Ileal bile acid transporter inhibition, CYP7A1 induction, and antilipemic action of 264W94

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Abstract 264W94 was designed to inhibit the ileal bile acid transporter (IBAT). Evaluated in vitro, 264W94 dose-dependently inhibited sodium-dependent uptake of 10 μM [^{3}H]taurocholic acid (TC) by rat and monkey brush border membrane vesicles with IC_{50}s of 0.24 μM and 0.41 μM, and had a competitive profile with K_i of 0.2 μM against TC in Chinese hamster ovary cells expressing human IBAT. In distal ileum in situ, 1–10 μM of 264W94 rapidly decreased uptake of 3mM TC by 24–39%, with corresponding decreases in biliary recovery. In rats and mice in vivo, oral 264W94 decreased absorption of TC analog, 23,25-^{75}Se-homocholic acid taurine (^{75}SeHCAT; quantitated in feces), with ED_{30} of 0.02 mg/kg bid. ^{75}SeHCAT traced through the GI-tract revealed that peak (97%) inhibition of ^{75}SeHCAT absorption by the distal quarter of small intestine occurred at 4 h after single dose of 264W94 (0.1 mg/kg). Inhibition of IBAT by 264W94 in rats was associated with compensatory, same-day, 4-fold induction of hepatic cholesterol 7α-hydroxylase (CYP7A1) activity, exhibiting normal diurnal fluctuation for 3 days of dosing. In diet induced hypercholesterolemic rats, 264W94 (0.03–1.0 mg/kg bid) dose-dependently reduced serum LDL+VLDL cholesterol up to 61%. In conclusion, 264W94 is a potent new cholesterol lowering agent that acts through inhibition of IBAT and exhibits activity in a human model.—Root, C., C. D. Smith, S. S. Sundseth, H. M. Pink, J. G. Wilson, and M. C. Lewis. **Ileal bile acid transporter inhibition, CYP7A1 induction, and antilipemic action of 264W94. *J. Lipid Res.* 2002.** 43: **1320–1330.**

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The value and safety of lowering plasma LDL cholesterol (LDL-C) in the treatment of cardiovascular disease has been established unequivocally. Clinical studies have shown that decreasing plasma LDL-C significantly reduces the coronary heart disease morbidity and mortality, and decreases the progression and increases regression of atherosclerotic lesions (1–5). The liver is the key organ for cholesterol homeostasis. Hepatocyte free cholesterol concentration is maintained by uptake of LDL-C from the plasma through regulation of the LDL receptor, cholesterol biosynthesis via HMG-CoA reductase, and cholesterol storage as cholesterol esters by ACAT, and through cholesterol secretion into bile, either as free cholesterol or as bile acids formed from cholesterol. Plasma LDL-C concentrations are strongly dependent on the rate of LDL-C clearance from the plasma, primarily by liver LDL receptors (6, 7).

Bile acids, having cholesterol as their biochemical precursor, play an important role in cholesterol homeostasis. Bile acids are synthesized in liver, secreted into the duodenum, and returned to the liver through the portal blood by way of a highly efficient intestinal reabsorption process (7–10). A sodium-dependent ileal bile acid transporter (IBAT), localized in the most distal part of the ileum (8, 9, 11), is thought to comprise the major channel for re-entry of bile acids into the portal blood in humans and other animals (7, 8, 10, 11). This enterohepatic circulation (EHC) of bile acids contributes to negative feedback regulation of bile acid synthesis by repression of hepatic cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme in the classical bile acid synthetic pathway (7, 12–15). Catabolism of cholesterol to form bile acids, as well as bile acid excretion, constitute a major route of elimination of cholesterol from the body (8, 13, 15). Enhancement of this route of elimination, by interruption of intestinal reabsorption and EHC of bile acids, either by partial

Abbreviations: BAS, bile acid sequestrants; BBMV, brush border membrane vesicles; CHO, Chinese hamster ovary; EHC, enterohepatic circulation; IBAT, ileal bile acid transporter; PIB, partial ileal bypass; ^{75}SeHCAT, 23,25-^{75}Se-homocholic acid taurine; TC, taurocholic acid.

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ileal bypass (PIB) surgery or by oral treatment with bile acid sequestrants (BAS), results in increased fecal bile acid wasting. While both PIB surgery and BAS treatment have adverse effect- or compliance-related drawbacks, this approach is a clinically proven means of lowering serum LDL-C and altering progression of atherosclerosis (2–5).

Accelerated fecal loss of bile acids with BAS or PIB treatment causes a compensatory increase in hepatic utilization of cholesterol for bile acid synthesis. Expression of hepatic CYP7A1, a member of a super-family of cytochrome P450 enzymes that catalyze α oxygenation of sterols (15, 16), and which plays a key role in maintaining liver cholesterol homeostasis and in determining plasma cholesterol levels (7, 12–15, 17), is upregulated (12, 15). Consequently, substrate cholesterol pools in the liver are consumed more quickly. In response, metabolic pathways that tightly maintain hepatic cholesterol levels, including de novo cholesterol synthesis and hepatic LDL receptor activity, are upregulated through intricately controlled transcriptional mechanisms (14, 18). Since plasma and newly synthesized cholesterol provide a cholesterol pool for bile acid synthesis (15, 16, 19), the latter increase in LDL receptor expression ultimately lowers circulating plasma LDL-C concentrations (15, 20, 21).

The goal of our research program, first articulated by Lack and Weiner in 1963 (22), has been to find, as a more palatable alternative to BAS, a specific competitive inhibitor of the ileal bile acid transporter (IBAT) system. Based on a prior lead compound 2164U90 (23, 24), 264W94 is a new, low molecular weight 1, 4-benzothiazepine derivative designed to be a specific inhibitor of IBAT. The purpose of the present research was to characterize the pre-clinical pharmacological effects of 264W94 on rodent and primate IBAT models in vitro, and on biliary, gastrointestinal, and fecal bile acid distribution, hepatic CYP7A1, and serum cholesterol levels in rodents in vivo.

METHODS

Materials

264W94 [(−)-(3R, 5R)-*trans*-3-butyl-3-ethyl-2,3,4,5-tetrahydro-7,8-dimethoxy-5-phenyl-1,4-benzothiazepine1,1-dioxide], having a molecular weight of 417.569 with the empirical formula of $C_{23}H_{31}NO_4S$, was synthesized at Burroughs Wellcome Co. (25). The chemical structure of 264W94 is shown in **Fig. 1**. A stable cell line consisting of the human ileal bile acid transporter gene expressed in Chinese hamster ovary (CHO) cells (referred to as CHO-hIBAT cells) was obtained from Dr. Paul Dawson (11, 26) at Wake Forest University School of Medicine in Winston Salem, NC. 23,25-^{75}Se-homocholic acid taurine (^{75}SeHCAT) and [4-^{14}C]cholesterol were obtained from Amersham International. [^{3}H]taurocholic acid (TC) (2.1–2.6 Ci/mmol) was obtained from New England Nuclear. Unlabeled TC (98% pure) was obtained from Sigma Chemical Co. Reagents for enzymatic assay of serum cholesterol fractions were obtained from Seragen Diagnostics.

