

Inhibition of ileal sodium-dependent bile acid transport by 2164U90

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Abstract Inhibition of the ileal bile acid active transport system, previously shown to be mechanism underlying the hypocholesterolemic activity of 2164U90 in rodents, was further characterized in isolated intestinal preparations from three species. 2164U90 inhibited sodium-dependent transport of taurocholic acid by Caco-2 cells and by monkey and human ileal brush border membrane vesicles in a concentration-dependent manner with IC_{50} s of 7 μ M, 5 μ M, and 2 μ M, respectively. In rat ileal brush border membrane vesicles, 2164U90 was a competitive inhibitor of sodium-dependent taurocholic acid uptake with an estimated K_i of $1.8 \pm 0.2 \mu$ M. In anesthetized rats, 5 μ M 2164U90 placed in the isolated distal ileum with 3 mM [³H]taurocholic acid decreased ileal uptake, transport into the bile, and transport rate of taurocholic acid by 31–35%. Stereospecificity of inhibition by 2164U90 was demonstrated by the relative inactivity of three other possible stereoisomers in rat ileal sacs and brush border membrane vesicles. 2164U90 did not inhibit sodium-dependent glucose transport by monkey jejunal brush border membrane vesicles, indicating that 2164U90 may be specific for the bile acid transporter. ■ These results suggest that 2164U90 is a potent, selective, stereospecific, competitive inhibitor of the sodium-dependent bile acid transporter in the ileal mucosal cell brush border membrane.—Root, C., C. D. Smith, D. A. Winegar, L. E. Bricaddy, and M. C. Lewis. Inhibition of ileal sodium-dependent bile acid transport by 2164U90. *J. Lipid Res.* 1995. 36: 1106–1115.

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Interruption of the enterohepatic circulation of bile acids by decreasing intestinal bile acid reabsorption is recognized as a desirable approach to lowering plasma LDL cholesterol concentrations (1). Currently, the only approved drugs having this mechanism of action are colestipol and cholestyramine. Both are weak, nonspecific anion exchange resins that sequester bile acids in the gastrointestinal tract. The resulting decrease in bile acid reabsorption up-regulates the rate-limiting enzyme in bile acid synthesis, hepatic cholesterol 7 α -hydroxylase. Consequently, the substrate cholesterol pools in the liver are

depleted. This cholesterol depletion up-regulates hepatic LDL receptor synthesis, which ultimately lowers circulating plasma LDL cholesterol concentrations (2).

An approach taken by researchers in the development of hypocholesterolemic agents has been to interrupt the enterohepatic circulation of bile acids by specifically targeting the ileal bile acid active transport system. This saturable, bile acid specific, sodium-dependent transport system, confined to the most distal portion of the ileum, was first characterized by Lack and colleagues in the early 1960s (3, 4). Subsequent studies have indicated that a transporter associated with the ileal brush border membrane is responsible for the binding and active co-transport of bile acids with sodium ions (4–10). Photo-affinity labeling studies in rabbit (7) and rat (8, 9) ileal brush border membranes with photoreactive taurocholic acid derivatives, and expression cloning studies of a cDNA from hamster ileum (10), have identified integral membrane proteins that exhibit characteristics of the sodium-dependent bile acid transporter. This active transport system in the distal ileum is generally accepted to be the major route of bile acid reabsorption in humans and other animals (11–16).

Physical inactivation of this site of active reabsorption of bile acids by partial ileal bypass surgery on hypercholesterolemic patients is an effective means of lowering plasma LDL cholesterol. In a controlled clinical trial, this surgery produced a sustained lowering of plasma LDL cholesterol (38% after 5 years), a significant decrease (35% lower incidence) in the combined end point of death due to coronary heart disease and nonfatal myocardial infarction, and a significant slowing of the progression of

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; TC, taurocholic acid; BBMV, brush border membrane vesicles; RSS, residual sums of squares; BLM, basolateral membrane.

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atherosclerosis (17). These clinical findings advocate the hypothesis that transporter specific pharmacological inactivation of the ileal bile acid active transport system could produce similar beneficial effects in hypercholesterolemic patients, without the trauma of surgery or the unpleasant side effects of bile acid binding resins. In support of this concept, clinical studies on healthy subjects have indicated that ursodeoxycholic acid, which is absorbed via the transporter, competitively inhibits ileal absorption of endogenous bile acids, lowers plasma LDL cholesterol (18, 19), and up-regulates hepatic bile acid synthesis (20).

2164U90 is a new hypocholesterolemic compound that inhibits the ileal bile acid active transport system (21). This compound is a small, uncharged, lipophilic benzothiazepine derivative with a molecular weight similar to that of a bile acid. In vitro, low concentrations (0.3–30 μ M) of 2164U90 inhibited uptake and active transport of taurocholic acid by rat everted ileal sacs. In vivo, 2164U90 given orally for 2 days b.i.d. (3–30 mg/kg) inhibited absorption of a TC analog, 75 Se-homocholeic acid taurine, in normal rats and mice. Comparable doses of 2164U90 (1–25 mg/kg) lowered plasma LDL + VLDL cholesterol in diet-induced hypercholesterolemic animals. These results imply that inhibition of bile acid absorption via inhibition of the ileal bile acid active transport system underlies the hypolipidemic action of this compound.

