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European Journal of Pharmacology 539 (2006) 89–98
www.elsevier.com/locate/ejphar

Novel non-systemic inhibitor of ileal apical Na^+-dependent bile acid transporter reduces serum cholesterol levels in hamsters and monkeys

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Received 7 December 2005; received in revised form 30 March 2006; accepted 3 April 2006
Available online 7 April 2006

Abstract

1-{7-[(1-(3,5-Diethoxyphenyl)-3-{[(3,5-difluorophenyl)(ethyl)amino]carbonyl}-4-oxo-1,4-dihydroquinolin-7-yl)oxy]heptyl}-1-methylpiperidinium bromide, R-146224, is a potent, specific ileum apical sodium-dependent bile acid transporter (ASBT) inhibitor; concentrations required for 50% inhibition of [^{3}H]taurocholate uptake in human ASBT-expressing HEK-293 cells and hamster ileum tissues were 0.023 and 0.73 μM, respectively. In bile-fistula rats, biliary and urinary excretion 48 h after 10 mg/kg [^{14}C]R-146224, were 1.49±1.75% and 0.14±0.05%, respectively, demonstrating extremely low absorption. In hamsters, R-146224 dose-dependently reduced gallbladder bile [^{3}H]taurocholate uptake (ED_{50}: 2.8 mg/kg). In basal diet-fed hamsters, 14-day 30–100 mg/kg R-146224 dose-dependently reduced serum total cholesterol (~40%), high density lipoprotein (HDL) cholesterol (~37%), non-HDL cholesterols (~20%), and phospholipids (~20%), without affecting serum triglycerides, associated with reduced free and esterified liver cholesterol contents. In normocholesterolemic cynomolgus monkeys, R-146224 specifically reduced non-HDL cholesterol. In human ileum specimens, R-146224 dose-dependently inhibited [^{3}H]taurocholate uptake. Potent non-systemic ASBT inhibitor R-146224 decreases bile acid reabsorption by inhibiting the ileal bile acid active transport system, resulting in hypolipidemic activity.

Keywords: Apical sodium-dependent bile acid transporter; Enterohepatic circulation; Hypocholesterolemic agent; Monkey; Hamster; Cholesterol

1. Introduction

Atherosclerosis is a disease of the vascular wall leading to myocardial infarction, heart failure, peripheral vascular disease, and stroke (Glass and Witztum, 2001). Although multiple risk factors have been identified contributing to atherosclerotic lesion formation, its growth is initiated and sustained by increased levels of low density lipoprotein (LDL), and low and/or dysfunctional high density lipoprotein (HDL). Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (statins) are currently the mainstay of dyslipidemia management for prevention of cardiovascular disease (Adult Treatment Panel III, 2001) with recent data indicating additional benefits of further lipid lowering at higher dosage (Cannon et al., 2004; Grundy et al., 2004; Heart Protection Study Collaborative Group, 2002; LaRosa et al., 2005; Mosca et al., 2004). Occasionally serious side effects occur, such as myopathy (rhabdomyolysis), which can cause acute renal failure. Statins also elevate serum transaminases in a small percentage of patients. Although little evidence suggests

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doi:10.1016/j.ejphar.2006.04.005

progression to liver disease (Tolman, 2002), persistent transaminase elevation is implicated. To limit such potential side effects, some clinicians may combine a lower dose of statin with a cholesterol absorption inhibitor such as ezetimibe (McKenney, 2005). The relative safety and efficacy of this and other strategies to lower LDL and increase HDL cholesterol levels, including fibrates, HDL cholesterol mimetics, nicotinic acid derivatives, and inhibitors of cholesterol ester transfer protein in combination with a low-dose statin, need further evaluation. Much effort is directed to developing agents acting via other hypocholesterolemic mechanisms to use with statins.

One area of focus is the bile acid system. Bile acids play a critical role in the intestinal absorption of fat and cholesterol. After fulfilling their function as detergent agents in the intestinal lumen, they are absorbed by active ileal uptake and recycled in the enterohepatic circulation. Bile acid sequestrants such as anion exchanging resins have been used to treat hypercholesterolemia and hyperlipidemia with a good safety record for over two decades (Hoeg, 1991; Melian and Plosker, 2001), and in combination with statins have achieved synergistic reduction in blood cholesterol levels (Leren et al., 1988; Vega and Grundy, 1987). Limitations of bile acid sequestrant therapy include discomfort owing to their bulkiness as well as constipation, which contribute to poor patient compliance. Therefore, it is desirable to develop hypocholesterolemic agents that interrupt the reabsorption of bile acids at more tolerable therapeutic doses.

Ileal apical Na^+-dependent bile acid transporter (ASBT), which plays a critical role in the reabsorption of bile acids in the ileum in humans and animals (Dietschy et al., 1993; Hofmann, 1999; Mitropoulos et al., 1973; Shneider, 2001), is considered an attractive target for a new class of cholesterol-lowering drugs (Zhang et al., 2002). ASBT inhibitors would increase excretion of bile acids, causing increased catabolism of hepatic cholesterol to bile acids, and subsequently reducing hepatic and eventually serum cholesterol levels. Moreover, ASBT inhibitors would not be absorbed since bile acid binding to ASBT occurs within the lumen of the most distal part of the ileum. Thus, a non-systemic ASBT inhibitor would carry low risk of potential systemic toxicity and drug–drug interaction (Huang et al., 2005; Tremont et al., 2005). Human ASBT is expressed at the highest levels in the distal half of the ileum and in the kidney (Craddock et al., 1998). Gene cloning and expression techniques have facilitated rapid screening of potential drugs and several compounds have been identified (Hara et al., 1997; Lewis et al., 1995; Root et al., 2002; West et al., 2002).

Absorbed bile acids are transferred to the hepatic portal circulation where they are taken up into hepatocytes by another bile acid transporter, Na^+-taurocholate co-transporting polypeptide (NTCP) (Meier et al., 1997). Recently, we synthesized a novel series of ASBT inhibitors having potent cholesterol-lowering activity in vivo by screening with hamsters and human ASBT (*h*ASBT)- or human NCTP (*h*NCTP)-expressing HEK-293 cells. Some derivatives, designed with poor absorbability and greater molecular mass than that of "Lipinski's Rule of Five", were previously reported to reduce serum cholesterol levels in hamsters fed a chow diet (Kurata et al., 2004).