Animals

All studies using live animals were conducted in compliance with standard and humane animal care principles adopted in June 1994 by the Federation of American Societies for Experimental Biology.

Ileal brush border membrane vesicles

Preparation of ileal brush border membrane vesicles. Ileal brush border membrane vesicles (BBMV) were prepared from distal ileum of male Sprague-Dawley rats (CD, Charles River, 250–300 gm) or Cynomolgus monkeys by subcellular fractionation using the Ca^{++} precipitation method (27, 28). Monkey BBMV were stored in 50% glycerol under liquid N_2 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). Vesicle protein concentration was determined after completion of uptake measurements.

TC uptake measurements in BBMV. TC uptake by rat BBMV was determined at room temperature using a rapid filtration technique (24). Test compounds were dissolved in 100% ethanol and diluted in a [^{3}H]TC-containing incubation buffer. Equivalent ethanol was added to control incubation buffers. TC uptake was initiated by the addition of 50 μl of the vesicle suspension to 150 μl of the [^{3}H]TC-containing incubation buffer. Final concentrations of components in the incubation medium were as follows: 1.0–1.2 mg/ml vesicle protein, 10 μM [^{3}H]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 264W94, and 1% ethanol. Uptake was terminated at 30 s 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 chilled, prewetted fiberglass filters (1 μm pore-size) under vacuum in a Brandel 24-manifold filtration apparatus, 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. Passive uptake of TC incubated for 30 s in the absence of sodium was relatively small, ranging among preparations from 10–15% of total uptake in the presence of sodium. Passive uptake of TC was equal in same preparations incubated

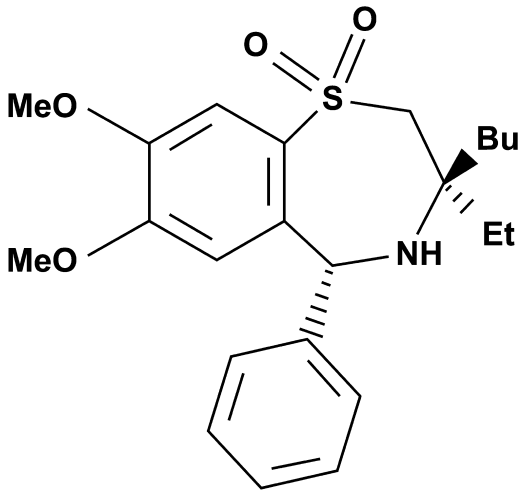

Fig. 1. Chemical structure of 264W94.

with 0 to 10 μM inhibitor 264W94. Thus, under these assay conditions, the inhibitor had no effect on passive uptake.

TC uptake by monkey 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, and filtration was performed with 0.45 μm filters, in a single manifold filter holder. The IC_{50} values were determined from the linear portion of the plot of percent inhibition as a function of the log of the inhibitor concentration.

Human IBAT

Human IBAT cDNA expressed in CHO cells. Stable CHO-hIBAT cell stocks were routinely grown in DMEM/F12 containing 10% FBS and 700 μg/ml Geneticin to retain *hIBAT* expression. For transport studies, CHO-hIBAT cells were plated at a density of 50,000 cells per well in a 24 well format, and cultured for 48–72 h at 37°C under 5% CO_2, 95% air.

TC transport assays in CHO-hIBAT cells. Immediately prior to TC transport assays, cells were washed twice in HBSS, buffered to pH 7.4 with Hepes-Tris (HBSSH) at 37°C. Cells were incubated for 10 min in 0.2 ml of incubation medium consisting of HBSSH containing 5 μM TC (0.5 μCi/ml [^{3}H]TC), 0.2% DMSO, and 264W94 (0, 0.1, 0.25, and 0.5 μM) at 37°C in the 5% CO_2 incubator. Incubation was terminated by placing the 24-well plate in an ice-water bath. The radiolabeled TC incubation medium was aspirated, and cells were washed three times with HBSSH containing 1 mM unlabeled TC at 4°C. Excess liquid was blotted away, and the cells were lysed in 0.1 N NaOH. Cell associated radioactivity was determined by liquid scintillation counting. A correction for non-IBAT-specific uptake was made by subtraction of cell associated radioactivity in non-transfected CHO cells incubated as above in the last four wells of each 24 well plate. Total cell protein was measured using a Bio-Rad protein assay kit with γ-globulin as standard. The IC_{50} was determined by linear regression of a plot of percent inhibition as a function of the log of the inhibitor concentration.

Kinetic studies in CHO-hIBAT cells. Our preliminary work with CHO-hIBAT cells determined that the transport of 11–100 μM TC was linear for at least 12 min. For kinetic studies, initial uptake velocities were determined in a 10-min incubation period. Kinetic parameters were determined by using a 4 × 5 matrix of inhibitor (0, 0.1, 0.2, and 0.3 μM 264W94) and substrate (11, 14, 20, 33, and 100 μM [^{3}H]TC) concentrations, under the general assay conditions described above. Initial uptake velocities are expressed in picomoles per milligram of cell protein per minute. The K_m and V_{max} for TC transport in the absence of inhibitor were determined by nonlinear regression analysis for fit to a rectangular hyperbola with a linear component described by the Michaelis-Menten equation $v = (V_{max}\ [S])/([S]+K_m)$. The model of inhibition and K_i for 264W94 were estimated by graphical analysis of Lineweaver-Burk plots of 1/v as a function of 1/[S] in the presence and absence of inhibitor, and solution of the reciprocal velocity equation for competitive inhibition: $1/v = (K_m/V_{max})\ (1+[I]/K_i)\ (1/[S]) + 1/V_{max}$.

TC absorption by rat ileum in situ

TC absorption from the ileum isolated in situ in pentobarbital anesthetized bile fistula rats (male Sprague-Dawley; 275–325 gm) was determined according to methods previously described (24). Incubation medium consisting of 3 mM [^{3}H]TC in 0.9% NaCl, 0.1 M sodium phosphate (pH 7.0), and 1% DMSO with or without 1 or 10 μM 264W94 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. Total transport of TC absorbed from the ileum was determined from the cumulative appearance of radioactivity in the bile. Both TC uptake and transport were expressed in 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 our previous studies (24). 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 min 3–7). Rate of transport was expressed in nanomoles per centimeter of ileum per min. Statistical comparisons were made using one-way ANOVA.

