

# Selective inhibition of cholesterol synthesis in liver versus extrahepatic tissues by HMG-CoA reductase inhibitors

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**Abstract** Hepatic specificity of inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase may be achieved by efficient first-pass liver extraction resulting in low circulating drug levels, as with lovastatin, or by lower cellular uptake in peripheral tissues, seen with pravastatin. BMY-21950 and its lactone form BMY-22089, new synthetic inhibitors of HMG-CoA reductase, were compared with the major reference agent lovastatin and with the synthetic inhibitor fluvastatin in several *in vitro* and *in vivo* models of potency and tissue selectivity. The kinetic mechanism and the potency of BMY-21950 as a competitive inhibitor of isolated HMG-CoA reductase were comparable to the reference agents. The inhibitory potency (cholesterol synthesis assayed by  $^3\text{H}_2\text{O}$  or [ $^{14}\text{C}$ ]acetate incorporation) of BMY-21950 in rat hepatocytes ( $\text{IC}_{50} = 21 \text{ nM}$ ) and dog liver slices ( $\text{IC}_{50} = 23 \text{ nM}$ ) equalled or exceeded the potencies of the reference agents. Hepatic cholesterol synthesis *in vivo* in rats was effectively inhibited by BMY-21950 and its lactone form BMY-22089 ( $\text{ED}_{50} = 0.1 \text{ mg/kg p.o.}$ ), but oral doses (20 mg/kg) that suppressed liver synthesis by 83–95% inhibited sterol synthesis by only 17–24% in the ileum. In contrast, equivalent doses of lovastatin markedly inhibited cholesterol synthesis in both organs. In tissue slices from rat ileum, cell dispersions from testes, adrenal, and spleen, and in bovine ocular lens epithelial cells, BMY-21950 inhibited sterol synthesis weakly *in vitro* with  $\text{IC}_{50}$  values 76- and 188-times higher than in hepatocytes; similar effects were seen for BMY-22089. However, the  $\text{IC}_{50}$  ratios (tissue/hepatocyte) for lovastatin and fluvastatin were near unity in these models. ■ Thus, BMY-21950 and BMY-22089 are the first potent synthetic HMG-CoA reductase inhibitors that possess a very high degree of liver selectivity based upon differential inhibition sensitivities in tissues. This cellular uptake-based property of hepatic specificity of BMY-21950 and BMY-22089, also manifest in pravastatin, is biochemically distinct from the pharmacodynamic-based disposition of lovastatin, which along with fluvastatin exhibited potent inhibition in all tissues that were exposed to it. — Parker, R. A., R. W. Clark, S-Y. Sit, T. L. Lanier, R. A. Grosso, and J. J. K. Wright. Selective inhibition of cholesterol synthesis in liver versus extrahepatic tissues by HMG-CoA reductase inhibitors. *J. Lipid Res.* 1990. 31: 1271–1282.

**Supplementary key words** BMY-22089 • BMY-21950 • lovastatin • fluvastatin • tissue selectivity • hepatic selectivity • ileal cholesterol synthesis • lens epithelial cells

The liver is the primary organ for regulation of total body cholesterol homeostasis in mammalian systems. Hepatic coordination of cholesterol biosynthesis with assembly, secretion, and uptake of plasma lipoproteins depends in part on cellular mechanisms coupling the activities of the key enzymes of sterol synthesis with the receptors governing lipoprotein clearance (1). Thus an important target for pharmacological regulation of plasma LDL cholesterol is liver 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme in the pathway of cholesterol biosynthesis. The rationale for the use of HMG-CoA reductase inhibitors in the treatment of hypercholesterolemia has been convincingly established by the recent clinical successes of lovastatin and its relatives (2). The efficacy, tolerance, and defined mode of action of these naturally derived products of microbial fermentation has led to a continuing search for new inhibitors of the reductase.

The recent discussion in the literature regarding the possibility of deleterious side effects of HMG-CoA reductase inhibitors in therapeutic situations (2–4) centers in part upon the degree to which these agents exert their inhibitory influence in extrahepatic tissues. Beyond the requirements of cholesterol for cell membranes, interference with isoprenoid metabolism has been demonstrated to alter fundamental cellular processes such as S-phase DNA synthesis and progression through the cell cycle (5). Lovastatin at relatively high concentrations decreased the replicative capacity of endothelial cells, smooth muscle cells, and fibroblasts (6). Recently, pravastatin, a hydroxylated analogue of lovastatin, was shown to inhibit potently in both ileum and liver in *ex vivo* studies in rats while ex-

Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; LDL, low density lipoprotein; HPLC, high performance liquid chromatography.

hibiting weaker effects in other tissues (7). In contrast, lovastatin was highly inhibitory in all tissues examined in this study. Furthermore, the study by Mosley et al. (3) demonstrated that cholesterol synthesis in rat ocular lens in vivo was acutely inhibited by high oral doses of lovastatin but not pravastatin, while hepatic sterol synthesis was maximally inhibited by either drug. The basis for these differences appears to be that cellular penetration of pravastatin in most peripheral tissues is lower than that of lovastatin (3, 7). On the other hand, efficient hepatic first-pass extraction leading to low blood levels may provide the pharmacodynamic equivalent of tissue selectivity for HMG-CoA reductase inhibitors (8). This was evident in the recent study by Germershausen et al. (9) in which the peripheral tissue concentration of lovastatin was markedly lower than the hepatic concentration in orally dosed rats. However, when toxicology can be linked to mechanism of action, the concentration of peripherally distributed inhibitor is predictive of peripheral organ toxicity only if the intrinsic sensitivity of a given tissue to inhibition is also taken into account. In at least some instances in humans, blood levels of lovastatin have been atypically high and correlated with the incidence of side effects such as myolysis (10). Chronic toxicological doses of lovastatin, which generate high blood drug levels, have been reported to produce cataracts in dogs (2, 8).

Recently, the synthesis and evaluation of a new series of inhibitors resulted in the identification of BMY-21950 as a highly potent competitive inhibitor of HMG-CoA reductase (11–13). The strikingly narrow structure-activity relationships of BMY-21950 analogues led to the conclusion that this compound is very nearly optimized

with respect to potency within this series (13). Upon examination of the effects of this agent in vivo, we observed potent inhibition of cholesterol biosynthesis in liver but not in the intestine, in contrast to nondiscriminatory inhibition by lovastatin. In cell-based assays in vitro, BMY-21950 and BMY-22089 blocked sterol synthesis in liver cells at low concentrations yet were ineffective as inhibitors of cholesterol synthesis in several extrahepatic tissues. The present report elucidates the biochemical profile of these agents in a series of models that provide a means to compare and contrast the fundamental differences between them and the major reference agents lovastatin, pravastatin, and fluvindostatin (XU-62320) (Fig. 1).

## METHODS

### Inhibitor preparations and other reagents

Lovastatin lactone (mevinolin) was obtained from Merck. Fluvindostatin Na salt, (Sandoz XU-62320), BMY-21950 Na salt, and BMY-22089 lactone were synthesized at Bristol-Myers Company, Pharmaceutical Research and Development Division, Wallingford, CT. The dihydroxyacid sodium salts of lovastatin and of chiral BMY-22089 were prepared from the corresponding lactones as follows: 25 mM lactone solutions in DMSO were mixed with 2 equivalents of NaOH in an equal volume of water, heated at 40°C for 45 min, diluted to 5.0 mM in water with adjustment to pH 7.85 with HCl, and stored at –25°C for up to 4 months. The other inhibitor solutions were always prepared fresh as water solutions of Na salts (5.0 mM) on the day of use.