Herein, we report the in vitro and in vivo pharmacological profiles of R-146224, a new ASBT inhibitor synthesized in our laboratory, and its effects on bile acid metabolism. We also present pharmacokinetic analysis using [^{14}C]labeled R-146224 and demonstrate the potential of R-146224 as a hypocholesterolemic drug in vivo.

2. Materials and methods

2.1. Test compounds

R-146224, 1-{7-[(1-(3,5-diethoxyphenyl)-3-{[(3,5-difluorophenyl)(ethyl)amino]carbonyl}-4-oxo-1,4-dihydroquinolin-7-yl)oxy]heptyl}-1-methylpiperidinium bromide, and its derivatives, R-146119 and R-151005 (Fig. 1), were synthesized at the Medicinal Chemistry Research Laboratories, Sankyo Co., Ltd. The molecular weights of R-146224, R-146119, and R-151005 are 785, 812, and 828 Da, respectively. [^{14}C]R-146224 (Code No. CFQ13129) was synthesized at Amersham Pharmacia Biotech UK Ltd. (Little Chalfont, Buckinghamshire, UK). Its chemical structure and the labeled position are shown in Fig. 1. The specific radioactivity was 2.11 GBq (57 mCi)/mmol, and the radiochemical purity was guaranteed to be more than 98% (98.6% by thin-layer chromatography, 98.6% by high performance liquid chromatography (HPLC)). Methyl 1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate (S-8921, Shionogi's ASBT inhibitor) was synthesized at Chemtech Labo., Inc. (Tokyo, Japan). For preparation of the dosing solution to animals, each compound was dissolved in distilled water (dosing vehicle). A stock solution of each compound was prepared for cellular and tissue assays in dimethyl sulfoxide and stored at 20 °C. Dilutions with dimethyl sulfoxide for all assays were prepared freshly prior to use. All reagents and solvents used were those commercially available, and their grades were of either extra pure, guaranteed, or HPLC grade.

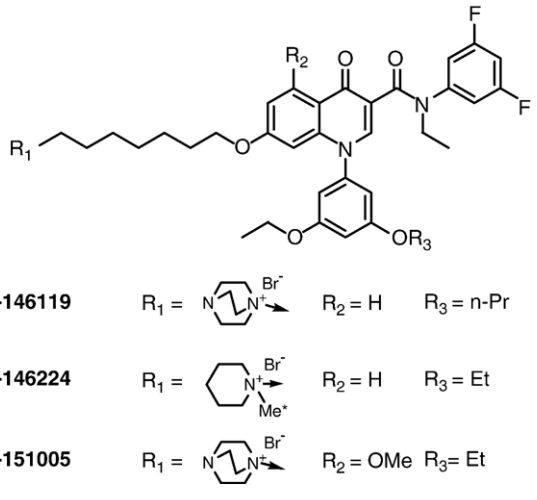

Fig. 1. Chemical structures of ASBT inhibitors.

2.2. Chemicals

L-[U-^{14}C]Alanine (6.29 GBq/mmol), L-[1-^{14}C]ascorbic acid (222 MBq/mmol), D-[8,9-^{3}H(N)]biotin (2090.5 GBq/mmol), [^{3}H(G)]daunomycin (684.5 GBq/mmol), [^{3}H(G)]digoxin (1369 GBq/mmol), D-[^{14}C]glucose (555 MBq/mmol), and [^{3}H(G)]taurocholic acid (74 GBq/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [Carboxy-^{14}C]Nicotinic acid (1.85 GBq/mmol) and [^{3}H(G)] riboflavin (vitamin B2 (1983.2 GBq/mmol)) were from Moravek Biochemicals Inc. (Brea, CA). [3,5,7,9-^{3}H]Folic acid potassium salt (888 GBq/mmol) was purchased from Amersham Pharmacia Biotech UK Ltd. (Little Chalfont, Buckinghamshire, UK). [^{3}H(G)]Thiamine hydrochloride (370 GBq/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All other chemicals used were of reagent grade.

2.3. [^{3}H]Taurocholate uptake into hASBT and hNCTP HEK-293 cells

The cell line HEK-293 (ATCC CRL 1573) was from the American Type Culture Collection (Manassas, VA). The *h*ASBT HEK-293 and *h*NCTP HEK-293 cells were stably transfected clones isolated as described below. Cells were grown in Dulbecco's modified Eagle's medium containing 4500 mg/l D-glucose, 10% (v/v) fetal bovine serum, 1% nonessential amino acids, 10 mM HEPES, 100 U/ml penicillin, and 100 mg/ml streptomycin (Life Technologies Ltd., Paisley, Scotland). Cells were grown until confluence in a monolayer at 37 °C in a humidified atmosphere of 5% CO_2. The medium for transfected cell lines was supplemented with 250 mg/ml of Geniticin (G-418) (Life Technologies, Inc., Grand Island, NY).

The cDNAs for *h*ASBT and *h*NCTP were obtained by polymerase chain reaction with pairs of primers (sense primer for *h*ASBT, 5′-AAC GTT GCT TAA CTC AAC CAG C-3′, and antisense primer for *h*ASBT, 5′-CTC GTC TGT TTT GTC CAC TTG A-3′, corresponding to 512–533 and 1649–1670 in the cDNA sequence of *h*ASBT (Craddock et al., 1998), respectively; sense primer for *h*NCTP, 5′-CAG CAA GAA CTG CAC AAG AAA C-3′, and antisense primer for *h*NCTP, 5′-GTA TTT GAG TAA TGG GTT AAA CAT ATA TAT TGA TT-3′, corresponding to 13–34 and 1460–1494 in the cDNA sequence of *h*NCTP (Hagenbuch and Meier, 1994), respectively) after reverse transcription of poly (A)-rich RNA from human intestine. Each amplified cDNA was purified by a Qiagen PCR Purification Kit and subcloned into the TA cloning vector PCR3.1 using a Eukaryotic TA Cloning Kit (Invitrogen, Carlsbad, CA). The ASBT and the NCTP inserts were verified by dideoxy sequencing.