The goal of the present work was to further characterize the mechanism of action of 2164U90 with isolated intestinal preparations. We have examined the effects of 2164U90 on taurocholic acid transport by rat everted ileal sacs, Caco-2 cells, and rat, monkey, and human ileal brush border membranes in vitro, and on isolated rat ileum in vivo. We present evidence here that this compound is a potent, selective, stereospecific, competitive inhibitor of the ileal sodium-dependent bile acid transport system.

METHODS

Materials

2164U90 [(–)-(3R,5R)-*trans*-3-butyl-3-ethyl-2,3,4,5-tetrahydro-5-phenyl-1,4-benzothiazepine 1,1-dioxide], 2163U90 [(+)-(3S,5S)-*trans*-3-butyl-3-ethyl-2,3,4,5-tetrahydro-5-phenyl-1,4-benzothiazepine 1,1-dioxide], 1357U88 [(+)-*trans*-3-butyl-3-ethyl-2,3,4,5-tetrahydro-5-phenyl-1,4-benzothiazepine 1,1-dioxide], and 1370U88 [(+)-*cis*-3-butyl-3-ethyl-2,3,4,5-tetrahydro-5-phenyl-1,4-benzothiazepine 1,1-dioxide] were synthesized in the Division of Organic Chemistry at Burroughs Wellcome Co. The chemical structures of these compounds are shown in Fig. 1. [3 H]taurocholic acid (2.1–2.6 Ci/mmol), [14 C]taurocholic acid (46.7 mCi/mmol), and [3 H]glucose (15.5 Ci/mmol) were obtained from New England Nuclear. Unlabeled taurocholic acid (98% pure) and chenodeoxycholic acid (97% pure) were obtained from Sigma Chemical Co. Ursodeoxycholic acid and

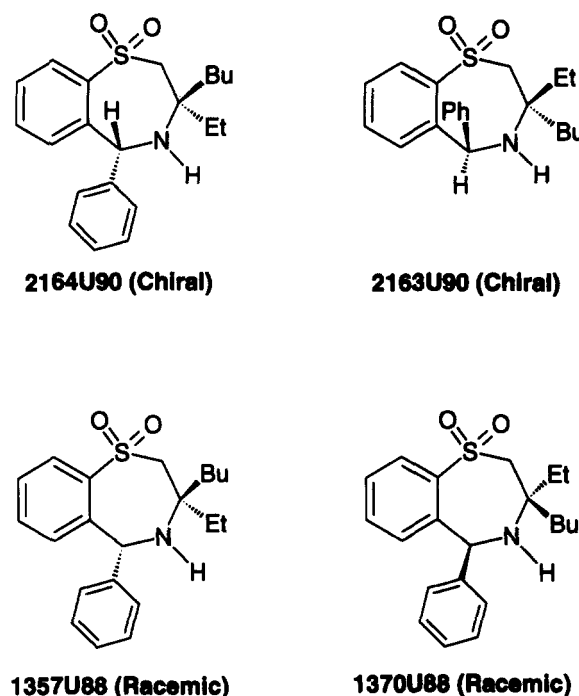


Fig. 1. Chemical structures of 2164U90, 2163U90, 1357U88, and 1370U88.

tauroursodeoxycholic acid were obtained from Steraloids, Inc.

Rat everted ileal sacs

Everted ileal sacs 5–6 cm long (four sacs per ileum, arranged according to a 4 × 4 Latin square) were prepared from male rats by the method of Wilson and Wiseman (22). The mucosal uptake and mucosal to serosal active transport of taurocholic acid, at an initial concentration of 0.37 mM, were determined in a 30-min incubation period by a previously described modification (21) of the procedure of Lack and Weiner (23).

Caco-2 cells

Cell culture. Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD) and used between passages 9 and 28. Cell stocks were maintained in 75 cm² flasks at 37°C in a humidified atmosphere of 5% CO₂, 95% air. The culture media consisted of high glucose (4.5 g/l) Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μ g/ml streptomycin. For transport studies, Caco-2 cell suspensions were plated onto 12-mm-diameter Transwell polycarbonate membranes (3.0 μ m pore size, Costar) at a density of 6.3 × 10⁴ cells/cm². The Transwell inserts were placed in 12-well culture plates with 0.5 ml media in the upper (apical) compartment and 1.5 ml media in the lower (basolateral) compartment. Apical and basolateral culture media were replaced every other day.

Transport studies. Cells grown on Transwell inserts for 21 or 22 days were used for taurocholic acid (TC) transport studies. Three monolayers were used for each condition. Complete medium in both apical and basolateral compartments was removed and replaced with pre-warmed transport medium consisting of Hank's balanced salt solution containing 25 mM glucose and 10 mM HEPES buffer, pH 7.4. Passive transport was determined by substituting KCl for NaCl in the transport medium. After a 30-min equilibration period at 37°C in a CO₂ incubator, 2164U90 or 2163U90 dissolved in 95% ethanol was added to the apical compartment for an additional 30 min. Control cells received equivalent ethanol (0.2%). Transport studies were initiated by addition to the apical compartment of [¹⁴C]TC to make 5 μM. Sampling of the basolateral compartment was done at 30, 60, 90, and 120 min. Transport was stopped by removing the insert from the multiwell dish and withdrawing the apical media. Cells were lysed in 0.2 N NaOH and protein concentration was determined. Total radioactivity in aliquots of the apical media, basolateral media, and cell lysates was determined using a Packard TRI-CARB liquid scintillation analyzer. Transport was linear for at least 60 min in all experiments. Results are expressed as picomoles of TC transport per milligram of protein per hour. The integrity of representative cell monolayers was confirmed by measurement of very low (<1% of applied dose) transcellular flux of 100 μM [¹⁴C]mannitol substituted in the apical media in place of [¹⁴C]TC exactly as described above for [¹⁴C]TC.