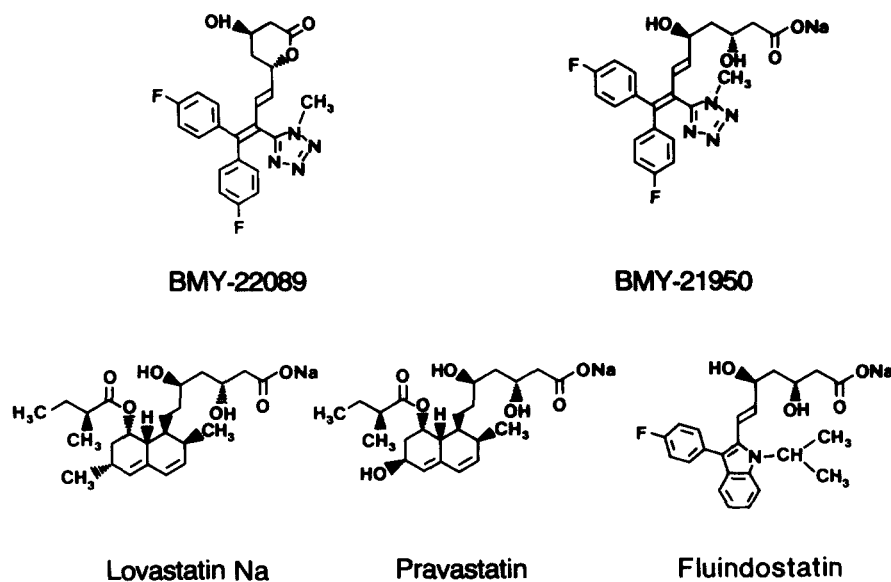


Fig. 1. Structures of the HMG-CoA reductase inhibitors discussed in this report.

All radiochemicals were obtained from New England Nuclear. Reagents and biochemicals, including collagenase for hepatocyte isolation, were from Sigma.

#### HMG-CoA reductase enzyme assay: IC<sub>50</sub> and kinetic studies

Evaluation of the 50% inhibitory concentration (IC<sub>50</sub>) of inhibitors and kinetic studies used the microsomal (97 kilodalton subunit), fully dephosphorylated form of rat liver HMG-CoA reductase, prepared as described by Parker, Miller, and Gibson (14) from cholestyramine-treated rats. IC<sub>50</sub> assays were conducted under time- and enzyme concentration-linear conditions as described (13, 14) with NADPH held at 1 mM and initial d,1-HMG-CoA concentration 0.33 mM; d,1-[3-<sup>14</sup>C]HMG-CoA was used at 1 μCi/μmol. Assays were conducted in buffer A (50 mM imidazole-HCl, pH 7.2, 250 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 5.0 mM DTT, and 20 μM leupeptin) with approximately 0.7 units (90 μg microsomal protein) of HMG-CoA reductase activity per assay. Enzyme plus inhibitor (100 μl) were preincubated at 37°C for 10 min followed by the addition (50 μl) of a mixture containing both substrates for a 10 min assay at 37°C. IC<sub>50</sub>s were calculated from linear regressions (least squares) of average percent inhibition (duplicates) versus log inhibitor concentration from at least 4 concentrations excluding values <10% or >90%; mean IC<sub>50</sub> values from multiple determinations are given in the results, with differences compared by *t*-test.

For kinetic studies, [3-<sup>14</sup>C]HMG-CoA was used at 2 μCi/μmol. The sequence of additions was: varied substrate + inhibitor + enzyme, followed by a 2-min preincubation at 37°C, followed by addition of co-substrate to initiate 2-min reactions. Initial evaluation of kinetic data was by Lineweaver-Burk plot and Dixon plot as described in the Results section. In order to accurately determine *K<sub>i</sub>*, the secondary plot method suggested by Dixon and Webb (15) was used. In this method, slope and intercept values were first determined by linear regression from a series of Lineweaver-Burk plots. The slope values were then plotted versus inhibitor concentration, yielding a straight line with I-axis intercept = -*K<sub>i</sub>* for competitive inhibition, according to the equation: Lineweaver-Burk Slope = (*K<sub>m</sub>*/*V<sub>m</sub>*)(1/*K<sub>i</sub>*)(I) + *K<sub>m</sub>*/*V<sub>m</sub>* (15).

#### Hepatocyte cholesterol biosynthesis assay

Primary rat hepatocytes were prepared for each experiment from nembutal-anesthetized normal or cholestyramine-fed (2% pure cholestyramine resin in normal chow) male Wistar rats weighing 180–260 g (Harlan, Indianapolis, IN). Rats were maintained on a reverse light cycle and hepatocytes were prepared 4 h into the dark phase. Parenchymal liver cells were obtained by the collagenase perfusion method described previously (16). Cell viability exceeded 95% initially and remained over 90%

throughout incubations. For the assay of cholesterol synthesis, aliquots of hepatocyte suspensions (approximately 100 mg wet wt in 2 ml) in Eagle's MEM with 26 mM NaHCO<sub>3</sub> and 15 mM HEPES, pH 7.4, and 2% w/v bovine albumin, were preincubated for 15 min with shaking under 95% O<sub>2</sub>/5% CO<sub>2</sub> in the presence of inhibitors or vehicle controls (0.5% v/v DMSO for lactones). Tritiated water (1 mCi/ml incubation volume) or [2-<sup>14</sup>C]acetate (1.5–2.5 mCi/mmol; 1–2 μCi/ml) was then added for a 45- to 90-min incorporation period (conditions established for time linearity). The reactions were terminated with 0.4 N perchloric acid. Total radiolabeled cholesterol was determined as digitonin-precipitable sterols from the nonsaponifiable lipid fraction after saponification in 90% methanol–0.3 N NaOH for 90 min at 70°C as described previously (16, 17). Precautions were taken to eliminate traces of digitonin-bound <sup>3</sup>H<sub>2</sub>O (17). Labeled fatty acids were estimated as the total saponified lipid fraction (16). Final digitonin-precipitated sterols were dissolved in toluene-based scintillation fluid and radioactivity was determined. The recovery of [<sup>14</sup>C]cholesterol internal standard in the tritiated water experiments was 84 ± 3%. Greater than 98% of the radioactivity in the digitonin-precipitable sterols was shown by HPLC to co-elute with cholesterol. IC<sub>50</sub>s were calculated from linear regressions of average percent inhibition (compared to vehicle controls) versus log inhibitor concentration using 4–7 inhibitor concentrations in each study, excluding values >90% or <10%. Mean IC<sub>50</sub> values from multiple experiments are given in the results with differences compared by *t*-test.

Cholesterol synthesis inhibition was also measured using dog liver tissue slices (0.2 mm thickness, 25 mg wet wt) (18). Slices were randomized for assay of inhibitors in 1.0 ml of the buffer described for hepatocytes. After a 10-min preincubation, cholesterol synthesis was assayed in duplicate by incorporation of [1-<sup>14</sup>C]acetate (1.8 mCi/mmol) for 60 and 120 min into digitonin-precipitable sterols. [<sup>14</sup>C]CO<sub>2</sub> production was determined by KOH trapping in incubation vessels as an index of slice thickness and metabolic variation. These studies were undertaken to provide an estimate of reductase inhibitor potency in a hepatic model independent of the collagenase treatment used for rat hepatocytes and to verify potency in a non-rodent species.

