0026-895X/07/7101-19–29$20.00
MOLECULAR PHARMACOLOGY

Mol Pharmacol 71:19–29, 2007

Vol. 71, No. 1
27896/3159254
Printed in U.S.A.

In Vivo Responsiveness to Ezetimibe Correlates with Niemann-Pick C1 Like-1 (NPC1L1) Binding Affinity: Comparison of Multiple Species NPC1L1 Orthologs[S]

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Received June 12, 2006; accepted September 27, 2006

ABSTRACT

Ezetimibe is the first in class 2-azetidinone that decreases plasma cholesterol by blocking intestinal cholesterol absorption. Ezetimibe effectively reduces plasma cholesterol in several species including human, monkey, dog, hamster, rat, and mouse, but the potency ranges widely. One potential factor responsible for this variation in responsiveness is diversity in ezetimibe metabolism. After oral administration, ezetimibe is glucuronidated. Both ezetimibe and the glucuronide lower plasma cholesterol; however, the glucuronide exhibits greater potency. Recent identification of Niemann-Pick C1 Like-1 (NPC1L1) as the molecular target of ezetimibe enables direct binding studies to be performed. Here, we report the cloning of NPC1L1 derived from multiple species and assess amino acid sequence homology among human, monkey, dog, hamster, rat, and mouse. The rank order of affinity of glucuronidated ezetimibe for NPC1L1 in each species correlates with the rank order of in vivo activity with monkey > dog > hamster and rat ≫ mouse. Ezetimibe analogs that bind to NPC1L1 exhibit in vivo cholesterol-lowering activity, whereas compounds that do not bind NPC1L1 are inactive. Specific structural components of ezetimibe are identified as critical for binding to NPC1L1. The results demonstrate that small variations in ezetimibe structure or in NPC1L1 amino acid sequence can profoundly influence ezetimibe/NPC1L1 interaction and consequently in vivo activity. The results demonstrate that the ability of compounds to bind to NPC1L1 is the major determinant of in vivo responsiveness.

Hypercholesterolemia is linked to cardiovascular disease, myocardial infarction, and stroke. Blood cholesterol levels are regulated by several components, including de novo synthesis, dietary cholesterol absorption, and biliary clearance and excretion. Alteration of the rate of any of these processes can drastically affect whole-body cholesterol levels. Several pharmaceutical therapeutics have been developed that inhibit cholesterol synthesis. These agents, collectively referred to as statins, inhibit the enzyme 3-hydroxymethylglutaryl coenzyme A reductase to effectively reduce blood cholesterol levels and represent the standard of care for treatment of dyslipidemia. A new class of cholesterol-lowering therapeutics, called 2-azetidinones, decreases plasma cholesterol levels by blocking intestinal absorption of cholesterol. Ezetimibe (Zetia; Merck/Schering-Plough, Kenilworth, NJ), the first-in-class representative of the 2-azetidinones, blocks both dietary and biliary cholesterol absorption in the

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.027896.
[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: NPC1L1, Niemann-Pick C1 Like-1; MES, 4-morpholineethanesulfonic acid; SR-BI, scavenger receptor class B, type I; PCR, polymerase chain reaction; HEK, human embryonic kidney; RACE, rapid amplification of cDNA ends; SCH, Schering Plough compound number; SCH60663, 1-*O*-[4-[*trans*-(2*S*,3*R*)-1-(4-fluorophenyl)-4-oxo-3-[3(*S*)-hydroxy-3-(4- fluorophenyl)propyl]-2-azetidinyl]phenyl]-β-D-glucuronic acid; SCH58235, 1-(4-fluorophenyl)-3(*R*)-[3(*S*)-hydroxy-3-(4- fluorophenyl)propyl)]-4(*S*)-(4-hydroxyphenyl)-2-azetidinone; SCH61159, 1-*O*-[4-[*trans*-(2-*S*,3*R*)-1-(4- fluorophenyl)-4-oxo-3-[3(*S*)-hydroxy-3-(4- fluorophenyl)propyl]-2-azetidinyl]phenyl-L]-3-*O*-(β-D-glucopyranosyl)-β-D-glucopyranose; SCH604813, (*R*)-[3-(4-fluorophenyl)-3(*S*)-hydroxypropyl]-4(*S*)-(4- hydroxyphenyl)-1-(4-iodophenyl)-2- azetidinone; SCH58832, *trans*-1-(4-fluoropheny)-3-[[2-(4-fluoropheny)-2-oxoethyl]thio]-4-(4-hydroxyphenyl)-2-azetidinone; SCH60179, 1-*O*-[4-[*trans*-(3*R*,4*S*)-1-(4-methoxyphenyl)-2-oxo-3-(3-phenylpropyl)-4-azetidinyl]phenyl]-2,3,4,6-tetra-*O*-(phenylmethyl)-β-D-glucopyranose; SCH50032, rel-(3*R*,4*S*)-4-(4- fluorophenyl-L)-1-(4-mehtoxyphenyl)-3-(3-phenylpropyl)-2-azetidinone; SCH354909, 1-*O*-[4-[1-[4-[3-[[3-(4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*S*-indacen-3-yl)-1-oxopropyl]amino]-1-propynyl]phenyl]-3(*R*)-[3(*S*)-hydroxy-3-(4-fluorophenyl)propyl]-2-oxo-4(*S*)-azetidinyl]phenyl]-β-D-glucopyranuronic acid; SCH610396, 1-*O*-[4-[1-[4-[3-[[3-(4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*S*-indacen-3-yl)-1-oxopropyl]amino]-1-propynyl]-phenyl]-3(*R*)-[3(*S*)-hydroxy-3-(4- fluorophenyl)propyl]-2-oxo-4(*S*)-azetidinyl]phenyl]-β-D-glucopyranuronic acid, methyl ester.