Total 24 h fecal excretion and enteric absorption of bile acids

Rats. Male Sprague-Dawley rats (CD, Charles River, 220–260 gm) were housed individually with free access to normal chow and water, and maintained on a 12 h light-dark cycle with lights on from 6 AM to 6 PM. The rats were divided into five treatment groups, consisting of six to eight rats per group. Rats were dosed by oral gavage with test compounds (0.003, 0.01, 0.03, or 0.1 mg/kg of 264W94) as a suspension in 0.5% methylcellulose (1 ml/100 gm body weight) at 9 AM and 3:30 PM for 2 days. The control group received 0.5% methylcellulose. ^{75}SeHCAT, a tri-hydroxy-bile acid analog, having transport characteristics similar to TC (23), was used as a tracer to label the bile acid pool. Two hours after morning dosing on day 2, the rats were given a trace amount (1.3 nmoles, ~65,000 dpm) of ^{75}SeHCAT orally in 1 ml saline. For the following 24 h, each animal's total fecal output was collected and placed into scintillation vials. Total fecal content of ^{75}SeHCAT in dpm was quantitated using a Packard AutoGamma 5,000 Series γ-counter. Both ^{75}SeHCAT excretion and absorption were expressed as a percentage of the total dpm in the oral ^{75}SeHCAT dose. ^{75}SeHCAT absorption was calculated as: ^{75}SeHCAT in oral dose minus ^{75}SeHCAT excretion in feces. Inhibition of ^{75}SeHCAT absorption was calculated as: [(control ^{75}SeHCAT absorption − treated ^{75}SeHCAT absorption)/control ^{75}SeHCAT absorption] × 100%. The ED_{30} value was calculated using the non-normalized data. Statistical comparisons were made using one-way ANOVA.

Mice. Male CD-1 mice weighing 22–28 gm were placed in individual cages and fed normal chow. The mice were were divided into five treatment groups, consisting of eight mice per group. The procedure was the same as described for rats except that the mice were given ^{75}SeHCAT (1.3 nmol) orally in 0.2 ml saline.

Time course of enteric ^{75}SeHCAT distribution

Male Sprague-Dawley rats weighing 220–260 gm, and housed as above, were divided into 12 groups, consisting of a control and a treatment group for each of six time points, and containing four to six rats per group. A trace amount (1.3 nmol) of ^{75}SeHCAT in 1 ml saline was administered orally, and then 24 h later, between 9 AM and 10 AM, a single dose of 0.1 mg/kg 264W94 in suspension with 0.5% methylcellulose (1 ml/100 g body weight), or vehicle alone for controls, was administered orally. Total fecal output of each animal was collected from the time of dosing. Treated and control rats were sacrificed with CO_2 and dissected at the following time points after dosing: .5, 1, 2, 4, and 6 h. The stomach, cecum, colon, feces, and liver were placed directly into scintillation vials. The small intestine was cut into eight equal lengths, and each segment was flushed twice with 10

ml cold saline into scintillation vials. The eight flushed and drained small intestinal segments were placed in separate vials.

Total content of ^{75}SeHCAT in the dissected tissues and their flushed contents, and in the feces, was measured in a γ-counter, and expressed as a percentage of the total dpm in the ^{75}SeHCAT dose. Measurements from the eight segments were combined into four consecutive quarters, each approximately 25 cm in length. ^{75}SeHCAT absorption by the fourth (most distal) quarter of the small intestine (the site of the ileal bile acid active transport system), was calculated as follows: [(^{75}SeHCAT content of third quarter − ^{75}SeHCAT content of fourth quarter)/ ^{57}SeHCAT content of third quarter] × 100%. Inhibition of ^{75}SeHCAT absorption in the fourth quarter of the small intestine was calculated as follows: [(control ^{75}SeHCAT absorption by the fourth quarter − treated ^{75}SeHCAT absorption by the fourth quarter) ÷ control ^{75}SeHCAT absorption by the fourth quarter] × 100%. Statistical comparisons were made using the paired *t*-test.

CYP7A1 enzyme activity

Male rats (Fisher 344, Charles River) weighing 220–260 gm were given free access to normal chow and water, and maintained on a 12 h light-dark cycle with lights on from 6 AM to 6 PM. The rats were allocated, three per group, into a total of 26 groups, consisting of paired control and treatment groups for each of 13 time points. The rats were given a single daily oral dose of 264W94 (0.3 mg/kg), or vehicle control (0.5% sodium bicarbonate) at 8 AM for up to 4 days. Treated and control rats were sacrificed with CO_2 at the following time points after the first 8 AM dose: 8, 12, 24, 36, 40, 48, 72, 80, 84, 96, 104, 108, and 120 h. (Animals sacrificed at 8 AM were not dosed on that morning.) The livers were immediately excised, rinsed with cold 0.9% saline, and snap-frozen in liquid nitrogen. Frozen livers were stored at −80°C until the time of microsome preparation.

Microsomes were prepared from frozen liver as previously described (29), in 100 mM HEPES (pH 7.4), 50 mM NaF, 1 mM EDTA, 1 mM PMSF, and 20% glycerol at a concentration of 20–30 mg/ml and stored at −80°C. CYP7A1 activity in microsomal fractions was assayed using [4-^{14}C]cholesterol solubilized in 2-hydroxypropyl b-cyclodextrin [Molecusol HPB (MHPB), Pharmatec] as substrate. To prepare substrate, oxidation products were removed from [4-^{14}C]cholesterol (52 mCi/mmol) by dissolving 250 μCi in 8 ml hexane, loading onto a silica Sep-Pak cartridge (Millipore), and eluting the cholesterol with 8 ml 2% 2-propanol in hexane. After drying under N_2, the cholesterol was dissolved in 45% w/v MHPB to ∼0.7 μCi/μl by stirring overnight in an amber vial at room temperature. The final radiolabeled cholesterol/MHPB solution was stored at 4°C until use.

Assays of CYP7A1 enzyme activity were carried out in 400 μl vol containing ∼200 μg microsomal protein, 2 μCi [4-^{14}C]cholesterol (final concentration = 100 μM), 8 mM NADPH, 1 mM EDTA, and 50 mM HEPES (pH 7.4). Reactions were initiated by adding NADPH, incubated 30 min at 37°C, and terminated by adding 0.4 ml of 0.9% saline and 4 ml Folch Reagent (chloroform-methanol, 2:1, v/v with 0.005% BHT added as an antioxidant). The organic phase was evaporated to dryness under N_2, resuspended in 250 μl 7.5% 2-propanol in hexane, and 100 μl was analyzed on a normal phase HPLC system (Waters mPorasil 3.9 × 300 mm silica column; mobile phase consisting of 7.5% 2-propanol, 92.5% hexane; flow rate equal to 1.1 ml/min) equipped with a Radiomatic FLO-ONE/Beta radiochromatographic detector, using FLO-SCINT V scintillation fluid at 1 ml/min, to quantitate the conversion of cholesterol to 7α-hydroxycholesterol. Retention time for cholesterol was equal to 6 min, and retention time for 7α-hydroxycholesterol was equal to 15 min. CYP7A1 activity is expressed as picomoles of cholesterol converted per milligram of microsomal protein per min. Statistical comparisons of CYP7A1 activity were made using the paired *t*-test.

