Glucuronidation Converting Methyl 1-(3,4-Dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate (S-8921) to a Potent Apical Sodium-Dependent Bile Transporter Inhibitor, Resulting in a Hypocholesterolemic Action

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ABSTRACT

Methyl 1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate (S-8921) is a novel inhibitor of the ileal apical sodium-dependent bile acid transporter (ASBT/SLC10A2) developed for the treatment of hypercholesterolemia. The present study investigated the hypocholesterolemic action of S-8921 glucuronide (S-8921G) in rats. The plasma concentration of S-8921G was higher than that of S-8921 after single oral administration of S-8921 in normal rats, and S-8921G was excreted into the bile (13% dose). Oral administration of either S-8921 or S-8921G reduced the serum total cholesterol, particularly non-high-density lipoprotein cholesterol, in hypercholesterolemic normal rats. In Gunn rats devoid of UDP glucuronosyltransferase-1A activity, S-8921G was undetectable both in the plasma and bile specimens, and only S-8921G administration significantly reduced the serum non-high-density lipoprotein cholesterol. An in vitro inhibition study showed that glucuronidation converts S-8921 to a 6000-fold more potent inhibitor of human ASBT (Ki = 18 nM versus 109 μM). S-8921G was detected both in the portal plasma and loop when S-8921 was administered into the loop of the rat jejunum, although the cumulative amount of S-8921G recovered in the bile was 5-fold greater than that in the loop. The uptake of S-8921G by freshly prepared rat hepatocytes was saturable, and sodium-dependent and -independent systems were involved. Organic anions, such as bromosulfophthalein, estrone 3-sulfate, and taurocholic acid, inhibited the uptake. These results suggest that UDP glucuronosyltransferase-1 isomers play a critical role in the hypocholesterolemic action of S-8921 by converting S-8921 to a more potent ASBT inhibitor, and organic anion transporter(s) are also involved in its pharmacological action through the biliary excretion of S-8921G.

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ABBREVIATIONS: LDL, low-density lipoprotein; ASBT, apical sodium-dependent bile acid transporter; S-8921, methyl 1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate; S-8921G, S-8921 glucuronide; TCA, taurocholate; GA, gum arabic; LC, liquid chromatography/chromatograph; MS/MS, tandem mass spectrometry; RSD, relative standard deviation; HDL, high-density lipoprotein; HEK, human embryonic kidney; PCR, polymerase chain reaction; h, human; UGT, UDP glucuronosyltransferase; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; PEG, polyethylene glycol; AUC, area under the curve; BSP, bromosulfophthalein; Mrp/MRP, multidrug-resistance protein; BCRP/BCrp, breast cancer resistance protein; OATP/Oatp, organic anion transporting protein; 2164U90, (−)-(3R,5R)-trans-3-butyl-3-ethyl-2,3,4,5-tetrahydro-5-phenyl-1,4-benzothiazepine 1,1-dioxide; SC-435, 1-[4-[(4R,5R)-3,3-dibutyl-7-(dimethylamino)-2,4,5-tetrahydro-4-hydroxy-1,1-dioxido-1-benzothiepin-5-yl]phenoxy]butyl]-4-aza-1-azoniabicyclo[2.2.2]octane methanesulfonate; S 0960, 7α,12α-dihydroxy-3β-[3α,7α,12α-trihydroxy-5β chol-an-24-amido]-5β-chol-an-24-oic acid; R-146224, 1-{7-[[1-(3,5-diethoxyphenyl)-3,3-dibutyl-7-(dimethylamino)-2,4,5-tetrahydro-4-hydroxy-1,1-dioxido-1-benzothiepin-5-yl]phenoxy]butyl]-4-aza-1-azoniabicyclo[2.2.2]octane methanesulfonate; S-8921G, S-8921 glucuronide; S-8921, methyl 1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate. Hyperlipidemia, especially hypercholesterolemia, is a major risk factor for atherosclerosis, leading to coronary heart disease. In clinical trials, reducing serum low-density lipoprotein (LDL) cholesterol has been demonstrated to reduce...
the incidence of coronary heart disease and to reverse ath-
ersclerotic lesions in hypercholesterolemic patients (Lipid
Research Clinics Program, 1984; Shepherd et al., 1995). The
hypercholesterolemic agents most commonly used are the
HMG-CoA reductase inhibitors (so-called statins). Statins
have a potent hypocholesterolemic action and few side ef-
effects, resulting in good patient compliance.