proximal jejunum in hamsters (Salisbury et al., 1995). Ezetimibe administration also prevents the development of atherosclerosis in ApoE knockout mice (Davis et al., 2000a,b, 2001a). In human studies, treatment with ezetimibe (10 mg/day) produces a decrease in cholesterol absorption of greater than 50% (Jeu and Cheng, 2003) and a consequent decrease in total blood cholesterol levels of 15 to 20% (Lipka et al., 2000; Bays et al., 2001; Dujovne et al., 2002; Knopp et al., 2003). Furthermore, coadministration of statins and ezetimibe produces dual-pathway inhibition resulting in an additive effect on plasma cholesterol reduction (Davidson et al., 2002; Gagne et al., 2002; Ballantyne et al., 2003; Kerzner et al., 2003; Melani et al., 2003). This therapeutic strategy is particularly effective because the two drug classes decrease cholesterol by distinct mechanisms, inhibition of cholesterol synthesis (statins), and inhibition of cholesterol absorption (ezetimibe).

Preclinical studies show that ezetimibe selectively lowers cholesterol absorption in hamster, mouse, rat, dog, and monkey (van Heek et al., 1997, 2001a,b,c; Davis et al., 2001b; Davis, 2001). Ezetimibe efficacy is species-dependent, and a rank order has been proposed previously (monkey > dog > hamster and rat ≫ mouse) based on a comparison of cholesterol-fed animal models (Burnett, 2004). Animal studies have also revealed that ezetimibe is glucuronidated after first-pass metabolism and that a cycle of interconversion between the glucuronidated and nonglucuronidated forms occurs in vivo. In humans, ezetimibe efficacy is also well-documented (Ballantyne, 2002; Sudhop et al., 2002). Similar to the pharmacokinetics observed in the animal studies, ezetimibe is also glucuronidated in humans and subjected to enterohepatic recirculation (Patrick et al., 2002). This pharmacokinetic characteristic of ezetimibe has complicated the interpretation of efficacy studies. Both the glucuronidated and nonglucuronidated forms of ezetimibe are active, but the glucuronidated form is more potent. Therefore, the ratio of glucuronidated-to-nonglucuronidated ezetimibe present in a particular species probably affects the observed in vivo efficacy. The dependence of the diverse efficacy among species on differences in target protein amino acid sequence or species-specific metabolism variations affecting drug pharmacokinetics parameters remains open to debate.