#### In vitro cholesterol synthesis in rat tissue slices and cell dispersions

The procedure for in vitro assays in rat ileum slices was adapted from the description by Spady and Dietschy (18). A portion of distal ileum (located 5–15 cm proximal to the coecum) was excised, rinsed, inverted, and rinsed again in incubation buffer (MEM containing 26 mM NaHCO<sub>3</sub> and 15 mM HEPES, pH 7.4, without albumin). The anatomical sequence of sliced segments (ap-

proximately 2 mm) was maintained during the assay with alternating controls and inhibitor additions to allow valid determination of inhibition despite a gradient of sterol synthesis activity along the ileum (18, 19). After a 15-min preincubation, cholesterol synthesis was assayed as described for hepatocytes, using [ $^{14}\text{C}$ ]acetate (5  $\mu\text{Ci}$  per assay, 1.8 mCi/mmol) in 0.5 ml incubation buffer. Data were normalized for slice variation by the Lowry protein assay (20) carried out on saponified mixtures.

Cell dispersions from rat testes and spleen were prepared by nonenzymatic methods (7). The tissues (pooled from three to five rats) were excised, rinsed, passed twice through a 100-mesh stainless steel filter (Collector, Thomas Scientific Co.) and suspended in MEM plus 15 mM HEPES, pH 7.4, and 2% BSA. Viability (trypan blue) exceeded 95% at zero time and 90% at the end of assays. The final cell dispersions (1.0 ml), completely disaggregated into single cells, were preincubated (160–200 mg/ml, w/v, for testes and 80–100 mg/ml for spleen) for 15 min at 37°C in the presence of inhibitor or vehicle. Cholesterol synthesis was then assayed by a 60–120 min incorporation of [ $^{14}\text{C}$ ]acetate (55 mCi/mmol, 5–10  $\mu\text{Ci}$ /ml), previously shown to give time linearity. All other conditions matched those of the hepatocyte procedures described above. Digitonin permeabilization (pretreatment of cells with 16  $\mu\text{M}$  digitonin) of testes cell preparations followed the procedure described by Fiskum et al. (21).

Rat adrenal cell dispersions were prepared by collagenase-digestion plus filtration (60- and 100-mesh) as described (22) using adrenals pooled from 16 rats. The dispersed cells were centrifuged (400 *g*, 4 min) and were again filtered through a 100-mesh screen. The final yield from 1050 mg adrenal tissue was 430 mg cells (wet wt); viability by trypan blue exclusion exceeded 90%. Incubations contained aliquots (7 mg wet wt) of adrenal cells suspended in 0.50 ml Eagle's MEM + 15 mM HEPES, pH 7.4, and 2% bovine serum albumin; a 10-min preincubation preceded a 120-min incorporation of [ $^{14}\text{C}$ ]acetate (55 mCi/mmol, 20  $\mu\text{Ci}$ /ml).

### Bovine lens epithelial cells

Bovine ocular lens epithelial cells were cultured essentially as described by Hitchener and Cenedella (23) from calf eyes obtained from a local slaughterhouse. The anterior capsules of lenses were excised and placed under coverslips in 3 ml of RPMI 1640 with 10% fetal bovine serum (FBS) and gentamycin in 100-mm plates. Primary culture of the explants continued for 10 days in a 5%  $\text{CO}_2$  incubator (37°C) at which point epithelial cell outgrowth was observed. Cells were subcultured by trypsinization of adherent cells and maintained in RPMI 1640 plus 15% FBS. The studies reported here were conducted within the first six passages of cells.

For assay of sterol synthesis in lens epithelial cells, cultures were grown to 70% confluency in six-well (35-mm diameter wells) plates containing 2.5 ml medium. Cells were induced for sterologenesi by changing growth medium at 16 h before assay to RPMI + 7% lipid-deficient serum prepared as described (24). Time-linear sterol synthesis assay conditions were established in preliminary studies (not shown). For inhibitor studies, cells were preincubated with compounds for 30 min followed by the assay of cholesterol synthesis by a 2-h incorporation of [ $^{14}\text{C}$ ]acetate (55 mCi/mmol; 5  $\mu\text{Ci}$  per well) into digitonin-precipitable sterols. [ $^3\text{H}$ ]Thymidine incorporation into DNA was used to estimate cell replication according to Sinensky and Logel (5).

### In vivo cholesterol synthesis in rats: liver and ileum

In vivo cholesterol synthesis was determined by a method similar to that described by Alberts et al. (25) in rats (250 g male Wistar, fasted for 15 h) dosed with BMY-21950 or lovastatin (20  $\mu\text{mol}$ /kg p.o.) or vehicle (0.25% methylcellulose in water) at 4 h into the dark cycle. One-half hour after dosing, [ $^{14}\text{C}$ ]acetate (1.8 mCi/mmol, 100–140  $\mu\text{Ci}$ /kg) was injected i.p. and incorporation over the next 1.0 h into cholesterol in liver, plasma, and ileum was determined. Liver and ileum homogenates were prepared in saline and used to isolate total labeled sterols, normalized to wet weight, by digitonin precipitation of the nonsaponifiable lipids using procedures adapted from those described above for hepatocytes.

## RESULTS

### Comparison of the intrinsic activities of HMG-CoA reductase inhibitors

The intrinsic activity and kinetic properties of the new HMG-CoA reductase inhibitor, BMY-21950, were first established using isolated rat liver microsomal enzyme. BMY-21950 (racemic) inhibited HMG-CoA reductase activity with an  $\text{IC}_{50}$  of  $43 \pm 10$  nM (Table 1). The chiral

TABLE 1. Intrinsic potencies of HMG-CoA reductase inhibitors

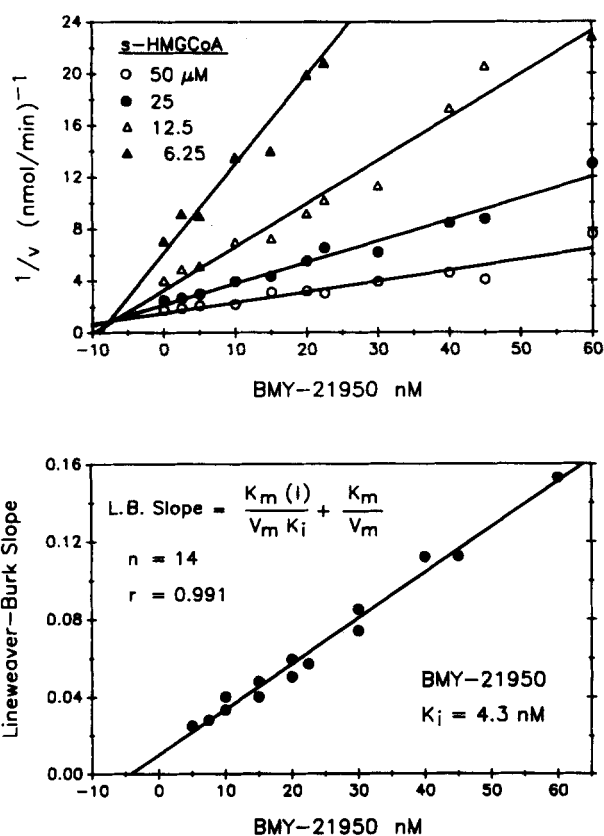
Inhibitor	n	$\text{IC}_{50}$ (nM)	Potency Relative to Lovastatin
BMY-21950	17	$43 \pm 10^a$	0.63
BMY-21950 [ $\alpha$ ] +	2	$19^b$	1.4
BMY-21950 [ $\alpha$ ] -	2	2300	0.015
BMY-22089	4	$1160 \pm 290$	0.023
Lovastatin Na*	11	$27 \pm 10^c$	1.0
Fluindostatin	4	$8 \pm 4^c$	3.4

The data represent  $\text{IC}_{50}$  values in nM (mean  $\pm$  SD for *n* determinations) for inhibition of rat liver microsomal HMG-CoA reductase activity. Compounds were tested as sodium salts of dihydroxyacids except BMY-22089 (lactone). BMY-21950 and BMY-22089 are racemic; BMY-21950 [ $\alpha$ ] + and [ $\alpha$ ] - represent the (+) and (-) enantiomers synthesized and tested separately. See Fig. 1 for structures.