As alternative pharmacological targets for the hypercholes-
teroletic effect, inhibition of intestinal absorption of choles-
terol and bile acids has attracted attention. Ezetimibe was
found to be a potent inhibitor of Niemann-Pick C1-like 1,
playing a significant role in the cholesterol absorption in the
small intestine (Garcia-Calvo et al., 2005), and combination
therapy with statins increased the effectiveness of LDL cho-
lesterol reduction (Gazi and Mikhailidis, 2006). Inhibitors of
the ileal apical sodium-dependent bile acid transporter
(ASBT/SLC10A2), a membrane transporter responsible for
the absorption of bile acids (Cradock et al., 1998), include
2164U90 (Lewis et al., 1995), S-8921 (Hara et al., 1997),
SC-435 (West et al., 2002), S 0960 (Schlattjan et al., 2003),
and R-146224 (Kitayama et al., 2006). Bile acids are synthe-
sized from cholesterol in the liver, and they are secreted into
the bile. More than 90% of the bile acids are reabsorbed from
the intestinal lumen in the ileum, and they are removed from
the blood by the first-pass effect in the liver, leading to
enterohepatic circulation (Hofmann, 1993). The bile acid pool
is closely regulated; thus, interruption of the enterohepatic
circulation of bile acids would lead to an increase in the
biosynthesis of bile acids from cholesterol (Packard and
Shepherd, 1982). Indeed, treatment with ASBT inhibitors
increases the fecal excretion of bile acids (Lewis et al., 1995;
Hara et al., 1997; Huff et al., 2002; Kitayama et al., 2006),
resulting in an induction of hepatic cholesterol 7α-hydroxy-
lyase (CYP7A), a rate-limiting enzyme of bile acid synthesis
(Higaki et al., 1998; Huff et al., 2002). ASBT inhibitors ex-
hibit a hypocholesterolemic effect (2164U90, S-8921,
R-146224, and SC-435). This is partly ascribed to the in-
crease in the bile acid synthesis in the liver and partly to the
increase in the uptake of plasma LDL cholesterol through the
up-regulation of LDL receptors due to partial depletion of
hepatic cholesterol, because SC-435 or S-8921 treatment
causes induction of hepatic LDL receptors (Higaki et al.,
1998; Huff et al., 2002). This latter finding is due to the
partial depletion of hepatic cholesterol (Packard and She-
pherd, 1982).

S-8921 is a water-insoluble compound with a clog P of 5.5.
Among the ASBT inhibitors, S-8921 is a weak inhibitor of
ASBT with an IC_{50} value of 66 μM (Hara et al., 1997),
whereas others range from 1.2 nM to 10 μM (Lewis et al.,
1995; West et al., 2002; Schlattjan et al., 2003; Kitayama
et al., 2006). Unlike 2164U90 and SC-435, which are compe-
titive inhibitors of ASBT (Root et al., 1995; Hallén et al., 2002),
the inhibition of ASBT by S-8921 is a mixture of competitive
and noncompetitive processes (Hara et al., 1997). Further-
more, it has been found that the glucuronide conjugate of
S-8921 (S-8921G) is a more potent inhibitor of taurocholate
(TCA) absorption than the parent compound (J. Ono, S.
Sakamoto, T. Ichihashi, M. Izawa, Y. Yano, T. Mizui, and K.
Hirano, unpublished data). S-8921G is the major metabolite
of S-8921 in the bile following oral administration, and rat
bile after oral administration of S-8921 inhibits the ileal
absorption of TCA (J. Ono, S. Sakamoto, T. Ichihashi, M.
Izawa, Y. Yano, T. Mizui, and K. Hirano, unpublished data).
The absolute bioavailability of S-8921 was only a few percent
in rats and dogs (Yamaguchi et al., 1998). After oral dosing of
[^14]CS-8921 in rats, the urinary and biliary excretion of the
total radioactivity was approximately 1 to 2% and 20 to 30%
dose, respectively, and, even in intravenous dosing, most of
the total radioactivity was excreted into the bile, although
the unchanged form of S-8921 was undetectable in the bile
(Yamaguchi et al., 1998). Enterohepatic circulation of the
total radioactivity of S-8921 was observed to a small extent
(6% dose) (Yamaguchi et al., 1998). In our preliminary study,
S-8921G was barely deconjugated, and it was absorbed when
S-8921G was instilled into the ileal loop (S. Sakamoto,
unpublished observation). However, most of the radioactivity
in the feces after oral administration of[^14]CS-8921 was ac-
counted for by S-8921 (K. Miyata, K. Nomura, and S. Hara,
unpublished observation). Thus, it is now considered that
S-8921G is converted to S-8921 by bacterial flora in the
cecum and/or the large intestine, and part of the S-8921 is
reabsorbed, which may result in the long-acting pharmacolo-
gical effect.

The purpose of the present study was to show the impor-
tance of S-8921G in the pharmacological action of S-8921.
In vivo pharmacological and pharmacokinetic properties of
S-8921 were compared between normal and UDP-glucurono-
syltransferase-1 (UGT1)-deficient rats (Gunn rats) (Burchell
et al., 1995). In addition, we compared the inhibition poten-
cies of S-8921 and S-8921G against human ASBT (hASBT)
using cDNA-transfected cells.

**Materials and Methods**

**Materials.** S-8921 (99.8%), S-8921G (99.7%),[^14]CS-8921 (2.16
MBq/mg), and[^14]CS-8921G (1.64 MBq/mg) were synthesized at
Shionogi & Co., Ltd. (Osaka, Japan). The chemical structures of
S-8921 and S-8921G are shown in Fig. 1.[^1][^H]TCA (74.0 GBq/mmol)
was purchased from PerkinElmer Life and Analytical Sciences
(Bos-
ton, MA). TCA and gum arabic (GA) were purchased from Sigma-
Aldrich (St. Louis, MO) and Nacalai Tesque (Kyoto, Japan), respec-
tively. Other chemicals were of analytical or reagent grade. Human
UGT Supersomes were obtained from BD Gentest (Woburn, MA).

**Animals.** All procedures for the animal experiments were ap-
proved by the Institutional Animal Care and Use Committee. Male
Wistar rats (Jcl:Wistar; 7–10 weeks of age) and Gunn rats (Slc:Gunn;
8–11 weeks of age) were purchased from CLEA Japan, Inc. (Tokyo,
Japan) and Japan SLC, Inc. (Tokyo, Japan), respectively. Male

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![Fig. 1. Chemical structures of S-8921 and S-8921 glucuronide.](image-url)
Sprague-Dawley rats (Slc:SD; 10–11 weeks of age) were purchased from Japan SLC, Inc. The animals were housed in a room with controlled light (on from 8:00 AM to 8:00 PM), temperature (23 ± 3°C), and humidity (30–70%). The rats were allowed free access to tap water and solid laboratory food.