Although ezetimibe was discovered and developed in the absence of a known molecular target, the ability of the drug

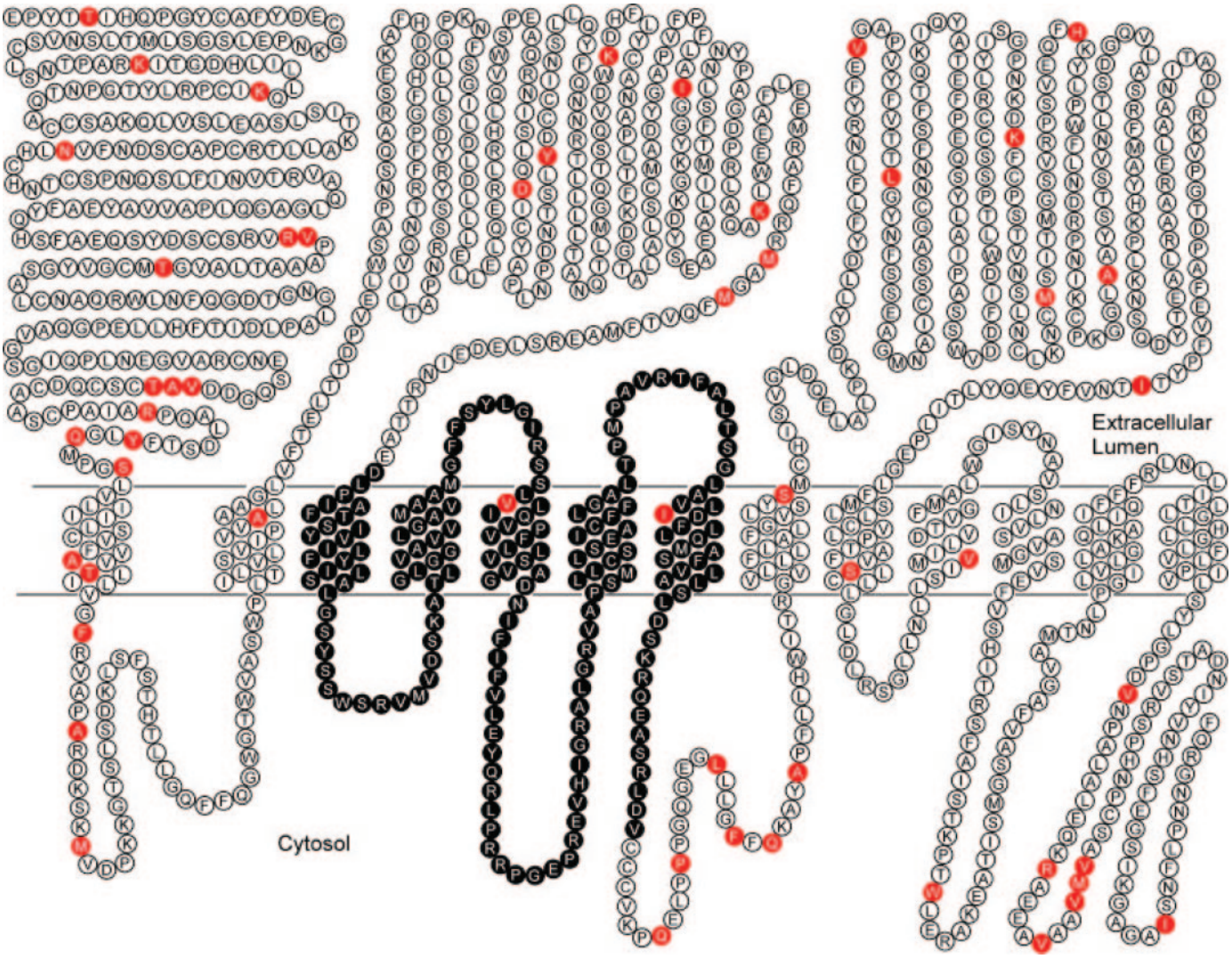


Fig. 1. Ball model of predicted membrane topology of human NPC1L1. Residues highlighted (black) identify the predicted sterol sensing domain (Carstea et al., 1997). Colored residues (red) identify amino acids that are not conserved between human and monkey proteins.

to block intestinal cholesterol absorption provided strong evidence favoring a protein-mediated cholesterol absorption mechanism over a passive diffusion model. The decade-long search for the target of ezetimibe recently culminated with the identification of the protein Niemann-Pick C1 Like-1 (NPC1L1) as a critical mediator of sterol absorption, regulator of whole-body cholesterol homeostasis, and molecular target of ezetimibe (Altmann et al., 2004; Davis et al., 2004; Garcia-Calvo et al., 2005).