Hypocholesterolemic activity

Hypercholesterolemia was induced in male Sprague Dawley rats (CD, Charles River, 270–310 gm) by a diet enriched in cholesterol and cholic acid. Prior to diet initiation, baseline blood samples were collected by cardiac puncture under halothane anesthesia. Serum was obtained for analysis of total cholesterol, HDL-C, and dextran precipitable LDL+VLDL cholesterol concentrations. The rats were divided into five groups, all with comparable mean baseline cholesterol concentrations, and with five animals in each group. Five days after baseline sampling of serum cholesterol the rats were put on a diet of ground Wayne Laboratory food mixed with cholesterol (0.4%) and cholic acid (0.2%) by weight, and fed ad libitum. Beginning on the same day as the diet, 264W94 (0.03, 0.1, 0.3, and 1.0 mg/kg) was administered by oral gavage as a suspension in 0.5% methylcellulose (1 ml/100 gm body weight) at 9 AM and 3 PM for 3.5 days. Control animals received cholesterol-cholic acid diet and 0.5% methylcellulose. Four hours after the final morning dose, the rats (fasted for 4 hours) were anesthetized and bled for determination of serum cholesterol. Serum cholesterol concentrations were determined enzymatically using reagents from Seragen Diagnostics. HDL-C was determined after selective precipitation of LDL+VLDL-C with dextran sulfate and magnesium sulfate. LDL+VLDL-C was determined from the difference between total cholesterol and HDL-C.

RESULTS

Ileal brush border membrane vesicles

In rat and monkey ileal BBMV, 264W94 at 0.1 to 10 μM decreased the sodium-dependent uptake of TC in a concentration dependent manner. The IC_{50} values for 264W94 against 10 μM TC are 0.24 μM and 0.41 μM for rat and monkey ileal BBMV, respectively (**Fig. 2**).

Human IBAT

In CHO-hIBAT cells, 264W94 at 0.1 to 0.5 μM inhibited human IBAT-specific transport of 5 μM TC by 14% to 75% in a concentration-dependent manner with IC_{50} of 0.25 μM.

Transporter specific TC transport by CHO-hIBAT cells in the absence of inhibitor was saturable, and the estimates for V_{max} and K_m were 448 ± 13.2 pmol·mg^{-1}·sec^{-1} and 22 ± 1.3 μM, respectively (**Fig. 3A**). Evaluation of initial velocities at various TC and 264W94 concentrations by Lineweaver-Burk plots of 1/v as a function of 1/[S] indicates that the mechanism of inhibition by 264W94 is primarily competitive (Fig. 3B). Assuming the competitive model described by the equation, $v = [V_{max}\ [S])/([S]+K_m\,(1+[I]/K_i)]$, and solving for K_i, the estimated K_i for 264W94 is 0.2 μM.

TC absorption by rat ileum in situ

Table 1 shows the effects of 264W94 on ileal uptake of 3 mM [^{3}H]TC, transport of TC into the bile, and rate of transport into the bile. 264W94 concentrations of 1 μM and 10 μM inhibited ileal TC uptake by 24% and 39%, respectively. Effects of 264W94 on TC transport into bile, and transport rate were comparable. The time course of

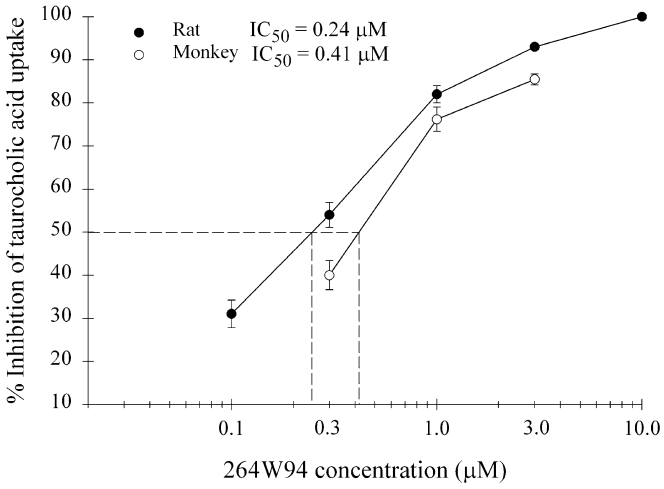

Fig. 2. Concentration-dependent inhibition by 264W94 of sodium-dependent taurocholic acid (TC) uptake by rat and monkey ileal brush border membrane vesicles (BBMV). TC uptake was determined in rat and monkey ileal BBMV preloaded with 280–300 mM mannitol, 10–20 mM Hepes-Tris (pH 7.4) and incubated in 100 mM NaCl (total uptake) or 100 mM KCl (passive uptake), 17.5–20 mM Hepes-Tris (pH 7.4), 80–85 mM mannitol, 10 μM [^{3}H]TC with 0–10 μM 264W94, and 1% ethanol for 30 s at room temperature. Sodium-dependent TC uptake was determined by subtraction of passive from total uptake. Results are expressed as percent inhibition of sodium-dependent TC uptake compared with control. Each data point represents the mean ± SE for four determinations.

the biliary recovery of TC taken up by the ileal lumen was similar in treated and control animals (**Fig. 4**). Of the total TC taken up by the ileum, 80–87% was recovered in the bile within 20 min in treated and control animals.

Total 24 h fecal excretion and enteric absorption of bile acids

Table 2 summarizes the effects of 264W94 on fecal excretion of a TC analog ^{75}SeHCAT in normal rats and mice. In both species, 264W94 administered orally at doses of 0.003 to 0.1 mg/kg b.i.d. for 2 days, increased fecal excretion of ^{75}SeHCAT in a dose-dependent manner. In rats, ^{75}SeHCAT fecal excretion was significantly increased 82%, 145%, and 232% relative to control, at doses of 0.01, 0.03, and 0.1 mg/kg, respectively. In mice ^{75}SeHCAT fecal excretion was increased 15%, 29%, and 50% relative to control, at doses of 0.01, 0.03, and 0.1 mg/kg, respectively.

Rats absorbed more ^{75}SeHCAT than mice, evident from the smaller percentage of the total ^{75}SeHCAT dose excreted by control rats compared with control mice. Expressed as inhibition of the absorbed dose of ^{75}SeHCAT used, these data indicate that 264W94 inhibited ^{75}SeHCAT absorption, in a dose-dependent manner, with ED_{30} values of 0.02 mg/kg in both rats and mice.