Ileal brush border membrane vesicles (BBMV)

Preparation of BBMV. BBMV were prepared from distal ileal tissue of male Sprague-Dawley rats (Charles River), Cynomolgus monkeys (Hazelton, Washington), or human surgical specimens (UCLA Medical Center) by subcellular fractionation, using the Ca²⁺ sedimentation method (24, 25). Monkey and human BBMV were stored in 50% glycerol under liquid N₂ until needed, and rat BBMV were freshly prepared on the day of use. The final pellet containing washed BBMV was resuspended immediately prior to use, in 280 mM mannitol, 20 mM HEPES-Tris, pH 7.4 (rat), or 300 mM mannitol, 10 mM HEPES-Tris, pH 7.4 (monkey and human). Vesicle protein concentration was determined.

Taurocholic acid (TC) uptake measurements in BBMV. TC uptake by rat BBMV was determined at room temperature using the rapid filtration technique adapted from Barnard and Ghishan (26). Test compounds were dissolved in 100% ethanol and added to a [³H]TC-containing pre-incubation buffer. Equivalent ethanol was added to control buffers (with no inhibitor). TC uptake was initiated by the addition of 50 μl of the vesicle suspension to 150 μl of the [³H]TC-containing pre-incubation

buffer. Final concentrations of components in the incubation medium were as follows: 1.3–1.8 mg/ml vesicle protein, 10 μM [³H]TC, 100 mM NaCl for total uptake or 100 mM KCl for passive uptake, 80 mM mannitol, 20 mM HEPES-Tris, pH 7.4, 0–10 μM test compound, and 1% ethanol. Uptake was terminated at 30 sec by dilution and vortexing with ice-cold stop solution containing 100 mM KCl, 80 mM mannitol, 0.1 mM unlabeled TC, and 20 mM HEPES-Tris, pH 7.4. The quenched vesicle mixture was quickly transferred onto a chilled, pretreated 0.45 μm filter, under vacuum in a single manifold filter holder, and washed three times with 4 ml of ice-cold stop solution. Total radioactivity on the washed filters was determined with a liquid scintillation analyzer. Correction was made for nonspecific retention of radioactivity by blank filters. TC uptake is expressed as picomoles per milligram of vesicle protein. Passive uptake (determined in the presence of KCl) was subtracted from total uptake (determined in the presence of NaCl) for a measurement of sodium-dependent TC uptake.

TC uptake by monkey and human BBMV was determined as described above, except that mannitol and HEPES-Tris concentrations in the incubation buffers were 85 mM and 17.5 mM, respectively.

Kinetic studies in rat BBMV. Our preliminary work with the rat BBMV preparations determined that the uptake of 10–300 μM TC was linear for at least 4 sec. For the kinetic study, initial uptake velocities were determined in a 3-sec incubation period. Short incubation periods were timed using a metronome. TC uptake was initiated by rapid vortexing of 10 μl of vesicle suspension with 30 μl of [³H]TC-containing pre-incubation buffer mixture in a high surface tension polystyrene tube according to the method of Stevens, Ross, and Wright (25). The final incubation mixture contained 2.5–3.0 mg/ml vesicle protein, 10–300 μM [³H]TC, 100 mM NaCl (or 100 mM KCl), 80 mM mannitol in 20 mM HEPES-Tris buffer, pH 7.4, with 0–6 μM 2164U90 and 1% ethanol. Incubation was stopped, and the quenched incubation mixture was filtered and washed, and TC uptake was quantitated as described above. Initial uptake velocities are expressed in picomoles per milligram of vesicle protein per second.

Kinetic constants were determined by nonlinear regression analysis according to the method of Motulsky and Ransnas (27) which uses a 1/v² weighting factor (28). The data were tested for fit to curves described by the following equations:

1. No inhibitor
$$v = \frac{V_{max}[S]}{[S] + K_m}$$
2. Competitive inhibition
$$v = \frac{V_{max}[S]}{[S] + K_m(1 + [I]/K_i)}$$

$$\text{3. Noncompetitive inhibition} \quad v = \frac{V_{\max}[S]}{[S](1 + [I]/K_i) + K_m(1 + [I]/K_i)}$$

$$\text{4. Uncompetitive inhibition} \quad v = \frac{V_{\max}[S]}{[S](1 + [I]/K_i) + K_m}$$

Statistical comparisons of uptake velocities, curve-fitting, and estimations of kinetic constants were generated with the NLIN Procedure computer program from SAS Institute, Inc. (29). The program gives a *P*-value for accepting the fit of the data to one model versus another by performing an approximate *F*-test, which compares the residual sums of squares (RSS) from the nonlinear regression analyses.