\*Values with different superscripts are different at  $P < 0.05$ .

(+) isomer ( $IC_{50} = 19 \text{ nM}$ ) inhibited with approximately twice the potency of the racemate, while the (-) isomer was essentially devoid of activity. Thus, the intrinsic potency of BMY-21950 is within the range exhibited by lovastatin and fluvindostatin (Table 1) when tested as Na salts of the dihydroxy acids. These compounds, sharing with mevalonate (the product of HMG-CoA reductase) a 3,5-dihydroxy carboxylate moiety, can be reversibly converted to  $\delta$ -lactones (Fig. 1). The lactone counterparts tested directly were weak inhibitors of the isolated enzyme relative to the salts of the dihydroxyacid forms, as seen for BMY-22089 (the lactone of BMY-21950; Table 1).

Microsomal HMG-CoA reductase isolated from gerbil liver, dog liver, and human hepatoma HepG2 cells was also tested for sensitivity to inhibition by BMY-21950 and the reference agents. Similar relative potencies of the inhibitors were found using enzyme from these sources as for rat liver reductase activity (data not shown). For example, BMY-21950 (chiral) and lovastatin were equipotent with  $IC_{50}$  values of 20 nM versus HepG2 enzyme.



**Fig. 2.** Mechanism of HMG-CoA reductase inhibition by BMY-21950. Left panel: Dixon plot for BMY-21950. Right panel: secondary plot of Lineweaver-Burk slopes. This secondary plot depicts slope values calculated by linear regression from a series of Lineweaver-Burk plots, as suggested by Dixon and Webb (15).  $K_i$  was calculated by this method according to the formula:  $L. B. \text{ Slope} = \frac{K_m (I)}{V_m K_i} + \frac{K_m}{V_m}$  (15). For  $n = 14$  determinations,  $r = 0.991$ ; substituting  $K_m = 28 \mu\text{M}$  and  $V_m = 12.8 \text{ nmol/min/mg}$  yields  $K_i = 4.3 \text{ nM}$  for chiral BMY-21950.

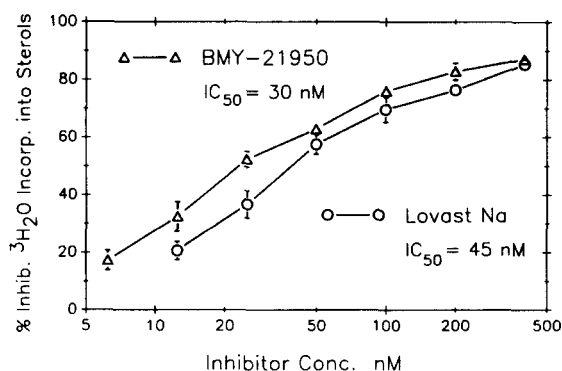
## Evaluation of the kinetic mechanism of BMY-21950

HMG-CoA reductase catalyzes a complex two-substrate reaction (26), which requires that substrate saturation analysis be conducted for both NADPH and HMG-CoA. The kinetic studies used microsomal HMG-CoA reductase prepared from livers of cholestyramine-fed rats. When NADPH was the variable substrate with HMG-CoA saturating, the apparent  $K_m$  for NADPH was 44  $\mu\text{M}$  and was not affected by 100 nM BMY-21950 (data not shown), while  $V_{max}$  was decreased. Thus BMY-21950 inhibits HMG-CoA reductase noncompetitively with respect to NADPH.

With HMG-CoA as the variable substrate and NADPH constant and saturating, competitive inhibition by BMY-21950 was evident. This finding was reasonable on structural grounds and in view of the known competitive inhibition mechanism for lovastatin (25). The Dixon plot (Fig. 2, upper panel) shows the intersecting pattern diagnostic of competitive inhibition versus HMG-CoA. As suggested by Dixon and Webb (15), a secondary plot method (Lineweaver-Burk slope vs inhibitor concentration) was used to accurately determine  $K_i$  from multiple double reciprocal plots (Fig. 2, lower panel). The linearity of this plot ( $n = 14$ ,  $r = 0.991$ ) confirms a competitive inhibition mechanism for BMY-21950 versus HMG-CoA. By this method, the  $K_i$  for the active enantiomer of BMY-21950 was 4.3 nM. Under these conditions,  $K_m$  for HMG-CoA was  $28 \pm 3 \mu\text{M}$  ( $n = 5$ ) ( $V_{max}$  was 12.8 nmol/min per mg protein). These findings indicate that chiral BMY-21950 binds rat liver HMG-CoA reductase with an affinity 6500 times higher than that of the natural substrate (i.e.,  $K_m/K_i = 6500$ ).

## Inhibition of cholesterol synthesis in primary rat hepatocytes

The effectiveness of the inhibitors in suppressing liver cholesterol biosynthesis was assessed using incorporation of tritium from tritiated water into digitonin-precipitable sterols in isolated rat hepatocytes. In the experiments depicted in Fig. 3, BMY-21950 inhibited the rate of cholesterol synthesis in a concentration-dependent manner with a calculated  $IC_{50}$  of 30 nM, while lovastatin  $\text{Na}^+$  salt inhibited with an  $IC_{50}$  of 45 nM. The slopes of the concentration-dependency curves for these agents were parallel. Comparative studies with the reference agents were continued using the less hazardous precursor [ $2\text{-}^{14}\text{C}$ ]acetate. As given in Table 2, BMY-21950 and BMY-22089 were equipotent, and exhibited  $IC_{50}$  values using [ $2\text{-}^{14}\text{C}$ ]acetate virtually identical to those obtained by the tritiated water method. These findings indicate that the lactone ring is readily hydrolyzed in hepatocytes to generate the active 3,5-dihydroxy acid form of the inhibitor. Chiral BMY-22089 ( $IC_{50} = 13 \text{ nM}$ ) was nearly twice as potent as the racemate. Using either method, fat-



**Fig. 3.** Cholesterol synthesis inhibition by BMY-21950 and lovastatin in primary rat hepatocytes. Sterol synthesis in hepatocytes was assayed by the incorporation over 90 min of tritiated water into digitonin-precipitable sterols. Mean percent inhibition  $\pm$  standard deviation from two experiments, assayed in duplicate for each inhibitor concentration, is given in the graph. [ $^3\text{H}$ ]Sterols synthesized in controls equalled 42,500 and 30,200 dpm/assay for the two experiments.

ty acid synthesis was unaffected by BMY-21950 under conditions producing up to 90% inhibition of cholesterol synthesis (data not shown). The cumulative mean  $\text{IC}_{50}$  values (Table 2) indicate that racemic BMY-21950 ( $\text{IC}_{50} = 21$  nM) inhibited cholesterol synthesis more potently ( $P < 0.03$ ) than lovastatin Na<sup>+</sup> salt ( $\text{IC}_{50} = 39$  nM). Fluvindostatin was less potent ( $P < 0.01$ ) in the hepatocyte assay than BMY-21950 and BMY-22089; the greater apparent potency of fluvindostatin in the isolated enzyme assay was not observed in the hepatocyte cholesterol synthesis inhibition assay.

