoA reductase inhibitor that structurally belongs to the same class of compounds as CP-83101 [34], however, exhibits greater hepatoselectivity than mevinolin, with respect to adrenals and testes [34], suggesting the potential for compounds of this class to exhibit greater hepatoselectivity than the compactin-like class of HMG-CoA reductase inhibitors.

The tissue distribution of fluindostatin (SRI-62320), a synthetic HMG-CoA reductase inhibitor [33] that is structurally similar to BMY-22089 and CP-83101, has not been formally reported. However,

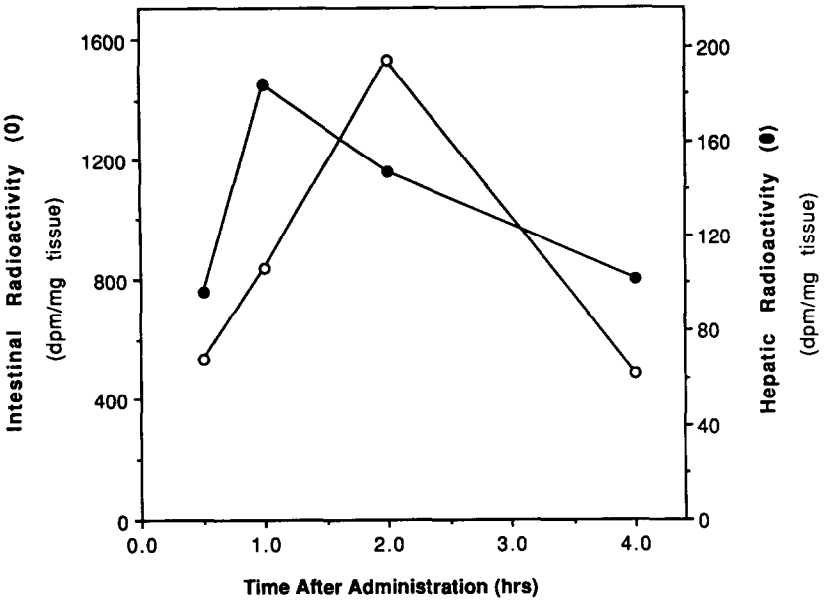

Fig. 10. Comparison of intestinal and hepatic radioactivity as a function of time after oral [^{3}H]CP-83101 administration. The experimental conditions are as described in the legend of Fig. 5. In addition to 400 mg liver samples, a 1 cm segment of the small intestine, 3 cm from the stomach-intestine interface, was also obtained and subjected to tissue oxidation, as described in Materials and Methods. Radioactivity obtained following tissue oxidation is expressed per unit tissue weight. Key: (○) intestinal and (●) hepatic radioactivity per mg tissue.

the drug has been reported to be selectively taken up by the liver first pass, and rapidly excreted via the bile into the feces.* The relative degree of specificity of SRI-62320 relative to CP-83101 and other HMG-CoA reductase inhibitors thus remains uncertain.

In view of the potential for adverse side-effects associated with systemic exposure of HMG-CoA reductase inhibitors [3, 15–20], the increased hepatoselectivity of BMY-22089, relative to mevinolin, suggests that compounds of the CP-83101/BMY-22089 class of HMG-CoA reductase inhibitors have the potential to be free from, or exhibit reduced severity of, many of the adverse side-effects noted following compactin or mevinolin treatment [3, 15–20]. However, one should regard with care the suggestion that relative differences in tissue distribution are an adequate representation of relative tissue exposures. For example, although increased rates of hepatic uptake can lead to relative differences in liver to tissue ratios, reduced rates of biliary clearance can also have similar effects on liver to tissue ratios without reflecting differences in tissue exposures. Indeed, an enhanced rate of biliary clearance could brand a compound as being less "hepatoselective" with respect to tissue ratios than a truly less hepatoselective compound with a relatively lower rate of biliary excretion.

With regard to metabolic disposition, all of these agents (including CP-83101) are highly hepatoselective [5, 34, 37, *], appear to be primarily excreted in the bile and eliminated in the feces [5, 38, *], undergo various degrees of enterohepatic recirculation [5, 38], and undergo various rates of metabolism to yield a variety of metabolites with reduced HMG-CoA reductase inhibitory activity [5, 39]. Whether differences in these parameters among the different HMG-CoA reductase inhibitors influence the degree to which they exhibit hepatoselectivity remains to be determined.

Finally, with regard to methodology, the ability to radiolabel CP-83101 by replacing the C-3 hydrogen with tritium in the final step of synthesis [29] is an important discovery in that, by this method, any synthetic HMG-CoA reductase inhibitor of this or other classes whose synthesis proceeds through a 3-keto intermediate [29] may be readily radiolabeled using this methodology. This method thus facilitates examination of the degree of hepatoselectivity of a variety of compounds and makes possible the usage of hepatoselectivity as a selection criteria for use in identifying HMG-CoA reductase inhibitors with improved properties. This study exemplifies the utility of this methodology and also demonstrates that tritium in this position in molecules of this class does not possess a high degree of lability and thus is not readily lost to the aqueous pool within the time frame of useful experimentation. Indeed, the rapidity with which and the extent to which CP-83101 was excreted into the bile, and the maintenance of tissue distribution over 24 hr, suggest that the tritium label has remained attached to the CP-83101 molecule or its metabolites throughout the course of these experiments.

* Engstrom RG, Weinstein DB, Kathawala FG, Scallen TS, Eskesen JB, Rucker ML and Miserendino R, Effects of XU-62320 (a potent HMG-CoA reductase inhibitor), on sterol synthesis *in vitro* and *in vivo* on serum lipids in rats and dogs, *The Aspen Bile Acid/Cholesterol Conference on Hepatic Lipoprotein and Cholesterol Metabolism*, August 21–24, 1987.

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