Glucose uptake by jejunal brush border membrane vesicles

Jejunal brush border membrane vesicles were prepared from jejunal tissue of cynomolgus monkeys as described above for ileal BBMV. Glucose uptake was determined using the rapid filtration technique adapted from Stevens et al. (25). Vesicles were preincubated on ice for 30 min with 1 or 10 μM 2164U90 in 1% ethanol. Control vesicles were preincubated in 1% ethanol. Uptake was initiated by addition of 10 μl vesicle suspension to 20 μl pre-incubation mixture containing [^3H]glucose or [^3H]glucose plus 2164U90 at room temperature. The final concentrations of components in the glucose incubation mixture were as follows: 5 mg/ml vesicle protein, 50 μM [^3H]glucose, 100 mM NaCl (or KCl), 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, 0–10 μM 2164U90, and 1% ethanol. Uptake was terminated at 5 sec by dilution and vortexing with 1 ml ice-cold stop solution containing 150 mM KCl, 10 mM HEPES-Tris, pH 7.4, and 1 mM phloridzin. A 0.9-ml aliquot of this mixture was pipetted onto a pre-wetted 0.45 μm cellulose nitrate filter and washed with 5 ml ice-cold stop solution under vacuum. Radioactivity on the washed filters was determined as described above for ileal BBMV. Results are expressed as picomoles of glucose per milligram of vesicle protein per second. Passive uptake (in KCl) was subtracted from total uptake (in NaCl) for a measurement of sodium-dependent glucose uptake.

Isolated rat ileum in vivo

TC absorption from the ileum isolated in situ in pentobarbital-anesthetized bile fistula rats was determined by modification of methods previously described (16). Incubation medium consisting of 3 mM [^3H]taurocholic acid in 0.9% NaCl, 0.1 M sodium phosphate (pH 7.0), and 1% DMSO with or without 5 μM 2164U90 was maintained at 37°C. To initiate the measurement of TC uptake by the ileum and transport into the bile, a 2-ml

bolus of incubation medium was injected into a flushed, isolated segment of distal ileum (~15 cm long, with vasculature intact). After a 4-min incubation period, the medium was aspirated. Bile was collected in timed intervals for a total of 50 min. Total uptake of TC from the ileal lumen was determined by the difference in radioactivity in aliquots of the incubation medium taken before and after incubation. Uptake is expressed as nanomoles per centimeter of ileum. Total transport of TC absorbed from the ileum was determined from the cumulative appearance of radioactivity in the bile. Transport is expressed as nanomoles per centimeter of ileum. No correction for a passive contribution to total TC transport was made, because passive transport of 3 mM TC from the jejunum, which may be used as an estimate of passive transport from the ileum, was only 0.7% of the total transport by the ileum in a previous study (16). The rate of TC transport into the bile was determined from the maximum rate of appearance of radiolabel in bile collected in 1-min intervals (during minutes 3–7). Rates of transport are expressed as nanomoles per centimeter of ileum per minute.

RESULTS

Rat everted ileal sacs

We showed previously that 2164U90 inhibited uptake and active transport of taurocholic acid (TC) by rat everted ileal sacs in a concentration-dependent manner with IC_{50}s of 4.0 μM and 1.5 μM , respectively (21). **Table 1** summarizes the effects of the (+) and (–) enantiomers of 2164U90, and the (+–)-*trans* and (+–)-*cis* geometrical racemates on mucosal uptake and mucosal to serosal active transport of TC by ileal sacs. 2163U90, the (+) enantiomer of 2164U90, had no significant effect on mucosal uptake or active transport of TC at concentrations up to 30 μM . At a concentration of 3 μM , 1357U88, the (+–)-*trans* racemic mixture (1:1) of 2164U90 and 2163U90, produced similar inhibition of TC uptake and active transport compared to 2164U90 at 3 μM . At 30 μM , 1370U88, the (+–)-*cis* racemate, which is diastereomeric with 1357U88, had a small effect on mucosal uptake of TC, but no significant effect on active transport.

Caco-2 cells

The effects of 2164U90 and 2163U90 on the sodium-dependent transport of TC by Caco-2 cells are summarized in **Table 2**. 2164U90 decreased the active transport of TC in a concentration-dependent manner with an IC_{50} of 7 μM . 2163U90 at 10 and 30 μM had no significant effect on TC active transport by Caco-2 cells.

Ileal brush border membrane vesicles (BBMV)

Table 3 summarizes the effects of 2164U90, 2163U90, 1357U88, 1370U88, and three naturally occurring bile

TABLE 1. Stereospecific effects of 2164U90 on uptake and active transport of taurocholic acid (TC) by rat everted ileal sacs

Compound	Conc.	Total Mucosal TC Uptake % Inhibition	Active TC Transport % Inhibition
	μM		
2164U90			
(-)- <i>trans</i> enantiomer	3	48.0 \pm 1.5 ^b	63.7 \pm 10.7 ^a
2163U90			
(+)- <i>trans</i> enantiomer	30	0.0 \pm 7.0	20.1 \pm 14.7 ^{ns}
1357U88			
(+)- <i>trans</i> racemate	3	41.4 \pm 4.4 ^b	59.7 \pm 3.6 ^b
1370U88			
(+)- <i>cis</i> racemate	30	18.2 \pm 5.1 ^b	28.1 \pm 12.0 ^{ns}

Distal ileal segments (5–6 cm) were everted and filled with 0.75 ml of oxygenated glucose-free Krebs bicarbonate buffer, pH 7.3, containing 0.37 mM [³H]taurocholic acid (serosal fluid). The sacs were incubated in 10 ml of the same buffer containing 0.37 mM [³H]TC with or without test compounds (mucosal fluid) for 30 min at 37°C. Total mucosal uptake of TC was determined from the disappearance of [³H]TC from the mucosal fluid. Active transport of TC was determined from the radioactivity recovered in the final serosal fluid, and expressed as the increase in the serosal:mucosal TC concentration ratio, relative to the initial ratio of 1.0. Each value represents the mean \pm SE for four or eight determinations. For control sacs, the mean mucosal TC uptake was 968.8 \pm 52.3 nmol, and the mean final serosal:mucosal TC concentration ratio was 2.22 \pm 0.19.