Time course of enteric ^{75}SeHCAT distribution

The time course of distribution of ^{75}SeHCAT in the small intestine and large intestine (combined cecum and colon) in control and in animals treated with a single dose

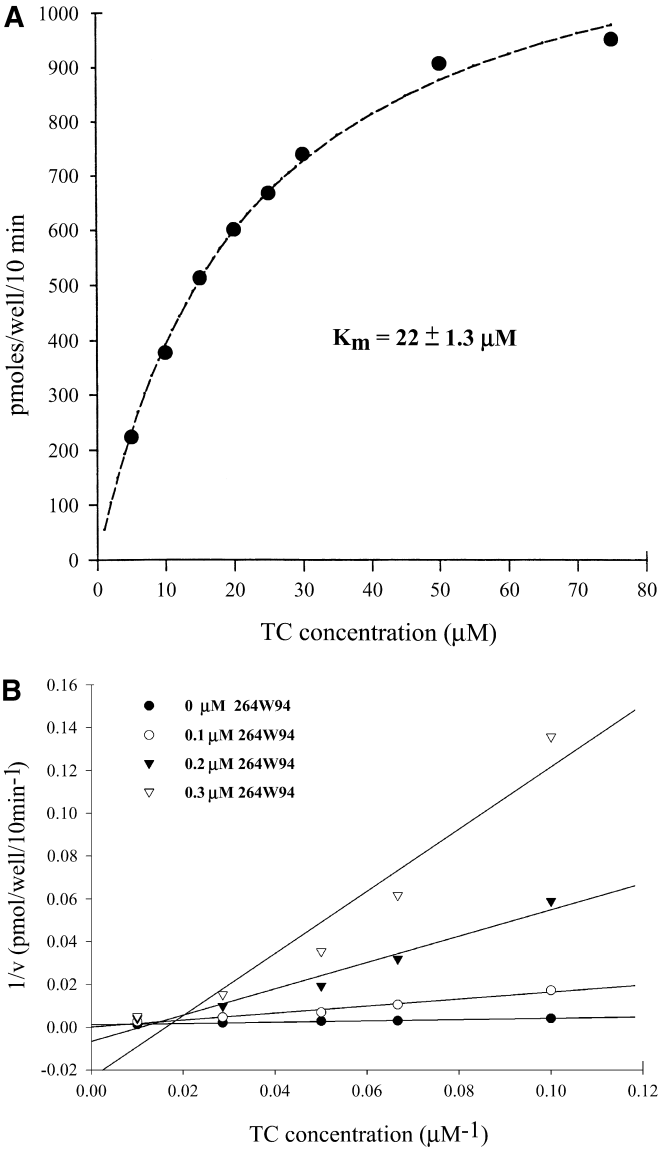

Fig. 3. Kinetic parameters of TC transport by a stable line of Chinese hamster ovary (CHO) cells expressing human ileal bile acid transporter (CHO-hIBAT cells) in the absence (A) and presence (B) of 264W94. CHO-hIBAT cells were incubated for 10 min at 37°C under 5% CO_2 and 95% air in 0.2 ml of incubation medium consisting of Hank's Balanced Salt Solution, buffered to pH 7.4 with Hepes-Tris, and containing [^{3}H]TC (11, 14, 20, 33, and 100 μM), 0.2%DMSO, and 264W94 (0, 0.1, 0.2, and 0.3 μM). After quenching of the incubation, CHO-hIBAT cell associated radioactivity was determined. Initial velocities are expressed as picomoles of cell protein per min. Each value represents the mean ± SE for four determinations. A: K_m and V_{max} for TC transport in the absence of inhibitor were determined by nonlinear regression analysis for fit to a rectangular hyperbola with a linear component described by the Michaelis-Menten equation $v = (V_{max}[S])/([S] + K_m)$. B: The model of inhibition and K_i for 264W94 were estimated by graphical analysis of Lineweaver-Burk plots of $1/v$ as a function of $1/[S]$ in the presence and absence of inhibitor, and solution of the reciprocal velocity equation for competitive inhibition: $1/v = (K_m/V_{max})(1+[I]/K_i)(1/[S])+1/V_{max}$.

TABLE 1. Effects of 264W94 on uptake and transport into the bile of taurocholic acid (TC) from the ileum in anesthetized rats

Treatment	Total Uptake (% inhibition)	Total Transport (% inhibition)	Transport Rate (% inhibition)
264W94 1 μM	25.4 (±3.3)**	26.1 (±3.6)**	34.1 (±7.1)**
264W94 10 μM	38.9 (±2.0)**	44.0 (±3.1)**	51.6 (±5.1)**

Ileal segments (~15 cm) isolated in situ in bile fistula rats, were incubated for 4 min with 2 ml of medium containing 3 mM [^{3}H]TC, 0.9% NaCl, 0.01 M sodium phosphate (pH 7.0), and 1% DMSO with or without 264W94 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 [^{3}H]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 six control or four treated animals.

**$P < 0.01$ (treated vs control by one way analysis of variance).

of 0.1 mg/kg 264W94 is shown in **Table 3**. All values are expressed as a percentage of the total ^{75}SeHCAT administered. In both control and treated rats, there was an increasing gradient of ^{75}SeHCAT content from the first (most proximal) through third quarters of the small intestine. In control rats at all time points, ^{75}SeHCAT content in the third quarter, relative to ^{75}SeHCAT content in the fourth (most distal) quarter of the small intestine decreased by 61% to 75%. In 264W94-treated rats, ^{75}SeHCAT content in the third quarter, relative to ^{75}SeHCAT content in the fourth quarter of the small intestine, decreased only 3% at 4 h after dosing. According to the calculation for inhibition of ^{75}SeHCAT absorption described in the Methods section, 264W94 inhibited ^{75}SeHCAT absorption in the distal quarter of small intestine by 83% and 97% (statistically significant at the level of $P < 0.01$) at the 2 h and 4 h time points, and by 52% at the 6 h time point ($P < 0.05$). There was a nonstatistically significant inhibition of absorption of 26% at the 1 h time point, and there was no effect at 0.5 h. At the 4 h and 6 h time points there were statistically significant ($P < 0.01$) increases of 159% and 84%, respectively, in ^{75}SeHCAT content in the large intestine of treated compared with control animals. Both inhibition of ^{75}SeHCAT absorption and increased ^{75}SeHCAT content in the large intestine peak at the 4 h time point.