**Pharmacokinetic Study of S-8921 and S-8921G in Rats.**

S-8921 was suspended in 5% gum arabic solution for administration. Rats were given a single oral dose of 10 mg/kg S-8921. The biliary excretion was determined in a separate experiment. For the plasma concentration-time study (n = 5/group; mean body weight, 310 g for Wistar rats and 292 g for Gunn rats), 5 ml of blood was collected from a jugular vein cannula at 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h after dosing. After blood sampling, the blood collected was replaced by 25 U/ml heparin-saline. Plasma was separated immediately, and it was stored at −80°C until quantitative determination. For the biliary excretion study (n = 5/group; mean body weight, 300 g for Wistar rats and 300 g for Gunn rats), bile was collected at 0 to 6 h and 6 to 24 h after dosing in a bottle containing 1 M acetic acid, pH 2.3 to 2.4, on ice. S-8921 and S-8921G were stable in the bile. Bile samples were stored at −80°C until quantitative determination. The concentrations of S-8921 and S-8921G in the plasma and bile were determined by LC/MS/MS after solid phase extraction using an SPE-C2 disk cartridge column (ANSYS Technologies, Inc., Lake Forest, CA). The recovery of S-8921 and S-8921G by solid phase extraction was more than 90%. The eluent was centrifuged, and 30 μl of the supernatant or the diluted supernatant was injected into the LC/MS/MS system. The LC/MS/MS system consisted of a model Alliance 2960 LC apparatus (Waters, Milford, MA) equipped with a model TSQ 8000 mass spectrometer (ThermoQuest, Waltham, MA). The analytical column was a YMC-Pak Pro C18 column (5 μm, 35 mm, 2.0 mm i.d.; YMC, Kyoto, Japan) kept in a column oven at 30°C. The mobile phase consisted of solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in acetonitrile). The flow rates from 0.0 to 1.1 min, from 1.1 to 3.2 min, from 3.2 to 8.0 min, and from 8.0 to 9.5 min were set to 1.0, 0.5, 0.3, and 1.0 ml/min, respectively. After a 1.1-min isocratic period at 30% B, a linear gradient was started toward 95% B at 3.0 min, kept at that composition until 7.5 min, and followed by an 8-min linear gradient period toward 30% B. The mass spectrometer was operated using the electrospray ionization source in the negative selected reaction-monitoring mode. The divert valve between 0 and 3.2 min, between 3.2 and 7.4 min, and between 7.4 and 9.5 min was set to on (wasted), off (introduced into the electrospray ionization interface), and on (wasted), respectively. Monitoring ions were 539.1 (precursor) → 477.1 (product) and 715.2 (precursor) → 539.3 (product) for S-8921 and S-8921G, respectively. The lower limit of quantitation for S-8921 and S-8921G was 0.5 and 0.5 ng/ml in plasma specimens and 10 and 20 ng/ml in bile specimens, respectively. 

**Pharmacological Effect of S-8921 and S-8921G in Rats.**

Animals were divided into the following eight dosing groups, so that each group (n = 8) had a similar baseline serum cholesterol concentration: 1) control, 5% gum arabic solution; 2) S-8921, 0.1 mg/kg; 3) S-8921, 1 mg/kg; 4) S-8921, 10 mg/kg; 5) S-8921G, 0.1 mg/kg; 6) S-8921G, 1 mg/kg; 7) S-8921G, 10 mg/kg; and 8) 5% gum arabic solution. S-8921 and S-8921G were suspended in 5% gum arabic solution. During the experiment, the animals were fed either an ordinary pellet diet (CA-1; CLEA Japan, Inc.) or a high-cholesterol diet (CA-1 containing 1% (w/w) cholesterol and 0.5% (w/w) sodium cholate; CLEA Japan, Inc.). Normal rats (Wistar rats) in dosing groups 1 to 7 were fed a high-cholesterol diet, and those in group 8 were fed an ordinary diet. Gunn rats were divided into groups 1, 4, 7, and 8. There was no statistically significant difference in basal body weights among the groups before the treatment with test compounds, and the mean body weight of normal rats and Gunn rats in each group was 302 to 312 g and 303 to 316 g, respectively.

S-8921 or S-8921G was orally administered once a day in the morning for 7 days. Blood samples (5 ml) were collected from the abdominal aorta under pentobarbital anesthesia the next morning following the final administration, and serum was separated by centrifugation at 4°C. Serum total cholesterol was determined from the mean value of the enzymatic method using a commercial kit (Pureauto S CHO-N; Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) on the day of blood sampling. A high-density lipoprotein (HDL) fraction was prepared from a 200-μl serum sample stored at 4°C, according to the method reported by Goldstein et al. (1983) with some modification. In brief, the serum (initial density = 1.006 g/ml) was adjusted to a final density of 1.063 g/ml by adding solid potassium bromide. Then, the serum was centrifuged at 42,000 rpm for 4 h at 10°C. After centrifugation, the upper fraction (80 μl) was aspirated to obtain the lower fraction that was designated as the HDL fraction (its density was assumed to be more than 1.063 g/ml). The serum HDL cholesterol level was calculated by multiplying the cholesterol level in the HDL fraction by the volume ratio (0.6) of the HDL fraction to the serum sample. The cholesterol level in the HDL fraction was quantified using an automated measurement apparatus (model 7070; Hitachi, Tokyo, Japan). The serum non-HDL cholesterol level was calculated by subtracting the HDL cholesterol level from the total cholesterol level.