^{ns}Not significant; ^a $P < 0.05$; ^b $P < 0.01$ (treated vs. control by one-way analysis of variance).

acids on the sodium-dependent uptake of TC by rat ileal BBMV. 2164U90 decreased the sodium-dependent uptake of TC by 44–96% at concentrations of 0.3–10 μM . 2163U90 at 10 μM had little effect on TC uptake. 1357U88 had effects similar to 2164U90. 1370U88 had some activity, but was much less potent than 1357U88 in rat BBMV. 2164U90 at 1 μM was as effective as the most potent bile acid, chenodeoxycholic acid at 10 μM , in inhibiting TC uptake.

Table 4 summarizes the effects of 2164U90 on the sodium-dependent uptake of TC by human and monkey ileal BBMV. 2164U90 inhibited the uptake of TC in a concentration-dependent manner with IC₅₀s of 2 μM and 5 μM for human and monkey, respectively.

The results of the kinetic study of inhibition of the sodium-dependent uptake of TC by 2164U90 in rat BBMV are shown in Fig. 2. The computer fit curves from the nonlinear regression analysis are superimposed over the actual data for initial uptake velocities. The competitive model was a significantly better fit for these data than the noncompetitive or the uncompetitive models. Sodium-dependent TC uptake in the absence of inhibitor was saturable, and the estimates obtained for the V_{max} and K_m were 202 \pm 15 pmol \cdot mg⁻¹ \cdot sec⁻¹ and 97 \pm 11 μM , respectively. The estimated K_i for 2164U90 was 1.8 \pm 0.2 μM .

The data did not converge for the noncompetitive equation, which indicates that the noncompetitive model does not reasonably fit the data (27). In the comparison of the data for fit to the competitive versus the uncompetitive

TABLE 2. Effects of 2164U90 and 2163U90 on sodium-dependent transport of taurocholic acid (TC) by Caco-2 cells

Compound	Concentration	% Inhibition (\pm SE)
	μM	
2164U90	30	74 \pm 7.2 ^b
	10	65 \pm 6.4 ^b
	3	34 \pm 2.7 ^a
	1	5 \pm 0.6
2163U90	30	17 \pm 4.2
	10	0 \pm 0.1

The cumulative transport of TC from the apical to basolateral media was determined at 37°C over a 1-h interval in cells grown for 21 or 22 days on Transwell filters. Total transport was determined in media consisting of Hanks' balanced salt solution containing 25 mM glucose and 10 mM HEPES buffer, pH 7.4. Passive transport was determined by replacing NaCl with KCl in the media. After a 30-min equilibration period in NaCl or KCl, test compounds dissolved in ethanol were added to the apical compartment for an additional 30 min. Control cells received equivalent ethanol (0.2%). Transport was initiated by addition to the apical compartment of [¹⁴C]TC to make 5 μM . Sodium-dependent transport was determined by subtraction of passive transport from total transport. Results are expressed as percent inhibition of sodium-dependent TC transport compared to control. For controls the mean sodium-dependent transport was 329.8 \pm 50.3 pmol \cdot mg⁻¹ \cdot hr⁻¹, and mean passive transport was 9.1% of the total. Each value represents the mean \pm SE for three to six determinations.

^a $P < 0.05$; ^b $P < 0.01$ (treated vs. control by one-way analysis of variance).

models, a P -value of > 0.999 was obtained in the approximate F-test, thus accepting the competitive model. Where two models (the competitive and uncompetitive equations) have the same number of parameters, a large

TABLE 3. Effects of 2164U90, 2163U90, 1357U88, 1370U88, and naturally occurring bile acids on the sodium-dependent uptake of taurocholic acid (TC) by rat ileal brush border membrane vesicles (BBMV)

Compound	Concentration	% Inhibition (\pm SE)
	μM	
2164U90	10	96 \pm 0.4 ^b
	3	84 \pm 1.1 ^b
	1	77 \pm 1.9 ^b
	0.3	44 \pm 2.6 ^b
2163U90	10	8 \pm 3.0 ^a
1357U88	3	85 \pm 1.5 ^b
	0.3	34 \pm 3.3 ^b
1370U88	3	22 \pm 3.0 ^b
Chenodeoxycholic acid	10	60 \pm 1.4 ^b
Ursodeoxycholic acid	10	49 \pm 1.2 ^b
Tauroursodeoxycholic acid	10	33 \pm 3.4 ^b

TC uptake was determined in rat ileal BBMV preloaded with 280 mM mannitol, 20 mM HEPES-Tris (pH 7.4) and incubated in 100 mM NaCl (total uptake) or 100 mM KCl (passive uptake), 20 mM HEPES-Tris (pH 7.4), 80 mM mannitol, 10 μM [³H]TC with or without test compounds for 30 sec at room temperature. Sodium-dependent uptake was determined by subtraction of passive uptake from total uptake. Results are expressed as percent inhibition of sodium-dependent TC uptake compared to control. Each value represents the mean \pm SE for four determinations.

^a $P < 0.05$; ^b $P < 0.01$ (treated vs. control by one-way analysis of variance).