In order to confirm the hepatic potency of BMY-21950 in an additional species, a dog liver tissue slice cholesterol synthesis assay was used with [ $1\text{-}^{14}\text{C}$ ]acetate incorporation (Table 2). In this system, BMY-21950 inhibited with

an  $\text{IC}_{50}$  of 23 nM (average of two experiments), and lovastatin inhibited with an  $\text{IC}_{50}$  of 35 nM. These dog liver  $\text{IC}_{50}$  values confirm the potencies using a model independent of the collagenase treatment required for hepatocyte isolation.

#### Inhibition in liver compared to ileum in vivo in rats

Using an in vivo rat model, acute inhibition of de novo cholesterol synthesis in liver was measured by [ $2\text{-}^{14}\text{C}$ ]acetate incorporation after oral administration of inhibitors. BMY-21950 inhibited formation of  $^{14}\text{C}$ -labeled cholesterol assayed in plasma and in liver with a 50% inhibitory dose ( $\text{ED}_{50}$ ) of 0.1 mg/kg (data not shown). For lovastatin Na, the  $\text{ED}_{50}$  was approximately 0.05 mg/kg, in agreement with the value reported by Alberts et al. (25) using a similar protocol. In the latter study, the  $\text{ED}_{50}$  for compactin was 0.29 mg/kg. In separate studies BMY-22089 lactone (0.05 to 0.5 mg/kg p.o.) was more effective than comparable doses of BMY-21950.

The acute rat model presented a possible approach to examining the question of differential inhibition by HMG-CoA reductase inhibitors in different tissues. Due to low rates of de novo synthesis, quantification of  $^{14}\text{C}$ -labeled sterols in most extrahepatic tissues is occluded by label contributed by the blood. However, the high blood background is negligible for the intestine as well as the liver. Cholesterol synthesis in a portion of the distal ileum of the rat may, under some conditions, exceed that in the liver on a per gram basis (19). When in vivo cholesterol synthesis in rat ileum was examined, a striking difference between BMY-21950 and lovastatin was observed. In the experiment depicted in Fig. 4 (upper panel), rats were dosed orally with 20 mg/kg BMY-21950 or 8 mg/kg lovastatin Na salt (viz., 20  $\mu\text{mol/kg}$  for the active enan-

TABLE 2. Hepatocyte cholesterol synthesis inhibition by HMG-CoA reductase inhibitors

Compound	Isolated Rat Hepatocytes <sup>a</sup>						Dog Liver Slices <sup>b</sup>	
	[ $^{14}\text{C}$ ]Acetate		$^3\text{H}_2\text{O}$		Cumulative		[ $^{14}\text{C}$ ]Acetate	
	n	$\text{IC}_{50}$ nM	n	$\text{IC}_{50}$ nM	n	$\text{IC}_{50}$ nM	n	$\text{IC}_{50}$ nM
BMY-21950	7	21 $\pm$ 4	4	22 $\pm$ 3	11	21 $\pm$ 3	2	23
BMY-22089	1	24	1	20	2	22		nd
BMY-22089 (+) <sup>c</sup>	2	13		nd		nd		nd
Lovastatin	6	32 $\pm$ 8	3	52 $\pm$ 9	9	39 $\pm$ 7 <sup>d</sup>	2	35
Fluvindostatin	5	52 $\pm$ 10 <sup>e</sup>		nd		nd		nd

The data represent  $\text{IC}_{50}$  values (nM, mean  $\pm$  SEM from n independent determinations). Cumulative  $\text{IC}_{50}$ s are the mean values from both isotopic methods in hepatocytes. All inhibitors except BMY-22089 (lactone) were tested as dihydroxyacid Na salts; BMY-21950 and BMY-22089 were tested as racemates except where footnoted; nd, not determined.

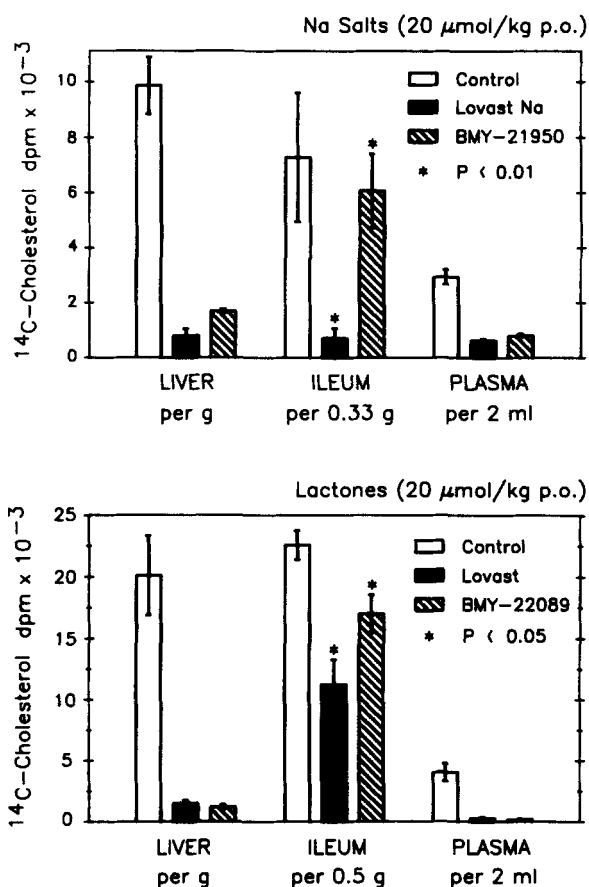
<sup>a</sup>Sterol synthesis was assayed in hepatocytes isolated from normal rats ([ $^{14}\text{C}$ ]acetate) or cholestyramine-fed rats ( $^3\text{H}_2\text{O}$  incorporation).

<sup>b</sup>Sterol synthesis was assayed in dog liver tissue slices using [ $^{14}\text{C}$ ]acetate incorporation.

<sup>c</sup>Chiral ( $[\alpha] + 240^\circ$ ) enantiomer of BMY-22089.

<sup>d</sup> $P < 0.03$  vs BMY-21950.

<sup>e</sup> $P < 0.01$  vs BMY-21950.