The *h*ASBT and *h*NTCP cDNA expression constructs were transfected into HEK-293 cells by LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA), as described by the supplier. The stable colonies were selected by growth of cells in 1 mg/ml G418 and were maintained in Dulbecco's modified Eagle's medium with 10% (v/v) fetal bovine serum and 0.5 mg/ml G418. Clones from the *h*ASBT- and *h*NCTP-transfected cells with the highest transport activities were designated *h*ASBT HEK-293 and *h*NCTP HEK-293, respectively.

Na^{+}-dependent taurocholate transport in *h*ASBT HEK-293 and *h*NCTP HEK-293 cells was investigated as previously described with minor modifications (Bhat et al., 2003). Briefly, cells were equilibrated at room temperature for 20 min at the time of the experiment and were then washed and incubated for 15 min at 25 °C with buffer containing 110 mM NaCl (with sodium) or choline chloride (without sodium), 4 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 50 mM mannitol, and 10 mM HEPES, pH 7.4. Cells were then washed and incubated with the same buffer containing the indicated concentrations of the test compounds dissolved in dimethyl sulfoxide [final concentration of dimethyl sulfoxide in incubation mixture: 0.1% (v/v)]. along with 1 μCi/ml of [^{3}H]taurocholic acid for 1 h. To terminate the transport process, cells were washed twice with ice-cold phosphate-buffered saline. The radioactivity remaining in the well was counted by a Packard Top-Count Scintillation Counter (PerkinElmer Life Sciences). The IC_{50} (the concentration inhibiting the taurocholate uptake by 50% of the control) was extrapolated from the consequent sigmoidal inhibition curves (GraphPad, PRISM, San Diego, CA).

2.4. [^{3}H]Taurocholate uptake into hamster ileum pieces (In vitro hamster everted ileum pieces experiments)

ASBT-mediated [^{3}H]taurocholate uptake was investigated as previously described with minor modifications (Kurata et al., 2004). The freshly obtained ileum segment from male Syrian golden hamsters (5–7 weeks old; Japan SLC, Inc., Shizuoka, Japan) was everted using a stainless steel rod and washed in ice-cold oxygenated Krebs–Ringer solution (115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM Na_2SO_4 and 25 mM $NaHCO_3$, pH = 7.4). The everted ileum was divided into about 5-mm pieces, which were randomized into several groups based on the gradient of ASBT activities. Next, tissue pieces were incubated for 5 min at 37 °C in oxygenated Krebs–Ringer solution supplemented with 37 μM [^{3}H]taurocholic acid, 2 mg/ml BSA (essential fatty acid free; Sigma Chemical Co., St. Louis, MO) and various concentrations of the test compounds dissolved in dimethyl sulfoxide [final concentration of dimethyl sulfoxide in incubation mixture: 0.1% (v/v)]. Nonspecific uptake was measured by the addition of 37 mM of non-radiolabeled taurocholate. After 5 min incubation, the pieces were washed 3 times in ice-cold Krebs–Ringer solution containing 2 mg/ml of BSA, weighed, and solubilized in NCS®-II, tissue solubilizer (Amersham Pharmacia Biotech, Arlington Heights, IL). Thereafter, radioactivity (dpm) of each specimen was determined in the liquid scintillation counter. Specific [^{3}H]taurocholate uptake was calculated by subtracting the nonspecific count from the total count and is expressed as dpm/mg of wet weight. Each value obtained for the test compounds is the mean of triplicate incubations. A validation study revealed that plotting the radioactivity of uptake of [^{3}H] taurocholate into hamster ileum pieces showed a decline in the slope of the transport rate, consistent with localization of ASBT in the intestine (data not shown).

2.5. [^{3}H]Taurocholate uptake and transport into enterohepatic circulation of bile acid in hamsters (in vivo)

All animal treatment protocols, including Sections 2.6, 2.7, and 2.8, were reviewed by and are in compliance with the guidelines established by Sankyo's Institutional Animal Care and Use Committee. Male Syrian golden hamsters at 7 weeks old were purchased from Japan SLC, Inc. (Shizuoka, Japan) and used after an acclimatization of at least 1 week. Five animals were housed per metal cage in a room controlled for temperature (21–25 °C), humidity (45–65%) and light (7 AM–7 PM). Food (commercial laboratory chow diet, F-2, Funabashi Farm Co., Ltd., Chiba, Japan) and water were given ad libitum during the entire experiment. R-146224 at various doses was orally administered by gavage in a volume of 2 ml/kg body weight at around 10:00 AM, followed 90 min later by [^{3}H]taurocholate administration in saline at a dose of 37 kBq/2 ml/kg body weight (approximately 1 μCi/body). Twenty-four hours after the [^{3}H]taurocholate dosing, the animals were anesthetized with diethyl ether and bled from the abdominal aorta. The liver was removed and gallbladder bile samples were collected. Some bile samples could not be collected due to insufficient volume. To determine the population dose of [^{3}H]taurocholate remaining in the gallbladder bile, 10 μl each of the bile and original dosing mixture were added directly to 10 ml of liquid scintillator (PICO-FLUOR™, Packard Instrument Co., Meriden, CT) and then the levels of [^{3}H] were determined using a liquid scintillation counter (2250CA, Packard Instrument Co., Meriden, CT). The levels of [^{3}H] in the samples were compared to those of the control group.

2.6. Substrate specificity: effects of R-146224 on absorption of vitamins and nutrients (in situ loop experiment)

Male Sprague–Dawley (S.D.) rats at 6 weeks old were purchased from Charles River Japan, Inc. (Yokohama, Japan) and used at 7 weeks old after an acclimatization of 1 week. Rats were fasted overnight before dosing of the test compound. R-146224 was dissolved in distilled water at a concentration of 1 mg/ml. Then, the solution was mixed with solutions of various radiolabeled compounds (100:0.3 (v/v)). After laparotomy under ethyl ether anesthesia, a midline abdominal incision was made. A jejune loop (for vitamin or drug injection) or an ileal loop (for taurocholic acid injection) of about 10 cm in length was prepared by ligatures at both ends. After the dosing solution was injected into the loop with a syringe, the loop was returned and the incision was sutured. One hour after the administration, the loop was removed and homogenized. A portion of each of the homogenized samples was analyzed by the liquid scintillation counter. The absorption ratio was calculated by the subtraction of the radioactivity remaining in the loop from that injected.