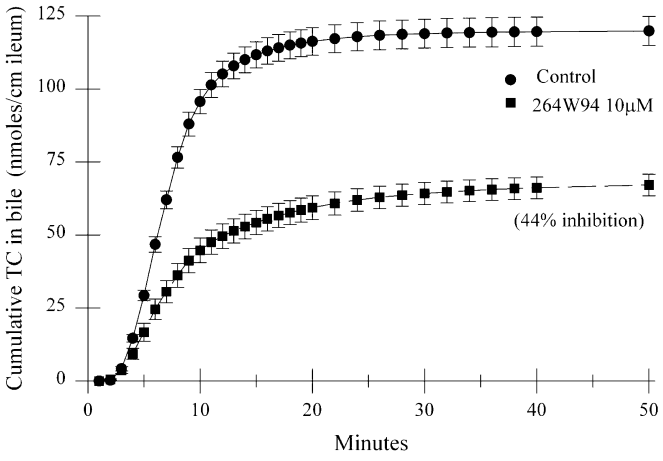

Fig. 4. Effects of 264W94 on transport into the bile of TC placed in the distal ileum of bile fistula rats. Isolated ileal segments ~15 cm long were incubated for 4 min with 2 ml of medium containing 3 mM [^{3}H]TC, 0.9% NaCl, 0.01 M sodium phosphate (pH 7.0), and 1% DMSO with or without 10 μM 264W94 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 six control or four treated animals.

CYP7A1 enzyme activity

Treatment with 264W94 (0.3mg/kg once daily p.o.) induced a 4.1-fold increase in CYP7A1 activity within 8 h following the initial dose (**Fig. 5**). This level of CYP7A1 induction (3.8-fold ± 0.61) was maintained throughout the dosing period, was statistically significant ($P < 0.01$), and did not return to control levels until 48 h after the last dose. During treatment, in both the control and 264W94-treated groups, a pronounced diurnal fluctuation in CYP7A1 activity of ~2-fold (range: 1.5 to 2.3) was maintained.

Hypocholesterolemic activity

Table 4 summarizes the effects of 264W94 on serum cholesterol in diet induced hypercholesterolemic rats. Administered orally to cholesterol-cholic acid-fed rats at doses of 0.03 to 1 mg/kg b.i.d. for 3.5 days, 264W94 dose-dependently attenuated diet-induced increases in serum LDL+VLDL-C, as well as the decrease in HDL-C, compared with the control hypercholesterolemic animals. Se-

TABLE 2. Effects of 264W94 on fecal bile acid excretion in rats and mice

264W94 Dose (mg/kg)	Rats ^{75}SeHCAT Excretion % Total Dose (± SE)	Mice ^{75}SeHCAT Excretion % Total Dose (± SE)
Control	19 ± 1.5	53 ± 2.4
0.003	22 ± 2.0	58 ± 3.0
0.01	35 ± 2.7**	60 ± 3.4
0.03	48 ± 6.3**	68 ± 3.6*
0.1	64 ± 4.5**	79 ± 1.5**

Rats and mice were dosed orally with 264W94 or control vehicle at 9:00 am and 3:30 pm for two days. At midday on the second day the animals were given a trace (1.3 nmol) of ^{75}SeHCAT, a TC analog. Total radioactivity in feces excreted over the next 24 h was determined. ^{75}SeHCAT excretion counted in the feces is expressed as a percentage of total ^{75}SeHCAT in the oral dose. Each data point represents the mean ± SE for six to eight animals.

**$P < .01$; *$P < .05$ (treated vs. control in paired t-test).

TABLE 3. Effect of 264W94 on time course profile of distribution of ^{75}SeHCAT in rat intestine

Group	Segment	Percent of Total ^{75}SeHCAT Recovered Post-Treatment ± SEM				
		0.5 h	1.0 h	2.0 h	4.0 h	6.0 h
Control						
	Small Intestine					
	1st Quarter	5.6 ± 0.5	3.6 ± 0.3	4.8 ± 0.4	4.9 ± 1.1	3.4 ± 0.5
	2nd Quarter	19.1 ± 1.5	12.9 ± 1.2	13.1 ± 1.1	11.2 ± 1.6	10.1 ± 1.6
	3rd Quarter	33.0 ± 2.3	27.1 ± 1.7	24.4 ± 2.1	36.3 ± 4.8	23.9 ± 4.2
	4th Quarter	11.6 ± 2.8	10.3 ± 1.5	9.5 ± 1.8	9.1 ± 1.9	8.8 ± 1.8
	Large Intestine	9.0 ± 1.8	13.8 ± 2.0	15.4 ± 2.3	16.0 ± 2.4	17.6 ± 2.1
Treated						
	Small Intestine					
	1st Quarter	5.0 ± 0.4	4.8 ± 0.6	2.2 ± 0.3	2.5 ± 0.6	3.0 ± 0.6
	2nd Quarter	14.6 ± 0.5	16.5 ± 2.4	7.2 ± 1.0	5.0 ± 1.0	7.1 ± 2.0
	3rd Quarter	25.8 ± 1.9	27.6 ± 2.5	25.6 ± 3.2	16.0 ± 2.5	16.1 ± 3.5
	4th Quarter	9.2 ± 1.0	15.0 ± 1.5	23.0 ± 2.3**	15.6 ± 1.5**	11.2 ± 1.4*
	Large Intestine	16.2 ± 2.1	13.1 ± 2.2	15.7 ± 2.4	41.5 ± 4.8**	32.3 ± 3.0**

Rat bile acid pools were labeled by oral administration of a trace amount (1.3 nmol) of ^{75}SeHCAT in 1.0 ml saline. Twenty-four hours later, the rats were given a single oral dose of 264W94, 0.1 mg/kg. The animals were sacrificed at the indicated times after 264W94 administration, and total content of ^{75}SeHCAT in gut segments (large intestine includes cecum and colon) was measured using a gamma-counter. Values are expressed as percentage of the total dose of radiolabel.

*$P < 0.05$, **$P < 0.01$ (treated vs control in paired t-test).

rum LDL+VLDL-C was significantly decreased 61%, 56%, and 40%, at doses of 0.3, 0.1, and 0.03 mg/kg, respectively. Significant increases in HDL-C of 75% and 60% were observed at 0.3 and 0.1 mg/kg, respectively. In this animal model, no additional effects were produced by the highest dose (1 mg/kg).

DISCUSSION

In the present work, we examined the effects of 264W94 in models of the rat, monkey, and human IBAT system in vitro, and in rodent models of bile acid absorption and cholesterol metabolism in vivo. Our results indicate that