TABLE 4. Effects of 2164U90 on the sodium-dependent uptake of taurocholic acid (TC) by human and monkey ileal brush border membrane vesicles (BBMV)

2164U90 Concentration μM	Human BBMV % Inhibition (\pm SE)	Monkey BBMV % Inhibition (\pm SE)
10	89 \pm 4.3 ^b	63 \pm 2.5 ^b
3	60 \pm 1.4 ^b	32 \pm 4.1 ^b
1	34 \pm 3.5 ^a	24 \pm 10.0 ^a

TC uptake was determined in ileal BBMV preloaded with 300 mM mannitol, 10 mM HEPES-Tris (pH 7.4) and incubated in 100 mM NaCl (total uptake) or 100 mM KCl (passive uptake), 17.5 mM HEPES-Tris (pH 7.4), 85 mM mannitol, 10 μM [³H]TC with or without 2164U90 for 30 sec at room temperature. Sodium-dependent uptake was determined by subtraction of passive uptake from total uptake. Results are expressed as percent inhibition of sodium-dependent TC uptake compared to control. Each value represents the mean \pm SE for four determinations.

^a $P < 0.05$; ^b $P < 0.01$ (treated vs. control by one-way analysis of variance).

P -value, >0.05 , indicates that the fit with the smaller RSS, the competitive model, is significantly better (27).

2164U90 had no significant effect on the passive component of TC uptake by rat BBMV measured in 100 mM KCl (data not shown).

Glucose uptake by monkey jejunal brush border membrane vesicles

2164U90 did not inhibit sodium-dependent glucose uptake in monkey jejunal brush border membrane vesicles at concentrations up to 10 μM (Table 5).

TABLE 5. Effect of 2164U90 on the uptake of glucose by the sodium-dependent glucose transporter in monkey jejunal brush border membrane vesicles (BBMV)

Incubation Medium	Glucose Uptake $\text{pmol} \cdot \text{mg}^{-1} \cdot \text{sec}^{-1}$ (\pm SE)
Control	40.9 \pm 2.8
1 μM 2164U90	39.9 \pm 0.9
10 μM 2164U90	38.6 \pm 2.3

Glucose uptake was determined in jejunal BBMV preloaded with 300 mM mannitol, 10 mM HEPES-Tris (pH 7.4), preincubated with or without 2164U90 for 30 min on ice, and then incubated in 100 mM NaCl (total uptake) or 100 mM KCl (passive uptake), 10 mM HEPES-Tris (pH 7.4), 100 mM mannitol, 50 μM [³H]glucose with or without 2164U90 for 5 sec at room temperature. Sodium-dependent uptake was determined by subtraction of passive uptake from total uptake. Each value represents the mean \pm SE for three determinations.

Isolated rat ileum in vivo

Five μM 2164U90 inhibited uptake by the ileal lumen, transport into the bile, and rate of transport of 3 mM [³H]TC equally by 31–35% (Table 6). The ratio of total uptake to total transport was equal for treated and control rats. The time course of the biliary recovery of TC taken up by the ileal lumen is shown in Fig. 3. In both treated and control animals, the initial rate of transport was linear from 3 to 7 min after delivery of the bolus of incubation medium. The rate started to decrease rapidly after 9–10 min, at which time the recovery in the bile was 85% of the total TC transported. This recovery was 95–97% complete at 15 min in both groups of animals.

Fig. 2. Competitive inhibition by 2164U90 of sodium-dependent taurocholic acid (TC) uptake by rat ileal brush border membrane vesicles (BBMV). TC uptake was determined in rat BBMV preloaded with 280 mM mannitol, 20 mM HEPES-Tris (pH 7.4) and incubated in 100 mM NaCl (total uptake) or 100 mM KCl (passive uptake), 20 mM HEPES-Tris (pH 7.4), 80 mM mannitol, 10–300 μM [³H]TC with 0–6 μM 2164U90 and 1% ethanol for 3 sec at room temperature. Initial velocities of sodium-dependent uptake were determined by subtraction of passive from total uptake velocities. Each data point represents the mean \pm SE for four to six determinations. V_{max} , K_m , and K_i and curve fitting were determined by nonlinear regression analysis as described in text.

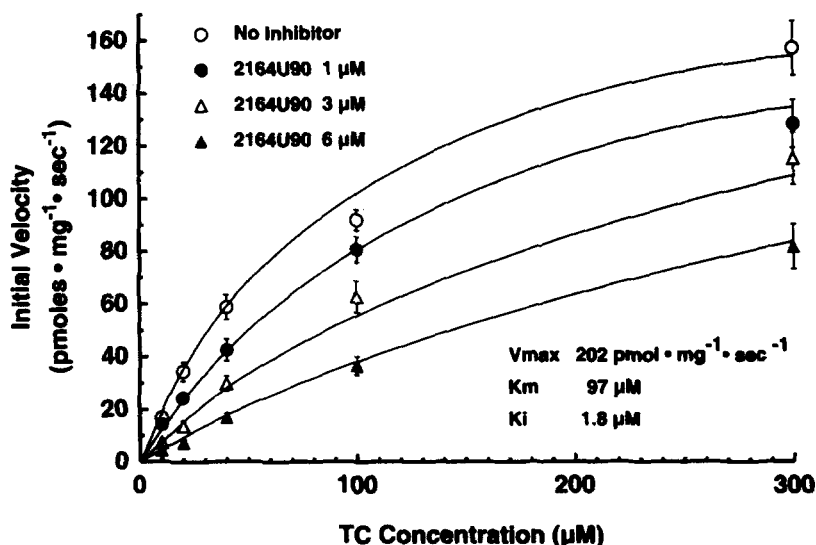


TABLE 6. Effects of 2164U90 on luminal uptake and transport into the bile of taurocholic acid (TC) from the ileum in anesthetized rats