2.7. Absorption of [^{14}C]R-146224 in rats

Rats underwent an abdominal surgical procedure for bile duct cannulation with a polyethylene tube (SP31, Natsume Seisakusho Co., Ltd., Tokyo, Japan) under diethyl ether anesthesia. The dosing solution prepared as mentioned above was orally administered to the bile-fistula rats using a metal stomach tube at a dose of 10 mg/kg. The rats were kept in Bollman cages (Sugiyama Gen Iriki Co., Ltd., Tokyo, Japan). Bile was collected at 4 °C for the periods of 0–1, 1–3, 3–6, 6–24, 24–30, and 30–48 h post-dose. Urine was also collected for the periods of 0–24 and 24–48 h post-dose.

Ten- or 20-μl aliquots of the bile were put in vials for liquid scintillation counting. Each bile sample was added with 0.5 ml of the tissue solubilizer NCS®-II, and mixed with 10 ml of a liquid scintillator for liquid scintillation counting, HIONIC-FLUOR™ (Packard Instrument Co., Meriden, CT). Aliquots of the urine (100 or 200 μl) were also put in vials and added with 1 ml of NCS®-II. The urine samples were mixed with 10 ml of HIONIC-FLUOR™. Radioactivity was determined by a liquid scintillation counter (2300TR, Packard Instrument Co., Meriden, CT).

The radioactivity of [^{14}C]R-146224 was multiplied by the total amount of the bile or urine collected at each period to calculate the radioactivity recovered in these excreta. For calculation of the excretion ratios (% of dose), the radioactivity recovered at each period was divided by the total radioactivity given to each bile-fistula rat.

2.8. Hypocholesterolemic activity of R-146224 in normocholesterolemic hamsters

Male golden Syrian hamsters at 7 weeks old were purchased from Japan SLC, Inc. (Shizuoka, Japan). Five animals were housed per metal cage in a room controlled for temperature (21–25 °C), humidity (45–65%) and light (7 AM–7 PM). Food (a commercial laboratory chow diet, F-2, Funabashi Farm Co., Ltd., Chiba, Japan) and water were given ad libitum during the entire the experiment. R-146224 dissolved in water (dosing vehicle) was administered daily at doses of 3, 10, 30, and 100 mg/kg/day to chow-fed male Syrian golden hamsters on 14 consecutive days by oral gavage at around 4:00 PM. Control animals received distilled water only. The animals were weighed daily and sacrificed on the morning after the 14th dose. Blood samples were taken from the abdominal aorta at 9 AM under ether anesthesia after 17-h fasting. The levels of serum total cholesterol and triglyceride were determined enzymatically using a HITACHI type 7250 automatic analyzer (Hitachi, Ltd., Tokyo, Japan). HDL cholesterol was measured after precipitation of very low density lipoprotein (VLDL) and LDL using a commercial kit (HDL-Cholesterol Precipitant Set, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Non-HDL cholesterol was calculated by subtracting HDL cholesterol from total cholesterol. Lipid in the liver was extracted as previously described (Folch et al., 1957). The lipid extract was evaporated under a stream of nitrogen gas, and redissolved with an aliquot of isopropyl alcohol. Total cholesterol, free cholesterol, and triglyceride concentrations were determined using appropriate chemical assay kits (Cholesterol C-II Test Wako, Free Cholesterol C Test Wako, Triglycerides E Test

Wako, respectively, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Esterified cholesterol was calculated as the difference between total and free cholesterol.

2.9. Hypocholesterolemic activity of R-146224 in normocholesterolemic monkeys

Animal experiments were carried out at the Tsukuba Research Laboratories in Hamri Co., Ltd. (Ibaraki, Japan) according to the guidelines provided by the Institutional Animal Care and Use Committee of Sankyo Co., Ltd. A total of 15 male cynomolgus monkeys at the ages of 3–5 years originating from Hamri Co., Ltd. were individually housed in metal cages in a room controlled for temperature (21–25 °C), humidity (45–65%), and light (8 AM–6 PM). They were maintained on a commercial laboratory chow diet fed twice a day. All animals were orally given distilled water as a dosing vehicle daily for 2 weeks before the drug treatment in order to stabilize their serum lipid concentrations. Blood was drawn from the radial vein of overnight-fasted monkeys on Days-7 and -1 before the initiation of R-146224 treatment to determine baseline total cholesterol concentrations. We defined the mean value of Days-7 and -1 as the baseline value. At the beginning of the dosing, the 15 monkeys were divided into three groups ($n=5$ in each group) on the basis of their baseline total cholesterol concentrations to make the mean values of the baseline total cholesterol concentrations of the groups almost equivalent. Subsequently, each group was assigned at random to the control, and R-146224 3 and 10 mg/kg groups. The animals were orally treated with R-146224 by intragastric intubation for 14 days. Blood samples were drawn from the radial vein of overnight-fasted monkeys around 24 h after administration. The levels of serum total cholesterol, HDL cholesterol, triglyceride, and phospholipid were determined enzymatically using the HITACHI type 7250 automatic analyzer. Non-HDL cholesterol was calculated by subtracting HDL cholesterol from total cholesterol.

2.10. [^{3}H]Taurocholate uptake into human ileum pieces (in vitro human ileum pieces experiments)

The inhibitory potency of R-146119, R-146224, and R-150761 have been tested on human ASBT in vitro by measuring the uptake of [^{3}H]taurocholate in 5×5 mm pieces of freshly obtained human ileal segments. The materials were obtained from the resection of e.g. colon tumor tissue, conducted according to the rules of the Medical Ethics Committee of the Leiden University Medical Center. [^{3}H]Taurocholate-uptake measurements in human ileal pieces are a slight modification of the method described for [^{3}H]taurocholate uptake into hamster ileum pieces in Section 2.4 and according to that recently published (Kurata et al., 2004). Incubations with the test compounds were performed in triplicate at 5 different concentrations. Each compound was tested in ileal segments from at least three different individuals. Average IC_{50} values have been calculated after curve-fitting of the individual dose–response curves.