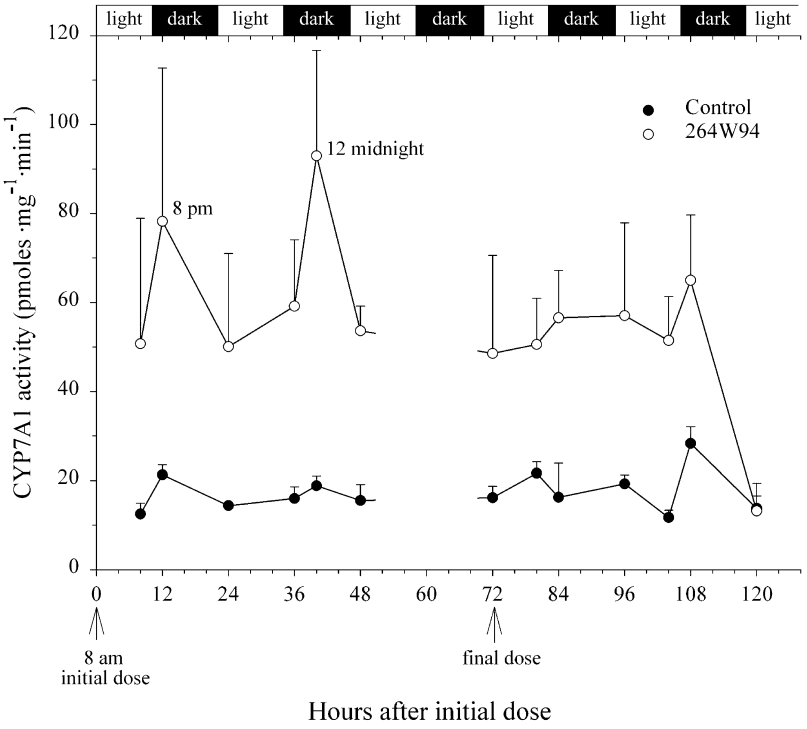

Fig. 5. Induction of hepatic cholesterol 7α-hydroxylase (CYP7A1) activity. Rats were dosed with 264W94 (0.3 mg/kg p.o.) once daily at 8 AM for up to 3 days. Liver microsomes were prepared from animals sacrificed at various time points during and after the dosing period. Microsomal CYP7A1 activity was assayed by measuring the conversion 4-^{14}C-labeled cholesterol (100 μM) to 7α-hydroxycholesterol in the presence of 8 mM NADPH and 1 mM EDTA in 50 mM HEPES (pH 7.4), during a 30 min incubation period at 37°C. 7α-hydroxycholesterol was quantified using HPLC. Each data point represents the mean ± SD for three treated and three control animals, each assayed in duplicate.

TABLE 4. Effects of 264W94 on serum cholesterol concentrations of diet-induced hypercholesterolemic rats

Group	Serum Cholesterol, mg/dl (± SE)		
	Total	HDL	LDL+VLDL
Baseline, All (pre-diet; pre-dose)	68 ± 2	34 ± 1	34 ± 1
Control (vehicle + diet)	134 ± 22	20 ± 2	113 ± 21
264W94, 0.03 mg/kg	91 ± 9	23 ± 1	68 ± 9*
264W94, 0.1 mg/kg	82 ± 5	32 ± 5*	50 ± 6*
264W94, 0.3 mg/kg	79 ± 4	35 ± 1*	44 ± 4*
264W94, 1.0 mg/kg	77 ± 5	34 ± 4*	43 ± 2*

Hypercholesterolemia was induced in rats by feeding a diet supplemented with cholesterol, 0.4% and cholic acid, 0.2%. Five days prior to initiation of diet and 264W94, baseline serum cholesterol values were obtained for all rats. The rats were divided into five groups, all with comparable mean baseline cholesterol concentrations. All rats were fed diet, and dosed orally with 264W94 or control vehicle at 9 AM and 3 PM for 3.5 days. Baseline values represent mean ± SE for all 25 rats in study. Values from final bleed performed 4 h after the last morning dose are expressed as mean ± SE for five rats per group.

*$P < 0.05$ (treated vs. control by one way analysis of variance).

264W94 inhibits sodium-dependent bile acid uptake by rat and monkey ileal BBMV in vitro, and competitively inhibits the human IBAT with sub-micromolar affinity. We offer evidence that the latter is the mechanism underlying the potent inhibition of bile acid absorption and hypocholesterolemic action observed here in our in vivo studies with 264W94.

The conservation and recycling of bile acids through the EHC is a multi-step system that begins with the efficient reabsorption of bile acids, primarily through sodium-dependent transport of bile acids across the apical (brush border) membrane of ileal cells by a transporter (8–11). In our in vitro preparations of rat and monkey BBMV, and in human IBAT, which simulate the first step in the EHC of bile acids, 264W94 decreased sodium-dependent TC transport with IC_{50} values of 0.24, 0.41, and 0.25 μM, respectively. These results suggest that the ileal apical membrane bile acid transporter, highly homologous (8, 11, 30) between rats and humans, is the site of action of 264W94 in rats, and predict that 264W94 might have similar action in monkeys and humans.

Kinetic analysis of the data from human IBAT expressed in a stable line of CHO cells (CHO-hIBAT cells) indicates that sodium-dependent TC transport is saturable with a K_m of 22 μM. This K_m value is similar to values, ranging from 14 μM to 52 μM, reported for IBAT in other systems (26, 30, 31), and to the K_m for rat ileal BBMV of 97 μM (24). The estimated K_i of 0.2 μM is consistent with the IC_{50} of 0.25 μM at 5 μM TC in human IBAT. The similar concentration-related effects and IC_{50} values for 264W94 on TC transport by human IBAT and rat ileal BBMV, suggest that the competitive inhibitory mechanism is the same as that characterized using rat BBMV for a related, but less potent compound, 2164U90 (24).

The results obtained in our in vivo models are consistent with inhibition of IBAT as the mechanism of hypocholesterolemic action of 264W94. First, in our in situ model with anesthetized rats, 264W94 applied directly to the site of action, the distal ileum, inhibited ileal uptake of TC, transport of TC into the bile, and the rate of transport of TC into the bile proportionally. Second, in rats and mice, 264W94 administered orally at doses of 0.003 to 0.1 mg/kg b.i.d. for 2 days inhibited absorption of a TC analog, ^{75}SeHCAT, in a dose-dependent manner with an ED_{30} of 0.02 mg/kg for both species. Third, hepatic enzyme CYP7A1, negatively regulated by bile acids in the EHC, and a key indicator of compensatory hepatic bile acid synthesis in response to increased bile acid excretion (12, 14, 15), was induced 4-fold in response to treatment with 264W94 (0.3 mg/kg q.d., p.o.) for 3 days in normal rats. Finally, in cholesterol-cholic acid-fed rats, doses of 264W94 (0.03 to 1.0 mg/kg b.i.d. orally for 3.5 days), comparable to those that inhibited bile acid absorption in normal rats, dose-dependently attenuated diet-induced increases in serum LDL+VLDL-C, as well as the decreases in HDL-C, compared with the control hypercholesterolemic animals.