**Construction of Stably Transfected HEK293 Cells Expressing hASBT.**

The hASBT gene was isolated by PCR using human ileum cDNA (Multiple Tissue cDNA panels; Clontech, Mountain View, CA). The gene was amplified using a forward primer containing a c-Myc site (5’-CACCATGCAAGGCTTAGCTAGGAGGAGCCCTGATGAATGATCCGA-3’) and a reverse primer containing an Apal site (5’-TTGGTGGGCCCCACTTTGATGATC-3’). PCR product was cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA). Then, the pENTR/D-TOPO vector was digested with NotI and Apal, and the ASBT cDNA was ligated into the NotI and Apal sites of the pcDNA3/FRT expression vector (Invitrogen). hASBT-expressing HEK293 cells (hASBT-HEK) were constructed by cotransfection of hASBT-pcDNA5/FRT and pOG44 vector (Invitrogen) into HEK293 cells (Fip-In-293 cell line; Invitrogen) using FuGENE6 (Roche Diagnostics, Indianapolis, IN), according to the manufacturer’s instructions, and by selection with 100 μg/ml hygromycin B (Invitrogen) for 2 weeks. We checked that transfection of ASBT was successful by reverse transcription-PCR.

**Inhibitory Effect on TCA Uptake into hASBT-HEK Cells.**

hASBT-HEK and mock-control cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 50 μg/ml hygromycin B (Invitrogen) at 37°C with 5% CO2 and 95% humidity. The cells were then seeded in 12-well plates at a density of 1.5 × 105 cells/well. After a 3-day culture period, the cells were washed twice and preincubated for 15 min at 37°C with transport buffer (137 mM NaCl, 4.17 mM KHCO3, 0.44 mM KH2PO4, 0.34 mM K2HPO4, 10 mM HEPES, 5.55 mM d-glucose, 1.26 mM CaCl2, 0.49 mM MgCl2, and 0.41 mM MgSO4·H2O pH 7.4). In the Na+-free buffer, choline chloride was used instead of NaCl.

Uptake was initiated by replacing transport buffer with 600 μl of substrate solution (100 mM Tris/HCl: 1, 5, and 10 μM) or substrate solution containing S-8921 (10, 100, or 200 μM) or S-8921G (10, 30, or 100 nM). After incubation for 30 s, the substrate solution was removed, and the cells were washed three times with 1 ml of ice-cold transport buffer and dissolved in 500 μl of 2 N NaOH. Aliquots (800 μl) were transferred to scintillation vials after adding 500 μl of 2 N HCl. The
radioactivity associated with the cells and incubation buffer was measured in a liquid scintillation counter (LS6000SE; Beckman Coulter, Fullerton, CA) after adding 5 ml of scintillation fluid (Clearsol-I; Nacalai Tesque) to the scintillation vials. The remaining 20 μl of supernatant was used to determine the protein concentration by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Glucuronidation with Human UGT Supersomes. The reaction mixture (0.5 ml) consisted of 0.1 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 0.1 mg of protein/ml human UGT Supersomes, 0.0125 mg of Brij 58, and 50 μM [14C]S-8921. The reaction was started by addition of 5 mM UDP-glucuronic acid and incubated for 1 h at 37°C. Then, the reaction was stopped by addition of 0.5 ml of ice-cold acetonitrile to the reaction mixture, and the mixture was kept in ice water for 15 min. Next, the reaction mixture was centrifuged at 3000 rpm for 10 min. Then, 50 μl of supernatant was injected into the LC/mass spectrometer fitted with a radiometric detector. The incubation was performed in duplicate.

An HPLC method for the analysis of S-8921 and its metabolites was developed using a Symmetry C8 column (5 μm, 150 mm, 4.6 mm i.d.; Waters). The mobile phase consisted of 0.1% acetic acid in distilled water and methanol. The programmed elution was isocratic with 63% methanol for the first 25 min, with a linear change to 73% methanol over the next 5 min, and then the program was maintained at 73% for 25 min. The eluent was monitored at 274 nm throughout the 55-min run time. The LC/mass spectrometer with a radiometric detector consisted of the following components: pump and autosampler (Agilent 1100 series; Agilent Technologies, Palo Alto, CA), Symmetry C8 analytical column (5 μm, 150 mm, 4.6 mm i.d.; Waters), LCQ DECA mass spectrometer (ThermoQuest), and radiometric detector (Packard Flow Scintillation analyzer; PerkinElmer Life and Analytical Sciences).

Absorption of S-8921 from the Loop in the Rat Jejunum and Ileum. The Sprague-Dawley rats were anesthetized with ethyl urethane. For the plasma collection group (n = 6; mean body weight, 406 g), a cannula was inserted into the portal vein for the collection of samples. For the bile collection group (n = 6; mean body weight, 380 g), both portal vein and bile duct cannulation was performed. Then, the animals were kept on a warm plate at 37°C. A closed loop (10 cm) of the jejunum or the terminal ileum was made by ligation at both ends. S-8921 (100 μM; 0.5 ml) dissolved in PEG400 and saline containing 0.1% DMSO [25/75 (v/v)] was instilled into the loop. At predetermined times after dosing, an aliquot of blood was collected from the portal vein. The blood was immediately centrifuged at 14,000 rpm for 3 min at room temperature to obtain plasma. At the end of the experiment, the loop was washed with saline and the washing solution was collected. Plasma, bile, and washing solution were frozen for storage until assay. S-8921G was barely absorbed and deconjugated to S-8921 when S-8921G (10 μM; 0.5 ml) dissolved in PEG400 and saline containing 0.1% DMSO [25/75 (v/v)] was instilled into the ileal loop.