2.11. Statistical analysis

Results are presented as mean±S.D. or mean±S.E.M. (n), or as percent inhibition compared with the mean vehicle-treated control value. The IC_{50} and ED_{50} values were calculated by nonlinear regression analysis using a computer program (GraphPad, PRISM, San Diego, CA). For most in vivo studies, drug-treatment values were compared with the vehicle-treatment control values by Dunnett's two-tailed t test using SAS System Release 8.2 (SAS Institute, Cary, NC). For the monkey study (Table 1), due to the heterogeneity of variance at the baseline ($P<0.01$ according to Levene's, Bartlett's and O'Brien's tests), observations on Days 7 and 14 were transformed to percentages of baseline values to provide homogeneity of variance. Repeated-measures analysis of variance (ANOVA), followed by Dunnett-type multiple comparison was performed on the transformed values. $P<0.05$ was considered to be significant.

3. Results

3.1. Inhibition of [^{3}H]taurocholate uptake into hASBT- and hNCTP-expressing HEK-293 cells

To characterize the pharmacological properties of R-146224, we first investigated its effect on [^{3}H]taurocholate uptake into

Table 1
Effect of R-146224 on serum lipid levels in monkeys

Parameter	Vehicle ($n=5$)	R-146224 3 mg/kg ($n=5$)	R-146224 10 mg/kg ($n=5$)
Baseline, mg/dl			
Total cholesterol	178±4	167±10	231±30
HDL cholesterol	77±9	71±4	59±7
Non-HDL cholesterol	101±10	97±10	173±35
Triglyceride	23±5	14±3	17±6
Phospholipid	203±18	182±13	201±28
7D-treated, mg/dl			
Total cholesterol	193±25 (109±16)	154±27 (91±11)	108±5 (52±9)[a]
HDL cholesterol	81±11 (111±19)	67±3 (96±4)	60±10 (113±25)
Non-HDL cholesterol	112±24 (121±34)	87±27 (86±22)	48±8 (34±9)[a]
Triglyceride	25±6 (126±34)	24±8 (247±135)	18±4 (203±88)
Phospholipid	217±24 (109±12)	192 ±14 (107±11)	156±21 (89±21)
14D-treated, mg/dl			
Total cholesterol	166±20 (94±12)	116±20 (69±9)	102±13 (50±12)[a]
HDL cholesterol	69±9 (94±16)	60±5 (84±4)	62±7 (114±20)
Non-HDL cholesterol	97±22 (106±30)	56±16 (58±14)	40±8 (32±13)[a]
Triglyceride	27±7 (136±36)	28±7 (264±119)	17±4 (175±81)
Phospholipid	194±17 (98±10)	166±18 (93±12)	155±19 (86±19)

R-146224 was orally administered to normocholesterolemic cynomolgus monkeys at doses of 0, 3, and 10 mg/kg for 14 days. The serum lipid levels were determined as described in the text. Results are presented as the mean± S.E.M. Percent of baseline is shown in parentheses. Significant difference from vehicle controls; [a]$P<0.05$ compared by repeated-measures ANOVA, followed by Dunnett-type multiple comparison.

*h*ASBT- and *h*NCTP-expressing HEK-293 cells. R-146224 inhibited [^{3}H]taurocholate uptake in a dose-dependent manner (results not shown). The derivatives, R-146119 and R-151005, also inhibited [^{3}H]taurocholate uptake. The estimated IC_{50} values of each compound are summarized in Table 2. R-146224 demonstrated potent inhibition of [^{3}H]taurocholate uptake into *h*ASBT-expressing HEK-293 cells with an IC_{50} value of 0.023 μM, whereas it exhibited weak inhibition of [^{3}H]taurocholate uptake into *h*NCTP-expressing HEK-293 cells with an IC_{50} value of 32 μM, indicating a >1400-fold selectivity. Of note, R-151005 exhibited better selectivity than that of R-146224.

3.2. Inhibition of [^{3}H]taurocholate uptake into hamster ileum pieces

The effects of R-146224 and its derivatives on [^{3}H]taurocholate uptake into hamster ileum pieces were studied. R-146224 dose-dependently inhibited [^{3}H]taurocholate uptake into hamster ileum pieces with an IC_{50} value of 0.73 μM. Both R-146119 and R-151005 also exhibited dose-dependent inhibition with IC_{50} values of 0.83 and 0.65 μM, respectively.

3.3. Inhibition of [^{3}H]taurocholate uptake and transport into enterohepatic circulation of bile acid in hamsters (in vivo)

R-146224 potently inhibited [^{3}H]taurocholate uptake into the gallbladder bile in hamsters in a dose-dependent manner (Fig. 2). The ED_{50} value calculated from the sigmoidal dose–response regression curve was 2.8 mg/kg/day.

3.4. Substrate specificity: effect of R-146224 on absorption of vitamins or nutrients (in situ loop experiment)

R-146224 at a concentration of 1.0 μg/ml significantly decreased [^{3}H]taurocholic acid absorption from the ileum loop. On the other hand, R-146224 had little effect on jejunal absorption of vitamins, amino acid (alanine), or sugar (glucose) involved at the transporter-mediated system, at the same concentration as that of R-146224 (Fig. 3). Taken together, R-146224 is a selective ASBT inhibitor in vivo.

3.5. Absorption of [^{14}C]R-146224

Biliary and urinary excretion ratios of the radioactivity in bile-fistula rats after oral administration of [^{14}C]R-146224 at a dose of 10 mg/kg are shown in Fig. 4. The cumulative recovery ratio of radioactivity in the bile, within 48 h post-dose, was 1.49±0.75%. The urinary excretion ratio was 0.14±0.05% within 48 h post-dose. Based on this result the absorption ratio was calculated as 1.63±1.78%, demonstrating that the oral absorption ratio of R-146224 is very low in rats.

Table 2
In vitro potency and selectivity of ASBT inhibitors

Compound	IC_{50} (μM)		Ratio
	ASBT	NTCP	(NTCP/ASBT)
R-146119	0.018	27	1500
R-146224	0.023	32	1400
R-151005	0.006	117	21,000

IC_{50} values of R-146119, R-146224, and R-151005 in [^{3}H]taurocholate uptake assays using human ASBT- and NTCP-expressing HEK-293 cells are summarized.