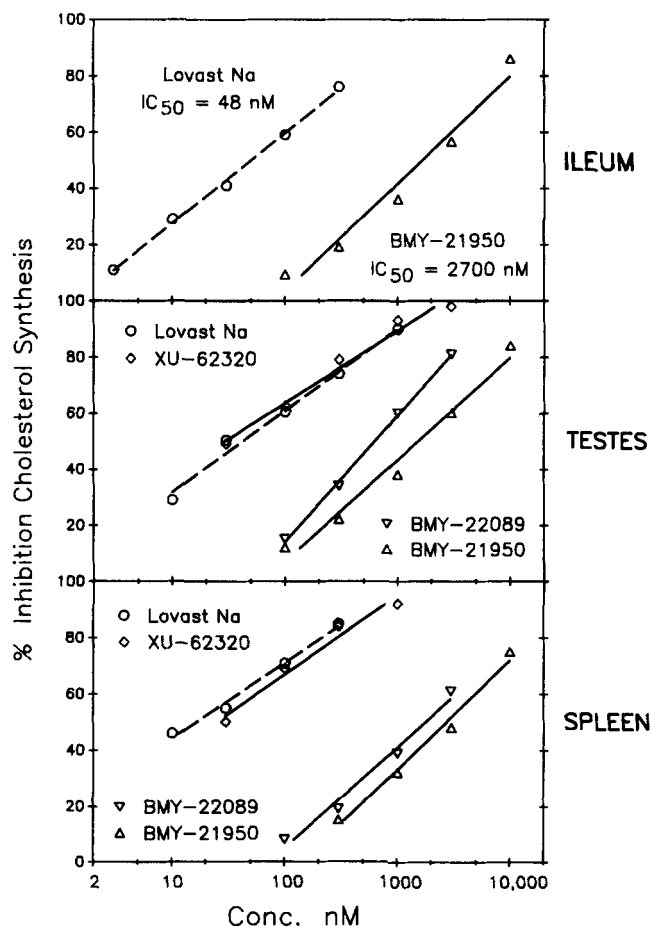
**Fig. 4.** Liver selective inhibition of sterol synthesis in vivo by BMY-21950 and BMY-22089. Inhibitors (20  $\mu\text{mol/kg}$ ) or methylcellulose vehicle control were administered p.o. to rats (upper panel,  $n = 4$  per group; lower panel,  $n = 6$  per group). Drugs were given as dihydroxyacid salt forms (upper panel) or as lactone forms (lower panel). Thirty min after dosing, [ $^{14}\text{C}$ ]acetate was injected i.p. for a 60-min incorporation, and radiolabeled sterols were quantified in liver, distal ileum, and blood plasma. Liver and ileum data were normalized on the basis of wet wt.

tiomer of BMY-21950 and for lovastatin). Liver sterol synthesis was suppressed 92% by lovastatin and 83% by BMY-21950; these values were reflected by comparable drops in [ $^{14}\text{C}$ ]sterols assayed in plasma. However, ileal synthesis was inhibited 90% by lovastatin Na but was essentially not inhibited (17%) by BMY-21950 ( $P < 0.01$  vs lovastatin). When the identical experiment was conducted using the lactone forms of the two drugs (Fig. 4, lower panel), both liver and plasma [ $^{14}\text{C}$ ]cholesterol were decreased 92% by lovastatin lactone and 95% by BMY-22089 (each at 20  $\mu\text{mol/kg}$  oral dose), while lovastatin lactone inhibited 50% in ileum compared to 24% inhibition by BMY-22089 ( $P < 0.05$  vs lovastatin). The greater ileal inhibition in vivo by the dihydroxyacid salt of lovastatin relative to the lactone form is consistent with the reported lower hepatic extraction and higher blood levels for the dihydroxyacid form of this drug (8). Moreover, the insensitivity of rat ileum to BMY-22089 and BMY-21950 (Fig. 4) suggests that the ileum may also discriminate these

agents from pravastatin since, according to the ex vivo study by Tsujita et al. (7), pravastatin inhibited both ileal and liver cholesterol synthesis in rats by greater than 93% at a dose similar to that used in the present study.

#### Specificity of reductase inhibitors in rat tissues in vitro

An in vitro approach could help distinguish between drug disposition versus differential cellular penetration as the basis for the apparent specificity of inhibition in liver over ileum (Fig. 4). Using a tissue slice method, the technical difficulty posed by the gradient of sterologenic activity along the ileal axis (19) was overcome by maintaining the anatomical relationship between slices and alternating control incubations with inhibitor treatment in the sequence. A striking difference in the sensitivity of rat ileum to lovastatin compared to BMY-21950 can be seen in the data (Fig. 5, top panel). The results indicate



**Fig. 5.** Selectivity of reductase inhibitors in rat tissues in vitro. Rat ileum tissue slices (upper panel), testes cell dispersions (center panel), and spleen cell dispersions (lower panel) were tested for sensitivity to HMG-CoA reductase inhibitors by assaying [ $^{14}\text{C}$ ]acetate incorporation into cholesterol. Conditions matched hepatocyte experiments (Table 2) as described in Methods. The data shown represent mean values from two experiments (ileum) or average percent inhibition (duplicates) from typical single experiments (testes and spleen).

that BMY-21950 ( $IC_{50} = 2700$  nM) inhibited 57-fold less potently than lovastatin (mean  $IC_{50} = 48$  nM) in rat ileum. The data, moreover, provide a basis for the *in vivo* findings given in Fig. 4, since the  $IC_{50}$  value for BMY-21950 is 129 times higher in ileum than hepatocytes *in vitro* compared to only 1.2 times higher in the case of lovastatin Na.

The possibility that BMY-21950 and BMY-22089, in comparison to the reference agents, inhibit selectively with respect to other tissues was further examined in a series of *in vitro* studies in isolated rat testes, spleen, and adrenal cell dispersions. The concentration dependency curves for BMY-21950 and BMY-22089 were shifted 1–2 orders of magnitude to the right of the lovastatin and fluvindostatin curves (Fig. 5, center and lower panels). The composite results of these *in vitro* studies are summarized in Table 3. The mean  $IC_{50}$  values for BMY-21950 were 149-, 86-, and 76-fold higher in spleen, testes, and adrenals, respectively, than in rat hepatocytes. The lactone BMY-22089 also exhibited mean  $IC_{50}$ s that were 79-, 24-, and 33-fold higher, respectively, than in hepatocytes. In contrast, lovastatin and fluvindostatin were essentially nonspecific with  $IC_{50}$  ratios (tissue/hepatocyte) near unity (Table 3).

#### Selectivity in cultured bovine ocular lens epithelial cells

Bovine ocular lens epithelial cells were cultured in monolayers and used to assay sterol synthesis inhibition. Table 3 includes the summarized  $IC_{50}$  values for the reductase inhibitors in this model. BMY-21950 and BMY-22089 were weakly inhibitory with  $IC_{50}$  values of 3940 nM and 1990 nM, respectively. Lovastatin Na and fluvindostatin were again potent with  $IC_{50}$  values close to their hepatocyte values.

dostatin were again potent with  $IC_{50}$  values close to their hepatocyte values.

Although the drug concentrations to which the ocular lens might be exposed *in vivo* are unknown, the potential significance of isoprenoid pathway inhibition in a dividing cell population is underscored by the experiment depicted in Fig. 6. Bovine lens epithelial cells grown in monolayers to 60–70% confluency were incubated with a series of concentrations of BMY-21950 or lovastatin Na. After 16 h of incubation, [ $^3$ H]thymidine incorporation into DNA over the next 8 h was assayed as an index of cell replication. In parallel cultures, cholesterol synthesis inhibition by [ $^{14}$ C]acetate incorporation was assayed during the initial 2 h of incubation. The results depicted in Fig. 6 indicate that conditions producing approximately 90% inhibition of sterol synthesis (approximately 1000 nM lovastatin) correlated with approximately 50% decrease in [ $^3$ H]thymidine incorporation. In contrast, at the same concentration (1000 nM), BMY-21950 inhibited sterologenesis less than 50% with virtually no detectable effect on DNA synthesis.