To understand the species-specific efficacy differences, we cloned and expressed several species orthologs of NPC1L1 and evaluated a collection of ezetimibe analogs using a newly validated fluorescent compound-based NPC1L1 binding assay. The binding affinities of the compounds at each species ortholog of NPC1L1 are compared with the ability to inhibit cholesterol absorption in vivo. We observe that NPC1L1 binding affinities correlate with in vivo efficacies for the compounds tested. Although species pharmacokinetic differences may contribute to the diversity of ezetimibe efficacy among species, the ability of compounds to bind to NPC1L1 is the major determinant of in vivo responsiveness.

Materials and Methods

Materials. The [^{3}H]ezetimibe glucuronide [1-([2,6–3*H*]-4-fluorophenyl)-(3*R*)-[3-(4-fluorophenyl)-(3*S*)-hydroxypropyl]-(4*S*)-[3,5-^{3}H]-4-hydroxyphenyl)-2-azetidinone, 34.5 Ci/mmol (Garcia-Calvo et al., 2005), was the kind gift of Merck Research Laboratories (West Point, PA). All other compounds were synthesized by Discovery Chemistry, Schering Plough Research Institute (Kenilworth, NJ) and are >99.9% pure. Compounds used in this study include SCH60663, SCH58235, SCH61159, SCH604813, SCH58832, SCH60179, SCH50032, SCH354909, and SCH610396.

cDNA Cloning. Cloning and sequencing of NPC1L1 from human (GenBank accession number AY437865), rat (GenBank accession number AY437867), and mouse (GenBank accession number AY437865) have been reported (Altmann et al., 2004). Jejunal enterocytes were isolated as described previously (Altmann et al., 2002) from freshly isolated tissue samples from rhesus monkey, cynomologus monkey, hamster, rabbit, and beagle dog. Isolated enterocytes

A

Percent Identity

Percent Divergence

	Human	Chimp	Monkey	Canine	Hamster	Mouse	Rat	Rabbit
Human		99.3	95.8	81.1	77.9	76.7	77.6	78.5
Chimp	0.7		95.7	81.1	77.8	76.7	77.5	78.4
Monkey	4.3	4.4		81.3	77.8	76.7	77.6	79
Canine	21.4	21.4	21.2		74.9	73.7	74.4	76.5
Hamster	26.1	26.2	26.2	29.6		88.4	88.1	75.5
Mouse	27.1	27.1	26.9	31.3	12.5		91.3	75.4
Rat	25.9	26.1	25.8	29.9	12.6	9.2		75.8
Rabbit	24.8	24.9	23.9	27.3	29.2	29.7	28	