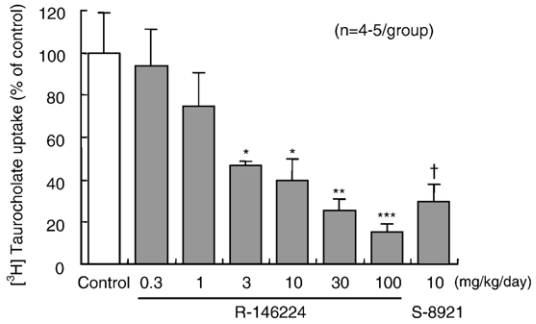

Fig. 2. Effect of R-146224 on [^{3}H]taurocholate uptake and transport into enterohepatic circulation of bile acid in hamsters (in vivo). The test compound was orally given at indicated doses. One and half hours later, [^{3}H]taurocholate was orally administered. Twenty-four hours after drug administration, gallbladder bile was collected and radioactivity was determined by liquid scintillation counting as described in the text. Data are represented as a percentage of control. Each bar represents the mean±S.E.M (N=4–5). *P<0.05, **P<0.01, and ***P<0.001, as compared with the control group using Dunnett's test. †P<0.05, as compared with the control group using Student's t test.

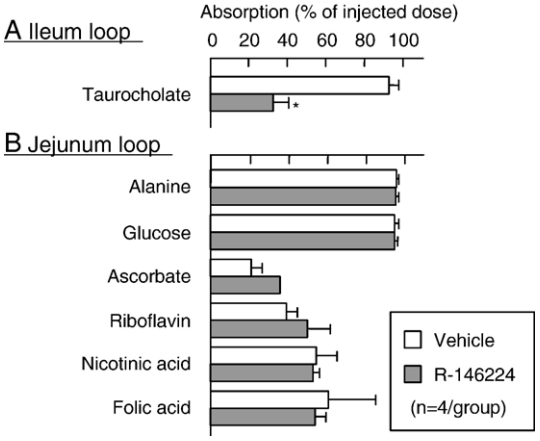

Fig. 3. Effect of R-146224 on absorption of vitamins and nutrients in rats (in situ loop experiment). Effects of R-146224 on absorption of vitamins and nutrients were investigated by an in situ loop method. A jejunal loop (for vitamin or drug injection) or an ileal loop (for taurocholic acid injection) of about 10 cm in length was prepared by ligature of both ends in rats. One hour after the administration of vitamins or nutrients with R-146224, the loop was removed and homogenized. The final concentration of R-146224 was 1.3 μM (1 μg/ml). The absorption ratio was calculated by the subtraction of radioactivity remaining in the loop from that injected. Each bar represents the mean±S.D. (N=4). *P<0.05, as compared with the control group using Student's t test.

3.6. Hypocholesterolemic effect of R-146224 in normocholesterolemic hamsters

To determine the effects of chronic treatment of R-146224 on serum lipids, R-146224 was orally administered to hamsters for

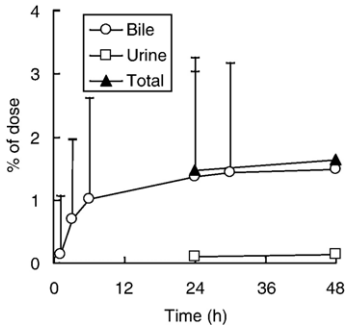

Fig. 4. Cumulative excretion of radioactivity in bile (open circles) and urine (open squares) after oral administration of [^{14}C]R-146224 at a dose of 10 mg/kg to bile-fistula rats. [^{14}C]R-146224 was administered to bile-fistula rats at a dose of 10 mg/kg using a metal stomach tube. Bile and urine were collected at 4 °C for indicated periods. The radioactivities of [^{14}C]R-146224 in the bile and urine were determined by liquid scintillation counting. For calculation of the excretion ratios (% of dose), the radioactivity recovered at each period was divided by the total radioactivity given to each bile-fistula rat. Each symbol and bar represents mean±S.D. (n=4).

14 days. No significant differences in food intake (measured daily) or in body weight (measured daily) were observed (data not shown). Once daily oral administration of R-146224 at 3–100 mg/kg resulted in a dose-dependent decrease in serum total cholesterol, non-HDL cholesterol, and HDL cholesterol (Fig. 5). Significant decreases were seen at the dose of 10 mg/kg. In addition, R-146224 reduced phospholipid levels at a dose of 100 mg/kg. On the other hand, R-146224 did not affect serum triglyceride levels. In the liver, R-146224 significantly reduced free cholesterol content in addition to drastic reduction in esterified cholesterol content. R-146224 did not significantly affect hepatic triglyceride content (Table 3).

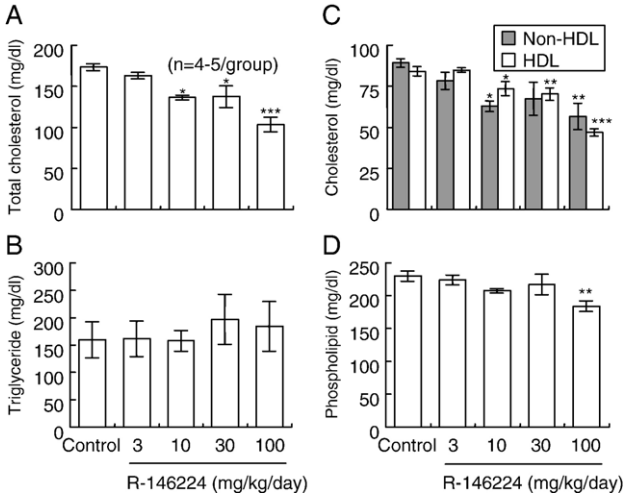

Fig. 5. Effect of R-146224 on serum lipid levels in hamsters. R-146224 was orally administered to normocholesterolemic Syrian golden hamsters at doses of 0, 3, 10, 30, or 100 mg/kg for 14 days. The levels of serum total cholesterol (A), triglyceride (B), HDL and non-HDL cholesterol (C), and phospholipid (D) were determined as described in the text. Results are presented as the mean±S.E.M (n=4–5). *P<0.05, **P<0.01, and ***P<0.001, as compared with the control group using Dunnett's test. No statistically significant differences were observed in triglyceride levels between the control and any R-146224 treatment group.