	Total Uptake	Total Transport	Transport Rate	Transport/Uptake
	nmol/cm	nmol/cm	nmol · cm ⁻¹ · min ⁻¹	
Control	151.6 ± 5.9	142.2 ± 6.2	21.9 ± 1.5	.926 ± .015
2164U90, 5 μM	104.6 ± 4.1 ^a	96.7 ± 5.3 ^a	14.3 ± 1.4 ^a	.924 ± .017
(Inhibition)	(31.0%)	(32.0%)	(34.8%)	(0.2%)

Ileal segments (~15 cm) isolated in situ were incubated for 4 min with 2 ml of medium containing 3 mM [³H]TC, 0.9% NaCl, 0.01 M sodium phosphate (pH 7.0), and 1% DMSO with or without 5 μM 2164U90 at 37°C. Bile was collected in timed intervals during and after incubation for a total of 50 min. Total uptake of TC from the ileal lumen was determined from the disappearance of [³H]TC from the incubation medium. Total transport of TC absorbed from the ileum was determined from the cumulative appearance of radioactivity in the bile. Transport rate was determined from the maximum rate of appearance of radiolabel in the bile from minutes 3–7. Values are the means ± SE for seven control or five treated animals.

^aP < 0.01 (treated vs. control by one-way analysis of variance).

DISCUSSION

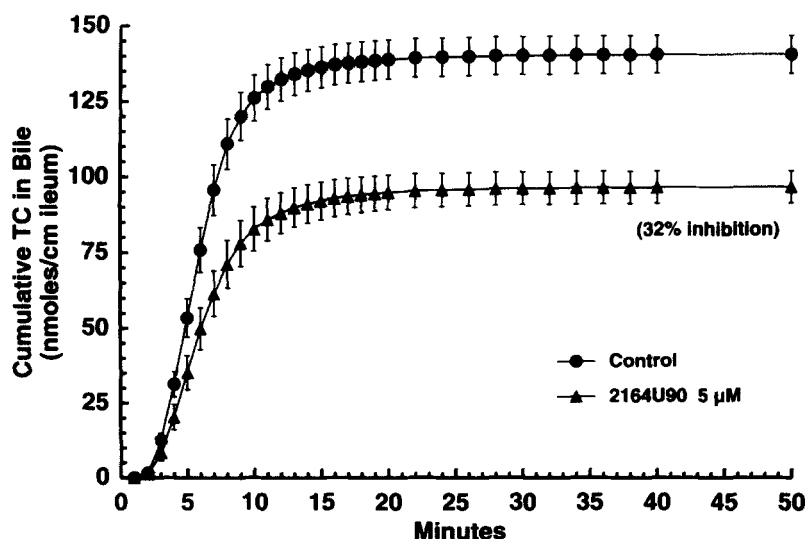
We have previously shown that 2164U90 lowered plasma LDL cholesterol in rats and mice by inhibition of the ileal bile acid active transport system (21). In the present study, we examined the effects of 2164U90 on sodium-dependent bile acid transport in rat everted ileal sacs, Caco-2 cells, and brush border membrane vesicles (BBMV) from rat, monkey, and human ileum in vitro, and on the rat ileum isolated in vivo. Our results indicate that 2164U90 is a potent, selective, stereospecific, competitive inhibitor of the bile acid active transport system in the distal ileum, and that the site of inhibition is likely to be the sodium-dependent bile acid transporter associated with the mucosal cell brush border membrane.

The absorption of bile acids across the ileal mucosal cell and into the bile is viewed as a multistep system that starts with sodium-dependent binding and active transport of

bile acids across the brush border membrane by a transporter (4, 5). This is followed by binding to cytosolic proteins for intracellular translocation to the basolateral membrane (BLM) (8), and transport across the BLM via a sodium-independent organic anion exchange system into the portal blood (8, 30, 31). Tight binding of bile acids to plasma proteins and efficient clearance by the liver may operate to maintain a downward bile acid concentration gradient from ileal cells all the way to the bile canaliculi (30). Our in vivo model represents the entire first pass intestinal-biliary absorption process for bile acids from the distal ileum. The in vitro preparations simulate events of ileal mucosal cell bile acid transport. Everted ileal sacs and Caco-2 cells are models of the whole ileal cell transport system. The ileal BBMV represent the first step in ileal cell bile acid transport.

In our initial study of rat everted ileal sacs, 2164U90 was a potent inhibitor of both total mucosal uptake and mucosal to serosal active transport of taurocholic acid

Fig. 3. Effects of 2164U90 on transport into the bile of taurocholic acid (TC) placed in the distal ileum of bile fistula rats. Isolated ileal segments ~15 cm long were incubated for 4 min with 2 ml of medium containing 3 mM [³H]TC, 0.9% NaCl, 0.01 M sodium phosphate (pH 7.0), and 1% DMSO with or without 5 μM 2164U90 at 37°C. Bile was collected in timed intervals during and after incubation for a total of 50 min. Transport of TC into the bile is expressed as nanomoles of TC per centimeter of ileum. Each data point represents the mean ± SE for seven control or five treated animals.