For the measurement of S-8921 or S-8921G, an aliquot (100 μl) of plasma, bile, and washing solution was added to 220 μl of acetonitrile. Then, the mixture was centrifuged at 14,000 rpm for 3 min at room temperature, and the supernatant (20 μl) was subjected to HPLC analysis under the following chromatographic conditions: pump model LC-10AD, UV detector model SPD-10AVp (λ = 260 nm), and controller model SCL-10Avp (Shimadzu, Kyoto, Japan); Cosmosil 5C18 AR-II column (150 mm, 3.0 mm i.d.; Nacalai Tesque); mobile phase, A: 0.1% acetic acid in water and B: 0.1% acetic acid in acetonitrile [A/B = 70/30–15/85 (v/v)]; and flow rate, 0.4 ml/min. The concentrations were determined by a nonvalidated HPLC method without an internal standard. The calibration curves for S-8921 and S-8921G were in the range 0.04 to 10 and 0.02 to 5 μM, respectively. The linearity of the analytical analysis was more than 0.99. The intra-assay bias and RSD at 0.4 and 0.2 μM for S-8921 and S-8921G ranged from -3.8 to 5.0% and from 14.3 to 9.2%, respectively.

Uptake of [14C]S-8921G into Freshly Isolated Rat Hepatocytes. Rat hepatocytes were prepared as described by Nezasa et al. (2003). Rat hepatocytes were preincubated in Krebs-Henseleit buffer for 10 min at either 37 or 4°C. To measure the uptake in the absence of Na+, sodium chloride and sodium bicarbonate in Krebs-Henseleit buffer were replaced with choline chloride and choline bicarbonate, respectively. The uptake study was initiated by adding an equal volume of substrate solution to the cell suspension. After incubation for 15 s to 5 min, the reaction was terminated by separating the cells from the reaction medium by centrifugal filtration (Yamazaki et al., 1993). After an overnight incubation in 2 N NaOH to dissolve the hepatocytes, the centrifuge tube was cut, and each compartment was transferred to a vial. The compartment containing the dissolved hepatocytes was neutralized with 50 μl of 2 N HCl, mixed with scintillation cocktail (Pico-Flour 40; PerkinElmer Life and Analytical Sciences), and the radioactivity was measured in a liquid scintillation counter (Tri-Carb 3100; PerkinElmer Life and Analytical Sciences). The concentration dependence of [14C]S-8921G uptake was investigated at 3.4 to 1000 μM after incubation in Krebs-Henseleit buffer for 30 s at 37°C. In addition, the inhibitory effect of various compounds [unlabeled S-8921G, 1 mM; bromosulphophthalein (BSP), 100 μM; estrone 3-sulfate, 100 μM; TCA, 300 μM; and S-8921, 100 μM] on 10 μM [14C]S-8921G uptake by hepatocytes was determined at 15 and 45 s.

Data Analysis. Pharmacokinetic analysis was performed using WinNonlin (Pharsight, Mountain View, CA) based on a noncompartment model with uniform weighting.

The apparent inhibition constant (Ki) for S-8921 and S-8921G was determined by WinNonlin using the equation for noncompetitive inhibition:

\[ v = \frac{V_{\text{max}} \times S}{K_m + S} + \frac{P_{\text{dis}}} {K_m + S} \]  

(2)

where, \( v \), \( V_{\text{max}} \), \( K_m \), and \( I \) are the Na+-dependent uptake of [3H]TCA, the maximal rate of TCA uptake, the apparent Michaelis-Menten constant, the concentration of [3H]TCA, and the concentration of inhibitor (S-8921 or S-8921G), respectively.

The kinetic parameters for hepatic uptake of [14C]S-8921G were calculated according to the following equation:

\[ v_0 = \frac{V_{\text{max}} \times S}{K_m + S} + P_{\text{dis}} \times \frac{S}{S} \]  

(2)

where, \( v_0 \) is the initial uptake rate (nanomoles per minute per milligram of protein), \( V_{\text{max}} \) is the maximal uptake rate (nanomoles per minute per milligram of protein), \( S \) is the substrate concentration (micromolar), \( K_m \) is the Michaelis constant of uptake rate (micromolar), and \( P_{\text{dis}} \) is the uptake clearance corresponding to the nonsaturable component (microliters per minute per milligram of protein).

Statistical Analysis. Statistical analysis for significant differences was performed using the Mann-Whitney test with the SAS system (SAS Institute, Cary, NC). Statistical significance was defined as \( P < 0.01 \) or \( P < 0.05 \).

Results

Pharmacokinetic Profile of S-8921 and S-8921G in Normal and Gunn Rats. The plasma concentrations of S-8921G after single oral administration of S-8921 at 10 mg/kg to normal rats were higher than those of S-8921 (Fig. 2a), and the AUCo–∞ of S-8921G was 2.6-fold higher than that of S-8921 (Table 1). S-8921 was not detected in the bile of normal rats until 24 h after oral administration, but 12.7 ± 2.5% dose was excreted into the bile as S-8921G (Table 1). The plasma level of S-8921 in Gunn rats was very high.
compared with that of normal rats, and the AUC_{0-H11009} of Gunn rats was approximately 9-fold higher than that of normal rats (Fig. 2b; Table 1). In Gunn rats, S-8921G was not detected in the plasma except for one point in one rat, and neither S-8921 nor S-8921G was detected in the bile of Gunn rats.