Table 3
Effect of R-146224 on hepatic lipid levels in hamsters

Treatment	Dose (mg/kg/day)	N	Cholesterol (mg/g liver)			Triglyceride (mg/g liver)
			Total	Free	Esterified	
Control	0	5	15.6±0.6	3.2±0.1	12.4±0.5	4.3±0.4
R-146224	3	4	15.7±0.9	3.3±0.2	12.3±0.8	4.2±0.4
R-146224	10	4	12.7±1.2	2.8±0.1[a]	10.0±1.1	3.6±0.2
R-146224	30	4	11.2±1.7	2.7±0.1[a]	8.5±1.7	4.3±0.5
R-146224	100	5	8.4±1.7[b]	2.5±0.1[b]	5.9±1.6[b]	4.1±0.3

R-146224 was orally administered to normocholesterolemic Syrian golden hamsters at doses of 0, 3, 10, 30, or 100 mg/kg for 14 days. The levels of hepatic lipids were determined as described in the text. Results are presented as the mean±S.E.M. (n=4–5). [a]P<0.05 and [b]P<0.01 as compared with the control group using Dunnett's test.

3.7. Hypocholesterolemic activity of R-146224 in normocholesterolemic monkeys

To investigate the hypocholesterolemic activity in normocholesterolemic monkeys, we administered R-146224 at doses of 3 and 10 mg/kg to cynomolgus monkeys for 14 days. No significant differences in food intake (measured daily) or in body weight (measured daily) were observed (data not shown). R-146224 at a dose of 10 mg/kg significantly reduced total cholesterol by 48% from the baseline on Day 7, and by 50% on Day 14. Furthermore, R-146224 at 10 mg/kg significantly reduced non-HDL cholesterol on Days 7 to 14. The decrease in non-HDL cholesterol was 66% from the baseline on Day 7 and 68% from the baseline on Day 14. On the other hand, there was no significant change in HDL cholesterol among the groups. These data indicate that R-146224 lowered serum total cholesterol mainly because of the reduction in non-HDL cholesterol in monkeys. There were no significant differences among the groups either in triglyceride levels or in phospholipid levels.

3.8. Inhibition of [^{3}H]taurocholate uptake into human ileum pieces

As shown in Fig. 6, R-146224 dose-dependently inhibited [^{3}H]taurocholate uptake into human ileum pieces with an IC_{50}

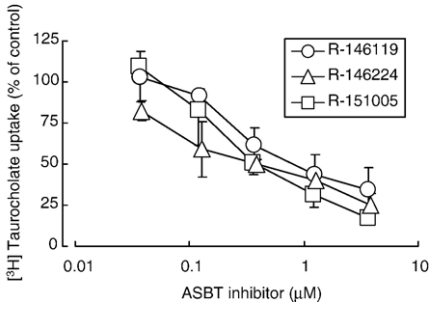

Fig. 6. Inhibition of [^{3}H]taurocholate uptake by ASBT inhibitors in human ileum pieces. Inhibition curves for ASBT inhibitors against [^{3}H]taurocholate uptake into human ileum pieces. Inhibition curves by R-146119 (open circles), R-146224 (open triangles), and R-151005 (open squares) are shown. Data are represented as a percentage of control. Each symbol represents the mean±S.E.M. of three separate determinations.

value of 0.46 μM. Both R-146119 and R-151005 also exhibited dose-dependent inhibition with IC_{50} values of 1.22 and 0.40 μM, respectively.

4. Discussion

Catabolism of cholesterol to bile acids and bile acid excretion constitutes a major elimination route of cholesterol from the body (Dietschy, 1968; Dietschy et al., 1966). Enhancement of this elimination route, by reducing the bile acid pool from the body, either by partial ileal bypass surgery or by oral treatment with bile acid sequestrants, results in increased fecal bile acid excretion. While these have disadvantages due to adverse effects and low compliance, they are clinically proven means of lowering serum LDL cholesterol and altering progression of atherosclerosis (Buchwald et al., 1990).

The studies described herein demonstrate R-146224 not only to be a potent inhibitor of ASBT in *h*ASBT-expressing HEK-293 cells, hamster and human ileum tissues, rat ileum loops and hamsters administered [^{3}H]taurocholate, but also to have good hypocholesterolemic activity in hamsters and monkeys. Five major observations have emerged from this study. The first major observation is that R-146224 is a potent and selective ASBT inhibitor. R-146224 inhibited the uptake of [^{3}H] taurocholate in *h*NCTP-expressing HEK-293 cells with an IC_{50} value of 32 μM, about 1400 times less than against *h*ASBT with an IC_{50} value of 0.023 μM. While R-145224 significantly inhibited bile acid absorption in the rat in situ loops, it did not affect absorption of vitamins and nutrients at the same concentration. Moreover, the pharmacokinetic analysis data using [^{14}C]labeled-R-146224 showed that the absorption rate of R-146224 is extremely low in bile-fistula rats. Total radioactivity absorbed, including that of the parent compound and metabolites, was less than 2% of dosage, indicating that R-146224 is a non-systemic compound, suitable for our aim. This also suggests that the potential effect of R-146224 on NCTP activity in vivo is negligible.