In our in vitro models, 264W94 is moderately more potent than a related 1,4-benzothiazepine derivative, 2164U90, which lacks the 7- and 8-methoxy groups (24). In monkey BBMV, the IC_{50} values for 264W94 and 2164U90 assayed for TC uptake under the same conditions were respectively 0.41 μM and 5 μM, representing a difference of ~10-fold. In rat BBMV, the IC_{50} values for 264W94 and 2164U90 were respectively 0.24 μM and 0.42 μM, representing only a 2-fold difference. However, 264W94 is a more potent inhibitor of ileal bile acid absorption in rats in vivo by ~500-fold compared with 2164U90. In rats, the ED_{30} for inhibition of ^{75}SeHCAT absorption by 264W94 is 0.02 mg/kg b.i.d. for 2 days, while a dose of 10 mg/kg 2164U90 was necessary to produce ~30% inhibition in the same model (23). 264W94 is also at least 150× more potent in lowering serum LDL+VLDL-C compared with 2164U90 in the same rat model of hypercholesterolemia. 264W94 (0.03 mg/kg b.i.d. for 3.5 days) resulted in a 44% decrease in LDL+VLDL-C, while 2164U90 (5.0 mg/kg b.i.d. for 3.5 days) decreased LDL+VLDL-C by 37% (23). A possible explanation for this disparity in the potency between 264W94 and 2164U90 could be differences in metabolism of the two compounds. It has been previously reported that 264W94 has as one of its major metabolites, an *O*-demethylated metabolite (in the 8-methoxy position), that is approximately 3× more potent than parent-compound 264W94

in inhibiting bile acid absorption in vivo (25, 32). Thus, the greater pharmacological effect of 264W94 could be attributed to the combination of 264W94 and one or more active metabolites. Another possible explanation for the disparity is that 264W94 or active metabolites formed in intestine, blood, or liver may undergo enterohepatic recirculation.

This work also provides additional insight into the time course of action of 264W94 in vivo. Compared with the in situ model where effects occurred within 4 min, the time of onset of inhibition of IBAT in rats, having bile acid pools equilibrated with ^{75}SeHCAT, was delayed by 1 h to 2 h when 264W94 was administered orally. The time of onset is most likely dependent on intestinal transit time for the active compound to reach the distal ileum, and is consistent with the physiological small intestinal transit time of 78 min reported for normal rats of comparable age (33). In the distal quarter of the small intestine, peak inhibition of bile acid absorption of 97% and 52% at 4 h and 6 h, respectively, after oral dosing with 264W94, was synchronous with the appearance of increased ^{75}SeHCAT content (159% and 84%, respectively) in the large intestine, and consistent with the 232% increase in fecal ^{75}SeHCAT excretion with a comparable dose in a 24 h period.

In our time-course study of hepatic microsomal CYP7A1 activity in rats, maximum basal induction was detected within 8 h after the initial dose of 264W94 (0.3 mg/kg). This elevation was maintained during 3 days of dosing, and did not return to baseline (control) levels until 48 h after the last dose. Evidence from prior studies of total biliary diversion or cholestyramine feeding in rats suggests that the maximum attainable induction of basal (midlight cycle) CYP7A1 activity by complete interruption of the EHC is 3- to 5-fold (12, 34–36). The 4-fold increase in CYP7A1 activity that we find with 264W94 is comparable to historical maxima. However, with cholestyramine feeding, about 5 days were required for CYP7A1 activity to reach a peak, and upon withdrawal, CYP7A1 levels dropped to baseline or below within 1 day (34, 37–40). The more rapid and prolonged effect of 264W94 on CYP7A1 activity compared with historical data on cholestyramine could be a reflection of greater potency, or pharmacokinetic properties, such as long half-life or longer residence time in the intestine resulting from EHC of active compound or metabolites.

CYP7A1 activity, under basal and dynamic physiological states, is thought to be regulated primarily at the transcriptional level. Recent research in nuclear receptor signaling indicates that *CYP7A1* is downregulated in the basal state in multiple species by bile acid receptor FXR (14, 18), and upregulated, at least in rats, by oxysterol receptor LXRα (14, 18, 41, 42). Our observation in rats that 264W94 stimulates rapid CYP7A1 induction, and washout results in eventual recovery of CYP7A1 to baseline, may be explained, respectively, by de-repression and re-repression of the *CYP7A1* gene in response to drug-induced alterations in hepatocyte bile acid concentrations and bile acid-FXR interactions (13–15, 18, 42). The diurnal fluctuation in CYP7A1 activity of approximately 2-fold that we observed in both control and 264W94 treated rats confirms prior evidence in rats and humans that there is a distinct circadian rhythm of bile acid synthesis that is independent of bile acid feedback regulation (29, 40, 43–46).

In conclusion, our results indicate that 264W94 is a rapid-onset, specific inhibitor of the IBAT system in rats and other animal models, and has a competitive profile with sub-micromolar affinity for human IBAT in vitro. We demonstrate that 264W94 is a potent LDL-C lowering agent in rats. Effective hypocholesterolemic doses are comparable to doses that produce IBAT inhibition in our bile acid fecal excretion and intestinal distribution studies, and that induce CYP7A1 activity, an indicator of upregulated bile acid synthesis. Taken together these results indicate that inhibition of the IBAT system is the mechanism underlying the hypocholesterolemic activity of 264W94. Efficacy in our pre-clinical rat studies, and activity in human IBAT in vitro suggest that 264W94 could be an effective LDL-C lowering agent in the clinic. With more stringent recommendations for LDL-C lowering in the new 2001 National Cholesterol Education Program guidelines, and recent concern over rhabdomyolysis with HMG-CoA reductase inhibitors (statins), particularly when used in combination with fibrates (1), there could be renewed interest in IBAT inhibitors as an alternative or complementary approach.

In the clinic, 264W94 might be expected to closely reproduce the effect of PIB surgery, as both treatments directly block the IBAT system in the distal ileum. Potential advantages of a specific pharmacological IBAT inhibitor over PIB surgery are that the effects could be readily reversible and titratable with dose. Inhibition of bile acid reabsorption using nonspecific BAS, for reason of a safe and complementary mechanism of action, has historically provided the cornerstone for most established combination drug regimens with statins, fibrates, and niacin. For example, combinations of statins and BAS produce additive effects in lowering LDL-C (1, 20, 21, 47–49). BAS therapy causes increased LDL receptor expression, as well as increased cholesterol biosynthesis, to compensate for the negative balance in hepatocyte free cholesterol resulting from increased cholesterol elimination through excretion of bile acids. This increase in cholesterol biosynthesis blunts some of the LDL-C lowering potential of BAS. When a statin and BAS are given together, the compensatory increase in cholesterol biosynthesis induced by BAS is inhibited by the statin, and additive reduction in hepatocyte free cholesterol results from the combined effects of inhibition of cholesterol biosynthesis and increased conversion of free cholesterol to bile acids. Consequently, greater hepatocyte LDL receptor expression causes a greater decrease in plasma LDL-C. Specific, small-molecule, competitive IBAT inhibitors like 264W94 might be expected to have additive effects similar to BAS in combination with statins. IBAT inhibitors might be used in combination with lower doses of statins to achieve optimal hypocholesterolemic activity with less risk of toxicity, or as a supplement to statins in patients with LDL-C levels not ad-

equately controlled by statins alone. In addition, IBAT inhibitors may have the potential to be used as monotherapy, or in combination with other antilipemic agents such as niacin or fibrates in patients with mixed dyslipidemia.

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