Glucuronidation of S-8921 with Human UGT Supersomes. [14C]S-8921 was incubated with nine human UGT Supersomes (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT1A10, UGT2B7, and UGT2B15) in the presence of UDP-glucuronic acid. S-8921 was converted to S-8921G, by incubating with UGT1A1, -1A3, -1A6, and -1A10-expressed microsomes (Table 2).

Effect of S-8921 and S-8921G on Serum Cholesterol in Gunn Rats. Feeding a high-cholesterol diet resulted in higher serum non-HDL cholesterol levels in both normal and Gunn rats (Fig. 3; control 5% GA). Serum non-HDL cholesterol in hypercholesterolemic normal rats treated with S-8921 (1 and 10 mg/kg p.o.) or S-8921G (1 and 10 mg/kg p.o.) was significantly reduced in comparison with the control group (Fig. 3a). In contrast, HDL cholesterol was slightly increased (Fig. 3a). In hypercholesterolemic Gunn rats, there was no significant reduction in serum non-HDL cholesterol by S-8921 (10 mg/kg p.o.) (Fig. 3b). However, S-8921G (10 mg/kg p.o.) significantly reduced serum non-HDL cholesterol (Fig. 3b).

Inhibitory Effect of S-8921 and S-8921G on hASBT. Expression of hASBT in HEK293 cells (hASBT-HEK) markedly increased intracellular accumulation of [3H]TCA (3.26 ± 1.75 versus 16.7 ± 0.7 μl/mg protein for 30 s in parent and hASBT-HEK cells, respectively). The uptake of [3H]TCA by hASBT-HEK cells exhibited sodium dependence. The uptake of [3H]TCA for 30 s was 16.7 ± 0.7 and 1.51 ± 0.57 μl/mg protein in the presence and absence of sodium ions, respect-

## Table 1

Pharmacokinetic parameters of S-8921 and S-8921G after oral administration of 10 mg/kg S-8921 in normal and Gunn rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Rat</th>
<th>Gunn Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S-8921</td>
<td>S-8921G</td>
</tr>
<tr>
<td>C_{max} (ng Eq. of S-8921/ml)</td>
<td>4.24 ± 1.62</td>
<td>23.7 ± 13.5</td>
</tr>
<tr>
<td>AUC_{0-H11009} (ng Eq. of S-8921 · h/ml)</td>
<td>110 (146)</td>
<td>383</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>3.95</td>
<td>5.98</td>
</tr>
<tr>
<td>X_{bile} (% of dose)</td>
<td>N.D.</td>
<td>12.7 ± 2.5</td>
</tr>
</tbody>
</table>

N.D., not detected; X_{bile}, cumulative amount excreted into the bile.

* These pharmacokinetic parameters were calculated by WinNonlin using the mean value of the plasma concentrations. The values in parentheses are the values as S-8921G (nanograms per milliliter or nanograms · hour per milliliter).

## Table 2

Production ratio of S-8921G with UGT Supersomes

Human UGT Supersomes (0.1 mg protein/ml) and 50 μM [14C]S-8921 were incubated for 1 h at 37°C. Data represent the mean of two incubations, and the values in parentheses represent the individual values.

<table>
<thead>
<tr>
<th>UGT Isoform</th>
<th>S-8921G</th>
<th>S-8921</th>
</tr>
</thead>
<tbody>
<tr>
<td>No enzyme</td>
<td>N.D.</td>
<td>100</td>
</tr>
<tr>
<td>1A1</td>
<td>7.11</td>
<td>(5.81, 8.40)</td>
</tr>
<tr>
<td>1A3</td>
<td>3.40</td>
<td>(2.90, 3.89)</td>
</tr>
<tr>
<td>1A4</td>
<td>N.D.</td>
<td>100</td>
</tr>
<tr>
<td>1A6</td>
<td>N.D.</td>
<td>100</td>
</tr>
<tr>
<td>1A8</td>
<td>20.4</td>
<td>(20.7, 20.0)</td>
</tr>
<tr>
<td>1A9</td>
<td>34.4</td>
<td>(37.1, 31.8)</td>
</tr>
<tr>
<td>1A10</td>
<td>5.43</td>
<td>(5.81, 5.04)</td>
</tr>
</tbody>
</table>

N.D., not detected.

---

Fig. 2. Plasma concentrations of S-8921 and S-8921G after oral administration of 10 mg/kg S-8921 in normal and Gunn rats. Following oral administration of 10 mg/kg S-8921, the plasma concentrations of S-8921 and S-8921G were determined at designated times in normal rats (a) and Gunn rats (b). Each time point and vertical bar represents the mean ± S.D. of five rats.
tively. Because TCA exhibits poor membrane permeability without the aid of membrane transporters, sodium-dependent uptake of $[^{3}H]$TCA in HEK293 cells expressing hASBT indicates that hASBT is correctly expressed in the plasma membrane.

Both S-8921 and S-8921G had an inhibitory effect on the uptake of $[^{3}H]$TCA by hASBT (Fig. 4). The apparent $K_{i}$ value of S-8921 and S-8921G on TCA uptake via hASBT was 109 ± 68 nM and 18.3 ± 6.5 nM, respectively. S-8921G exhibited a 6000-fold more potent inhibition of hASBT than S-8921. Dixon plots indicated that the type of inhibition of hASBT by S-8921G was noncompetitive (Fig. 4).