The second major observation is that R-146224 was found to be orally active in interrupting absorption and enterohepatic circulation of bile acids in vivo, and repeated administration reduced serum and hepatic cholesterol levels. R-146224 dose-dependently inhibited [^{3}H]taurocholate uptake into gallbladder bile in hamsters, a widely studied animal model whose cholesterol and bile acid metabolisms are similar to those in humans (Kris-Etherton and Dietschy, 1997; Suckling et al., 1991), with an ED_{50} value of 2.8 mg/kg. These data indicate that R-146224 dose-dependently inhibits ASBT, resulting in enhancement of bile acid excretion. Once daily oral administration of R-146224 by gavage for 14 days showed potent hypocholesterolemic activity without apparent systemic toxicity. Lipoprotein profiling showed that R-146224 reduced not only non-HDL cholesterol but also HDL cholesterol. This profile for cholesterol lowering in hamsters is comparable with that of bile acid sequestrants. The effect of cholestyramine on lowering plasma total cholesterol, HDL cholesterol, and non-HDL cholesterol concentrations has been previously reported (Groot et al., 1992; Suckling et al., 1991). The proposed mechanism responsible for the plasma LDL cholesterol lowering activity of cholestyramine treatment in humans and hamsters is an increase in clearance of lipoproteins via the LDL receptor mediated pathway. It has been also suggested that a decreased rate of VLDL cholesterol production is another possible mechanism for non-HDL cholesterol lowering in hamsters (Groot et al., 1992). Administration of R-146224 also resulted in the reduction in hepatic cholesterol content, which might be the result of increased hepatic bile acid synthesis. R-146224 also reduced HDL cholesterol levels in hamsters. The HDL from hamsters in which the HDL contains high apolipoprotein E concentrations may be taken up by the LDL receptors (Mahley et al., 1981). Apolipoprotein E has high binding affinity to the LDL receptors so HDL in hamsters may be removed from the circulation via LDL receptors. In general, HDL cholesterol is believed to be preferentially utilized for bile acid synthesis and biliary secretion. Cholesteryl esters in HDL are selectively taken up into hepatocytes via scavenger receptor class B type I (SR-BI) (Lewis and Rader, 2005). While there is some evidence that HDL-derived cholesterol may be preferentially utilized for bile acid synthesis or for direct biliary secretion, the fate of HDL-derived cholesterol within the liver is not entirely clear (Kozarsky et al., 1997; Plump et al., 1997; Robins and Fasulo, 1997). Based on this scenario, we postulate that SR-BI might be upregulated by some mechanism due to the cholesterol demand in hepatocytes for bile acid synthesis. The cardioprotective effect of HDL has been largely attributed to its role in reverse cholesterol transport, in which cholesterol that has been synthesized or deposited in peripheral tissues is returned to the liver for recycling or excretion in the bile. Cholesterol in the HDL is transported to the liver, via selective uptake of HDL cholesteryl esters by SR-BI ("direct" reverse cholesterol transport) or LDL by the LDL receptors in the liver after cholesteryl ester transfer to LDL by cholesteryl ester transfer protein ("indirect" reverse cholesterol transport) (Quintao, 1995). Consequently, ASBT inhibitors may have a potential to promote reverse cholesterol transport. Inconsistent with the effects of bile acid sequestrants and R-146224 in hamsters, R-146224 decreased non-HDL cholesterol without affecting HDL cholesterol in monkeys. It is well known that administration of bile acid sequestrants does not decrease HDL cholesterol in humans and in fact results in a small increase in HDL cholesterol (Melian and Plosker, 2001). Non-systemic ASBT has almost the same mechanism of action for LDL cholesterol lowering: inhibition of enterohepatic circulation of bile acid resulting in a reduced bile acid pool. Therefore, there may be a species difference in the effects of non-systemic ASBT inhibitors on serum lipoprotein cholesterols like bile acid sequestrants. Interruption of the enterohepatic circulation of bile acids in humans is associated with an increase in plasma triglyceride levels. Therefore, it is possible that ASBT inhibition would increase plasma triglyceride levels as a potential adverse effect. However, R-146224 did not affect triglyceride levels in hamsters or monkeys. It is presumably due to the duration of the treatment. Increased triglyceride levels may be observed with longer treatments. The results were inconclusive presumably due to the heterogeneity of variance. The effect of ASBT

inhibitors on plasma triglycerides in humans will need to be established.

The third major observation is how much inhibition of the transporter is necessary to cause a reduction in serum cholesterol levels. Once daily oral administration of R-146224 at 10 mg/kg significantly reduced serum cholesterol levels in hamsters, consistent with that for reduction of hepatic free cholesterol content, with a five times higher ED_{50} for inhibiting [^{3}H]taurocholate uptake. Studies on individuals with null mutations in ASBT might provide some insight into this discrepancy. Gene mutation of ASBT rendering ASBT incapable of transporting bile acids has been reported (Wong et al., 1995). Heterozygotes are expected to have half the ASBT-mediated bile acid transport activity of normal subjects, resulting in reduced plasma cholesterol levels. However, clinical data, mostly unpublished, are limited and do not clarify the relationship between the gene mutation of ASBT and serum cholesterol levels. This suggests that the lipid profile of heterozygotes may be normal, with a 50% genetic blockade of bile acid transport, either from enough capacity for the necessary transport or the normal allele being upregulated by some compensatory mechanism. In this case, pharmacologic blockade of more than 50% of the transport by R-146224 is required to cause an increase in bile acid excretion for cholesterol lowering. We did not determine bile acid excretion in this experiment. Further investigation is necessary to elucidate this.

The fourth major observation is that R-146224 was well tolerated in hamsters and monkeys even when administered at doses up to 100 mg/kg daily in hamsters or 10 mg in monkeys for 14 consecutive days. Theoretically, because ASBT inhibitors would increase the luminal concentration of free bile acids, treatment with ASBT inhibitors can cause intestinal adverse effects, particularly diarrhea and colon cancer. It was reported that the most common side effect of partial ileal bypass in the POSCH study was diarrhea (Buchwald et al., 1990). Although we have not seen any intestinal adverse effects of R-146224 in the various animal models tested, the potential for adverse side effects in humans needs to be evaluated.

The fifth major observation is related to the dose-related inhibition of R-146224 on [^{3}H]taurocholate uptake in human ileum pieces. Our data indicate that R-146224 would inhibit enterohepatic circulation of bile acids in humans since similar uptake rates of [^{3}H]taurocholate can be expected along the whole intestine, consistent with the ASBT expression pattern in the human ileum.

In summary, R-146224 appears to be a highly potent and non-systemic ASBT inhibitor that displays strong hypocholesterolemic properties in hamsters and monkeys. Moreover, R-146224 was demonstrated to work in human ileum pieces. These findings suggest that R-146224 has the potential to be an effective therapeutic agent for hypercholesterolemia in humans.

Acknowledgements

We thank Ms. Kayoko Ito and Ms. Naoko Ubukata for their expert technical assistance.

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