HMG-CoA reductase activity in microsomes isolated from rat testes and spleen was approximately equally sensitive to BMY-21950 as was liver microsomal enzyme (data not shown). In view of the above results, this suggests that different HMG-CoA reductase inhibitors may not gain equal access to the intracellular site of the enzyme in a given tissue. The experiments depicted in Fig. 7 suggest that the permeability of BMY-21950 into the intracellular compartment of nonhepatic cells is limited compared to lovastatin. Increasing the time of preincubation (Fig. 7, top panel) in testes cell dispersions increased the degree of inhibition by BMY-21950 while the effect of

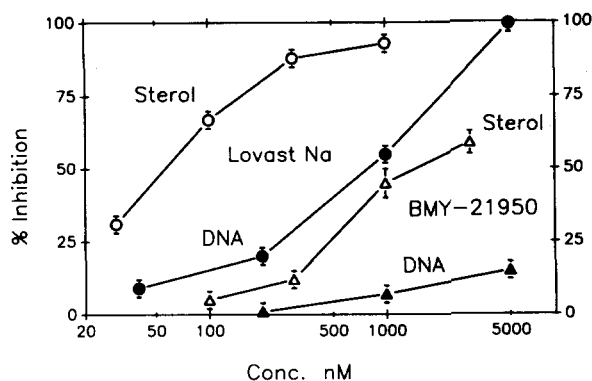
TABLE 3. HMG-CoA reductase inhibitor potencies in extrahepatic tissues *in vitro*

Inhibitor	$IC_{50}$ (nM) for Cholesterol Synthesis Inhibition				
	Spleen	Testes	Adrenal	Lens Epith.	Ileum
BMY-21950	3150 ± 359 n = 5 (149) <sup>a</sup>	1810 ± 199 n = 5 (86)	1600 n = 1 (76)	3940 n = 5 (188)	2700 n = 2 (129)
BMY-22089	1730 ± 20 n = 2 (79)	532 ± 68 n = 2 (24)	720 n = 1 (33)	1990 n = 4 (90)	nd
Lovastatin	45 ± 10 n = 5 (1.2)	50 ± 6 n = 5 (1.3)	<50 n = 1 (<1.3)	61 n = 3 (1.5)	48 n = 2 (1.2)
Fluvindostatin	62 ± 20 n = 3 (1.2)	87 ± 28 n = 3 (1.7)	<50 n = 1 (<1)	nd	nd

Inhibitors were tested as Na salts of dihydroxyacids except BMY-22089 lactone. Spleen, testes, and adrenal cell dispersions, and distal ileum tissue slices were from rat. Lens epithelial cell culture was derived from bovine ocular lens. Cells were preincubated with inhibitors for 15 min followed by assay of cholesterol synthesis by a 60-min [ $^{14}$ C]acetate incorporation. Values given as the mean (± SD)  $IC_{50}$  (nM) for sterol synthesis inhibition; n, number of experiments; nd, not determined.

<sup>a</sup>Values in parentheses are  $IC_{50}$  ratios for each tissue/rat hepatocytes. The hepatocyte  $IC_{50}$ s used to calculate the ratios are given in Table 2.





**Fig. 6.** Differential effects of reductase inhibitors on cholesterol synthesis and [<sup>3</sup>H]thymidine incorporation into DNA in bovine lens epithelial cells. Bovine ocular lens epithelial cells were incubated with the indicated levels of lovastatin Na or BMY-21950. [<sup>14</sup>C]Acetate incorporation during hours 1–2 was used to assay cholesterol synthesis (open symbols), while [<sup>3</sup>H]thymidine incorporation into DNA over hours 16–24 (closed symbols) provided an index of the effect on cell replication.

lovastatin remained constant. When the permeability of the testes cell plasma membranes was increased by pre-treating the cells with digitonin (Fig. 7, lower panel), the inhibition by BMY-21950 was again increased while the effect of lovastatin remained unchanged. These studies provide evidence for a cellular permeability-based mechanism underlying the apparent tissue selectivity of BMY-21950 and BMY-22089, in which the active site of the enzyme in nonhepatic cells is exposed to a substantially lower inhibitor concentration than is present in the extracellular environment.

## DISCUSSION

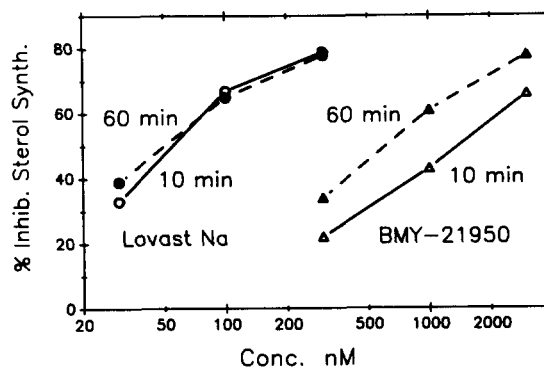
With respect to intrinsic potency, BMY-21950 and its lactone counterpart BMY-22089 represent nearly optimized compounds within this structural series of HMG-CoA reductase inhibitors. Potency is strikingly sensitive to the nature of the substituent at the position of the methyl-tetrazole (13) in the structure of BMY-21950 (Fig. 1). Essentially all of the activity of the racemic form of BMY-21950 can be accounted for by one enantiomer ( $[\alpha] = +240^\circ$ ), with a relative potency approximately 1.4 times that of lovastatin, a chiral product of microbial fermentation.

Kinetic studies were conducted using the native microsomal form of HMG-CoA reductase (subunit molecular mass = 97,000 daltons) prepared from livers of cholestyramine-induced rats. The mean  $K_m$  for S-HMG-CoA was  $28 \pm 3.1 \mu\text{M}$ . Under these conditions, the  $K_i$  for the active isomer of BMY-21950 was 4.3 nM. The ratio of  $K_m/K_i$  provides a way of comparing affinities of different inhibitors evaluated in different laboratories. Using the soluble (53,000 dalton subunit), proteolytic fragment form of HMG-CoA reductase, Alberts et al. (25)

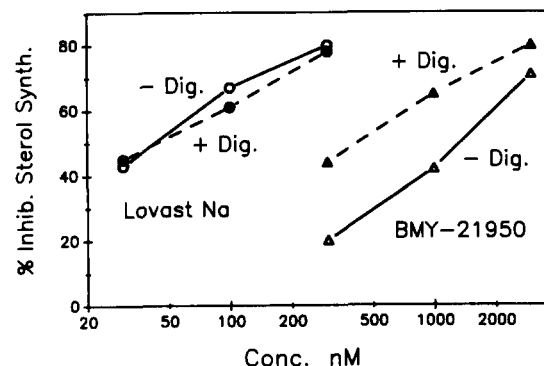
reported a  $K_m$  of 4.0  $\mu\text{M}$  for S-HMG-CoA and a  $K_i$  equal to 0.64 nM for lovastatin Na ( $K_m/K_i = 6200$ ). As noted by Grundy (27), the data indicate that the affinity of lovastatin for the active site of HMG-CoA reductase is 6200 times that of the substrate. Based on our kinetic studies, the active enantiomer of BMY-21950 has an affinity 6500 times that of HMG-CoA ( $K_m/K_i = 6500$ ) and therefore appears to bind the enzyme with approximately the same affinity as lovastatin. This conclusion is consistent with the comparative  $\text{IC}_{50}$  data determined in the isolated enzyme system (Table 1).