**S-8921G Concentration in the Portal Vein of Rats.** The concentrations of S-8921 and S-8921G in the portal vein were measured after S-8921 was applied to the loop of the jejunum and ileum. S-8921G was detected in the portal vein at 5 min after administration of 100 μM S-8921 into the intestinal loop, and the plasma concentration of S-8921G in the portal vein was higher than that of S-8921 at all time points (Fig. 5). In addition, S-8921G was detected in the lumen of both the jejunum and ileum. The concentrations of S-8921G in the lumen of the jejunum and ileum at 45 min after dosing were 7.60 ± 1.36 and 1.23 ± 0.99 μM (mean ± S.D.; $n = 3$), respectively. When S-8921 was applied to the loop of the jejunum in rats following bile duct cannulation, the concentration of S-8921G in the bile and intestinal lumen at 45 min after dosing was 1.68 ± 0.59 and 2.27 ± 0.95 μM (mean ± S.D.; $n = 3$), respectively. The volume of the bile collected during 45 min and in the intestinal lumen was 0.4 to 0.7 ml and 0.5 ml, respectively. Taking this into consider-
Inhibitory effect of various compounds on the uptake of $[^{14}C]$S-8921G by freshly isolated rat hepatocytes. (a) Uptake of 10 mM $[^{14}C]$S-8921G by freshly isolated rat hepatocytes was determined at 4 and 37°C. Open and closed circles represent the uptake of $[^{14}C]$S-8921G in the presence or absence of Na$^+$, respectively. Closed triangles represent the uptake of $[^{14}C]$S-8921G at 4°C. Each point and vertical bar represents the mean ± S.D. of three experiments. (b) Uptake of $[^{14}C]$S-8921G by freshly isolated rat hepatocytes was determined at concentrations ranging from 3.4 to 1000 nM for 30 s in the presence of Na$^+$. The relationship of the uptake to the substrate concentrations is shown by the Eadie-Hofstee plot. Lines represent the fitted curve obtained by nonlinear regression analysis. Each point and vertical bar represents the mean ± S.D. of three experiments.

TABLE 3
Inhibitory effect of various compounds on $[^{14}C]$S-8921G uptake into rat hepatocytes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% control</th>
<th>Uptake (μl/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Na$^+$)</td>
<td>100</td>
<td>3.2 ± 6.2</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>0</td>
<td>63.2 ± 6.2</td>
</tr>
<tr>
<td>4°C</td>
<td>100</td>
<td>41.3 ± 6.1</td>
</tr>
<tr>
<td>S-8921G, 1 mM</td>
<td>100</td>
<td>36.2 ± 6.9</td>
</tr>
<tr>
<td>BSP, 100 μM</td>
<td>100</td>
<td>51.7 ± 4.6</td>
</tr>
<tr>
<td>Estrone 3-sulfate, 100 μM</td>
<td>100</td>
<td>85.3 ± 1.8</td>
</tr>
<tr>
<td>TCA, 300 μM</td>
<td>100</td>
<td>77.3 ± 8.6</td>
</tr>
<tr>
<td>S-8921, 100 μM</td>
<td>100</td>
<td>96.0 ± 4.5</td>
</tr>
</tbody>
</table>

Discussion

S-8921 is a novel hypocholesterolemic agent and an inhibitor of the ileal ASBT/SLC10A2. In the present study, the contribution of S-8921G to the pharmacological effects after oral administration of S-8921 was investigated in rats.

Plasma concentrations of S-8921G after single oral administration of S-8921 were higher than those of S-8921 in normal rats (Fig. 2), and S-8921G was excreted into the bile in normal rats (Table 1). In Gunn rats, all the UGT1 isoforms of which are hereditarily deficient due to the mutation in the common region (Iyanagi, 1991), the plasma concentration of S-8921 was much greater than that in normal rats, and, particularly, S-8921G was barely detectable in both plasma and bile (Table 1). It is likely that S-8921G is mainly produced by UGT1 isoforms, and glucuronidation is the major mechanism limiting the oral absorption and elimination of S-8921 from the systemic circulation. It should be noted that the detailed pharmacokinetic analysis of S-8921 has not been performed in Gunn rats; thus, the possibility of adaptive changes in the metabolic enzymes and transporters cannot be excluded; these may also be part of the mechanism underlying an increase in the plasma concentration of S-8921. In vivo results were supported by an in vitro metabolism study using UGT Supersomes, which elucidated that UGT1 isoforms, except UGT1A4 and UGT1A6, converted S-8921 to its glucuronide (Table 2). Among these isoforms, UGT1A1, UGT1A8, and UGT1A10 are expressed in the small intestine, whereas UGT1A1, UGT1A3, and UGT1A9 are expressed in the liver (Strassburg et al., 2000; Tukey and Strassburg, 2000). These UGT1 isoforms could be involved in the intestinal and hepatic glucuronidation of S-8921, although the contribution of these isoforms to the glucuronidation of S-8921 remains to be elucidated in future studies.