(TC) with IC_{50} s of 4.0 and 1.5 μ M, respectively (21). Caco-2 cells are a human colon adenocarcinoma cell line that has been shown to express all of the salient features of the ileal bile acid transport system, including sodium-dependence (32, 33). The Caco-2 cells form confluent monolayers having intercellular tight junctions and distinct mucosal and basolateral polarity, similar to small intestinal epithelium. In the present study with Caco-2 cells, 2164U90 decreased the mucosal to basolateral active transport of TC in a concentration-dependent manner, with an IC_{50} of 7 μ M. These results extend the initial findings in rat everted ileal sacs to a human model of intestinal mucosal cell transport.

In an attempt to localize the site of interaction of 2164U90 with the multistep transport system for bile acids across the mucosal cell, we studied the effects of 2164U90 on TC uptake by brush border membrane vesicles from rat, monkey, and human ileum. With the different BBMVs preparations incubated under similar conditions, 2164U90 decreased sodium-dependent TC uptake with similar concentration-dependent effects for all three species. Kinetic analysis of the data from rat BBMVs showed that sodium-dependent TC uptake is saturable with a K_m of 97 μ M. This value is similar to values of 81 μ M, 36 μ M, and 37 μ M previously reported for rat, rabbit, and human BBMVs, respectively (6, 26, 34). The K_m for TC in rat BBMVs is also similar to the K_m of 33 μ M reported for the hamster ileal sodium-dependent bile acid transporter (IBAT), expressed in COS cells (10), and the K_m of 65 μ M reported for Caco-2 cells (33). Kinetic analysis of our data also determined that 2164U90 is a competitive inhibitor of the sodium-dependent uptake of TC by rat BBMVs with a K_i of 1.8 μ M. The similar effects of 2164U90 on sodium-dependent TC transport by BBMVs preparations from three very different species, including human, suggest that competitive inhibition of the sodium-dependent bile acid transporter in the ileal brush border membrane is the mechanism by which this compound decreases intestinal bile acid absorption.

The sodium-dependent glucose transporter is distinct from the bile acid transporter but shares some of the same requirements for substrate transport, such as the presence of sodium ions and essential thiol and amino groups (6, 35–37). We studied the effect of 2164U90 on the glucose transporter to determine whether the compound was likely to be a nonspecific inhibitor of other sodium-dependent intestinal transport systems. In monkey jejunal brush border membrane vesicles, concentrations of 2164U90 that completely blocked TC active uptake in rat ileal preparations did not inhibit glucose active transport. These results indicate that 2164U90 is not a general inhibitor of energy-requiring transport systems and suggest that it may be specific for the sodium-dependent ileal bile acid transporter.

2164U90 is one of four possible stereoisomers. 2164U90 is the (–)-(3R,5R) stereoisomer with the 3-butyl and 5-phenyl groups in the *trans* geometrical configuration. 2163U90 is the (+)-(3S,5S) stereoisomer, also with the (C-3/C-5) *trans* configuration. At concentrations that produced maximal inhibition with 2164U90, 2163U90 had little or no effect on TC uptake and transport by rat everted ileal sacs, Caco-2 cells, or rat ileal BBMVs. 1357U88, the (+–)-*trans* racemic mixture (1:1) of 2164U90 and 2163U90, was similar in potency to 2164U90 in inhibiting TC uptake and transport by rat ileal sacs and rat BBMVs. In contrast, 1370U88, the (+–)-*cis* racemate, had much less activity in rat ileal sacs and BBMVs compared to 1357U88. These results indicate that the interaction of 2164U90 with the ileal bile acid transport system, causing inhibition of active TC uptake, is stereospecific.

In our *in vivo* model, the active compound, 2164U90, was delivered directly into the putative site of action, the distal ileum, isolated *in situ* in anesthetized rats. Here 2164U90 at 5 μ M, a concentration active in the *in vitro* models, in a solution with a physiological concentration of 3 mM [3 H]TC, inhibited ileal uptake of TC, transport of TC into the bile and the rate of transport of TC into the bile by the same extent (31–35%). 2164U90 did not alter the time course of TC transport into the bile, which may indicate that the compound does not affect liver clearance of the total load of TC taken up by the ileal lumen. These results suggest that the apparent effects of 2164U90 on TC transport into the bile and rate of TC transport into the bile could be explained solely by specific inhibition of TC uptake at the ileal mucosal cell brush border membrane. Wess et al. (38) have used a similar *in situ* perfusion model in rats, also using 3 mM [3 H]TC as substrate, to demonstrate that a series of bile acid dimers are specific inhibitors of ileal bile acid transport. Their most potent three compounds, tested at 100 μ M, inhibited TC transport into the bile maximally by 23–59%.

In conclusion, our results indicate that 2164U90 is a potent, selective, stereospecific, competitive inhibitor of the sodium-dependent bile acid transport system of the ileum. The site of inhibition is likely to be the sodium-dependent transporter associated with the mucosal cell brush border membrane. 2164U90 inhibited sodium-dependent TC transport with comparable potency in ileal BBMVs preparations from three different species: rat, monkey, and human. Similarly *in vivo*, a low concentration of 2164U90 injected directly into the isolated distal ileum *in situ* in anesthetized rats, inhibited TC transport. The present work provides more extensive support for our previous evidence that selective inhibition of the ileal bile acid active transport system is the mechanism underlying the capability of this compound, given orally, to increase bile acid fecal excretion and lower plasma LDL cholesterol in rats and mice (21). ■

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