Liver parenchymal cells represent the primary target for HMG-CoA reductase inhibitors. The effectiveness of these agents in lowering LDL cholesterol in humans depends largely upon up-regulation of liver LDL receptors, a biochemical consequence of effective inhibition of cholesterol synthesis in hepatocytes (1). The assay of cholesterol synthesis through the incorporation of tritium from tritiated water is one of the most valid methods to evaluate inhibitors of cholesterol synthesis in cells (17). BMY-21950 was at least as potent as lovastatin as an inhibitor of cholesterol synthesis in the primary rat hepato-

### EFFECT OF PREINCUBATION TIME IN RAT TESTES CELLS



### EFFECT OF DIGITONIN-PERMEABILIZATION



**Fig. 7.** Effects of preincubation time and digitonin-permeabilization of rat testes cells on inhibition sensitivity. Rat testes cell dispersions were preincubated with inhibitors for 10 or 60 min (upper panel), or after pre-treatment of cells with or without 16  $\mu\text{M}$  digitonin (dig.) to permeabilize plasma membranes (lower panel). Cholesterol synthesis was then assayed by a 60-min [<sup>14</sup>C]acetate incorporation.

cyte model using either isotopic method. The lactone BMY-22089 was equipotent to BMY-21950, suggesting efficient conversion of the lactone to the active species.

The present studies provide the first demonstration of an HMG-CoA reductase inhibitor that exerts little or no significant effect in the ileum of the rat under conditions in which liver cholesterol synthesis is maximally inhibited (Fig. 4). While pravastatin was the first HMG-CoA reductase inhibitor shown to exhibit tissue selective properties, this agent inhibited both liver and ileal cholesterol synthesis strongly while affecting other tissues very weakly in the *ex vivo* study by Tsujita et al. (7). In the same study, using oral dose and time conditions similar to our own *in vivo* study, lovastatin (MB-530B) Na salt was highly inhibitory in all rat tissues examined including ileum. When fed chronically to rats (28), lovastatin induced large increases in HMG-CoA reductase in the intestine, indicative of pronounced inhibition of sterologenesis in this organ. Fluindostatin gave no evidence of tissue selectivity in our assays nor in the lens study of Mosely et al. (3). Although the effects of pravastatin in the models described in this report await future evaluation, the present studies do confirm and extend the concept of tissue selectivity associated with pravastatin but not lovastatin (3, 7, 29). Based on differential sensitivity to inhibition, BMY-21950 and BMY-22089 represent the first synthetic HMG-CoA reductase inhibitors that exhibit a very high magnitude of tissue selectivity while maintaining equal hepatic potency with lovastatin.

Cholesterol synthesis in the intestine of the rat and other species (including primates) follows a longitudinal gradient of activity with high rates exhibited in the distal ileum (18, 19). Sterol synthetic activity also varies along the villus-crypt axis (19, 28). The regulation and localization is complex, although under some conditions (e.g., fat feeding) intestinal cholesterol synthesis appears to subserve chylomicron formation (19). Simvastatin, a close analogue of lovastatin, was recently shown to affect cholesterol absorption in cholesterol-fed rabbits (30), suggesting a direct action within intestinal mucosal cells that alters cholesterol esterification efficiency. On the other hand, provision for membrane formation in the replenishment and differentiation of the mucosal epithelial layer is a major function of locally synthesized cholesterol in the intestine. Comparison of BMY-21950 (or BMY-22089) with lovastatin or fluindostatin may provide a useful approach to examine the function and significance of ileal sterologenesis under conditions in which hepatic synthesis can be selectively and maximally blocked.

Dividing mammalian cells require nonsterol isoprenes for DNA replication in addition to cholesterol for membrane synthesis. Studies of the ocular lens indicate that this organ, because of its isolation from circulating lipoproteins, may rely largely on *de novo* synthesis to supply

cholesterol for normal cell growth and differentiation, (23, 31). In vertebrates, all cells of the ocular lens are derived directly from a monolayer of epithelial cells at the anterior surface of the lens which continually differentiate to form the elongated, extremely cholesterol-rich fiber cells (23). Therefore, the lens epithelial cells are an interesting and relevant model for evaluation of cell selectivity of HMG-CoA reductase inhibitors. The findings presented here, as well as previously published results (3), indicate that the potential for interference in growth, differentiation, and maintenance of a tissue such as lens epithelium may be reduced with tissue-selective inhibitors such as BMY-21950 or pravastatin in comparison with lovastatin.

While the data of Fig. 4 suggest that the *in vitro* tissue selectivity of HMG-CoA reductase inhibitors may extend to the *in vivo* situation, further extrapolation of the data for BMY-21950 and BMY-22089 is tenable only to the extent that drug blood level and tissue distribution information becomes available. Because these data have not yet been obtained, direct comparisons cannot be made with other inhibitors at the present time. The distribution study of Germershausen et al. (9) showed that the plasma level of active inhibitor in rats dosed orally (25 mg/kg) with lovastatin peaked 1 h after dosing at 81 ng/g. This value is equivalent to approximately 200 nM, indicating that under these conditions *in vivo* the spleen and testes may be exposed to approximately 80% and 70% inhibitory levels of lovastatin, respectively, based on our *in vitro* profiles. The spleen and testes would require exposure to greater than 10,000 nM and 5,000 nM (5000 and 2500 ng/ml) levels of BMY-21950, respectively, for comparable levels of inhibition. However, until plasma drug concentration data for equally effective doses of the various inhibitors are available, conclusions regarding the relative degree of peripheral tissue inhibition *in vivo* may not be drawn. In this connection it is interesting to note the findings of a recent report (32) describing acute sterol synthesis inhibition and chronic HMG-CoA reductase induction in mononuclear leukocytes from humans treated with therapeutic doses of lovastatin. Finally, it remains possible that individual variation in blood levels of reductase inhibitors may be encountered in the general population. Thus, at any given plasma drug concentration, the selective inhibitors may manifest less inhibition in cells of the peripheral tissues than the nonselective agents.

The molecular mechanisms underlying selectivity for BMY-21950 and BMY-22089, as well as pravastatin (3, 7), appear to involve relatively low cellular penetration of the inhibitors in extrahepatic cells compared to liver. This explanation provides an important context for the recent report showing greater peripheral distribution of pravastatin than lovastatin (9). The methodology used in the latter study could not distinguish peripherally distributed

drug which remained excluded from the intracellular site of the enzyme (viz., interstitial) and thus was not inhibitory, from drug that had penetrated into cells and therefore was inhibitory. The biochemical basis for the selectivity of BMY-21950 is currently under further investigation in this laboratory. Structure-activity relationships for this property suggest that, in part, the hydrophilicity of the molecule resulting from the methyl-tetrazole moiety confers liver selectivity (R. A. Parker, S. Y. Sit, unpublished data). In consideration of potential side effects related to inhibition of isoprenoid synthesis in the tissues, the reductase inhibitor profiles in the present study indicate that, in addition to the pharmacodynamic-based disposition which imparts a semblance of liver specificity to lovastatin, the intrinsic sensitivity of extrahepatic tissues to inhibition should also be taken into account. ■

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