The hypocholesterolemic effect of S-8921 was examined in hypercholesterolemic rats. Feeding the high-cholesterol diet increased non-HDL cholesterol in rats (Fig. 3). The serum non-HDL cholesterol level following repeated oral administration of either S-8921 or S-8921G in hypercholesterolemic normal rats was significantly reduced compared with the control group, whereas HDL cholesterol was slightly increased by the treatment (Fig. 3a). In contrast, repeated oral administration of S-8921 had no significant effect in hypercholesterolemic Gunn rats, whereas S-8921G significantly reduced the serum non-HDL cholesterol level (Fig. 3b). Accordingly, glucuronidation is the critical step for the pharmacological action of S-8921 in rats, although S-8921G itself is also an inhibitor of ASBT. This was supported by the in vitro finding that S-8921G is a 6000-fold more potent inhibitor of hASBT than S-8921 (Fig. 4). In general, the glucuronides of xenobiotics are less active than their parent compounds, and glucuronidation is recognized as one of the major detoxification pathways. N-O-Glucuronides of hydroxamic acids, acyl
glucuronides of carboxylic acids, morphine 6-O-glucuronide, retinoid glucuronides, and D-ring glucuronides of estrogens are known to have greater pharmacological or toxicological activities than their parent compounds (Ritter, 2000). S-8921G is the first case in which glucuronidation of a phenolic compound results in more potent pharmacological activity than the parent compound.

To obtain an insight into the site of glucuronidation of S-8921, the formation of S-8921G was examined using the in situ loop method in the rat jejunum or ileum. Since S-8921G was detected both in the portal vein and the lumen of the jejunum or ileum after administration of S-8921 into the loop (Fig. 5), S-8921 undergoes glucuronidation in the intestinal epithelial cells. The cumulative amount of S-8921G recovered in the bile was almost 5-fold greater than that in the intestinal loop of the jejunum, suggesting that S-8921G is mainly absorbed into the portal vein at least in the jejunum. The amount of S-8921G recovered in the bile specimens includes S-8921G formed not only in the jejunum but also in the liver. Considering the higher concentration of S-8921G compared with S-8921 in the portal vein (Fig. 5), S-8921G formed in the liver will account for the smaller fraction of the recovery of S-8921G in the bile. Previously, it has been reported that the rat bile collected following oral administration of S-8921G could inhibit the ileal absorption of TCA (J. Ono, S. Sakamoto, T. Ichibashi, M. Izawa, Y. Yano, T. Mizui, and K. Hirano, unpublished data). It is possible that S-8921G excreted into the bile is also involved in the hypocholesterolemic effect in addition to S-8921 directly effluxed into the lumen from the intestinal epithelial cells.

The mechanism underlying the asymmetrical efflux of S-8921G in the jejunum remains unknown. Because the recovery was greater in the bile than in the lumen during the intestinal absorption study, the efflux transport across the basolateral membrane will be more effective than the luminal efflux. In the small intestine, Mrp3 protein is expressed in the whole segment, but the expression level is higher in the mid-to-distal region than the proximal region (Zelcer et al., 2006), whereas the mRNA expression level exhibits the opposite behavior (Maher et al., 2005). For the efflux transport of glucuronide conjugates in the luminal side, Mrp2 and BCRP have been shown to mediate the intestinal efflux of the glucuronide conjugates of xenobiotics (Adachi et al., 2005). Mrp2 expression decreases from the proximal to the distal region of small intestine (Maher et al., 2005), whereas Bcrp exhibits a complementary expression pattern (Tanaka et al., 2005). Further studies are necessary to investigate the involvement/contribution of these ATP-binding cassette transporter(s) in the efflux transport of S-8921G along with the glucuronidation activity.

The uptake of S-8921G was characterized using freshly isolated rat hepatocytes. The uptake of S-8921G was saturable and exhibited sodium dependence (Fig. 6). The uptake was inhibited by organic anions, such as BSP, estrone 3-sulfate, and TCA (Table 3). These results suggest that S-8921G absorbed into the portal vein is removed by hepatic organic anion transporters. Na+-taurocholate cotransporting polypeptide (SLC10A1) is responsible for the sodium-dependent uptake of bile acids (Hagenbuch and Meier, 1994), whereas organic anionic transport proteins (OATP/SLCO), such as Oatp1a1, Oatp1a4, and Oatp1b2 in rodents, and OATP1B1 and OATP1B3, account for the sodium-independent uptake of a variety of organic anions (Hagenbuch and Meier, 2003). Thus, these transporters may be responsible for the hepatic uptake of S-8921G. Because MRP2 is well known as the transporter involved in the biliary excretion of many glucuronides (Kusuhara and Sugiyama, 2001), it can be speculated that MRP2 is responsible for the biliary excretion of S-8921G. Further studies are necessary to identify the transporters that play key roles in the intestinal and hepatic disposition of S-8921G.

In conclusion, S-8921G produced by UGT1 isoforms (UGT1A1, UGT1A3, UGT1A8, UGT1A9, and UGT1A10) is a very potent inhibitor of hASBT, and it plays an essential role in the hypocholesterolemic effect of S-8921. The schematic diagram of the pharmacokinetics of S-8921 is shown in Fig. 7. After oral administration of S-8921, S-8921 is metabolized to its glucuronide by UGT1 isoforms in the intestine. A part of S-8921G is directly excreted into the intestinal lumen, and it inhibits ASBT in the ileum. In addition, S-8921 and its glucuronide are absorbed into the portal vein. S-8921G is taken up into the liver by transporter(s), and S-8921 is metabolized to its glucuronide by UGT1 isoforms in the liver. S-8921G, excreted into the bile, is also involved in the inhibition of ASBT in the ileum. Inhibition of ASBT leads to a reduction in serum total cholesterol, particularly non-HDL cholesterol (i.e., LDL cholesterol).

References
Cruddoeck AL, Martha WL, Rebecca WD, Lyndon CK, Holly CW, Melissa HW, and

![Fig. 7. Schematic representation of the disposition of S-8921 and S-8921G.](image-url)


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