B

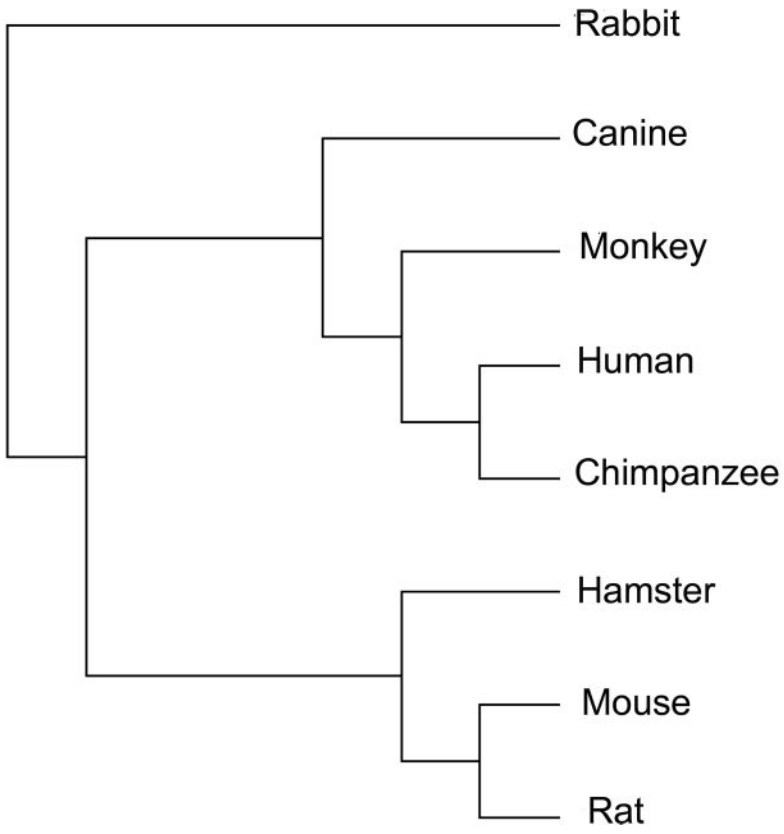
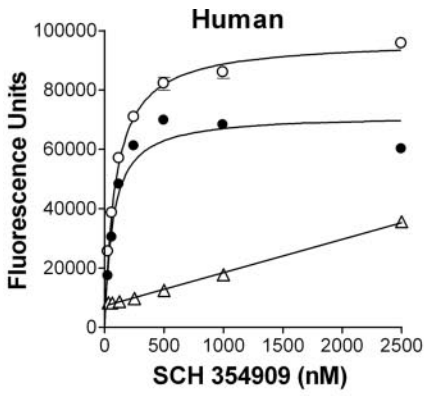
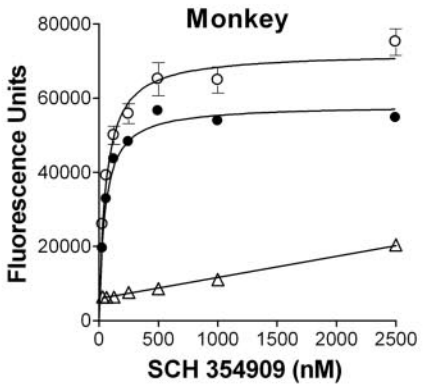
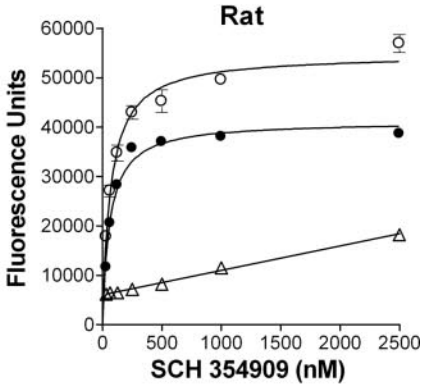
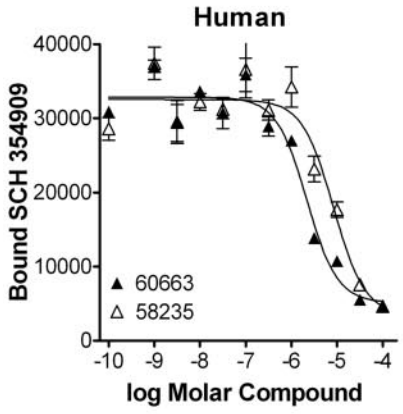


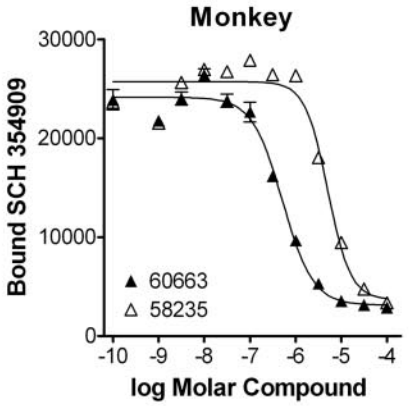
Fig. 2. Progressive multiple amino acid sequence alignment using the Clustal W method (Thompson et al., 1994). A, NPC1L1 amino acid sequence pair distances between species comparing the percentage of identity and percentage of divergence. B, phylogenetic tree representation of amino acid sequence alignment using Treeview (Page, 1996).

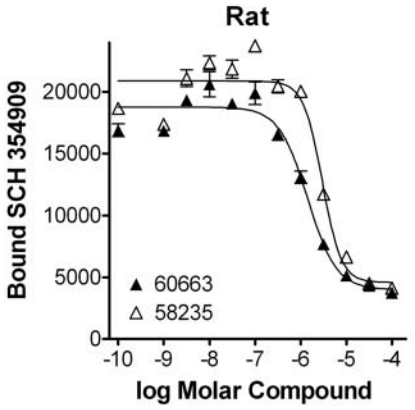


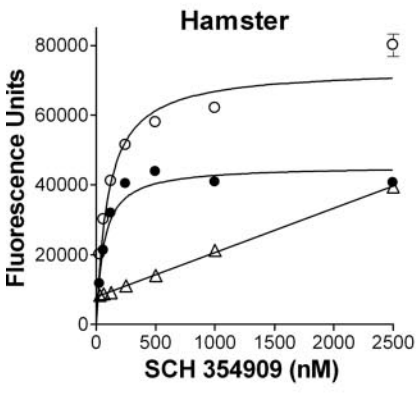


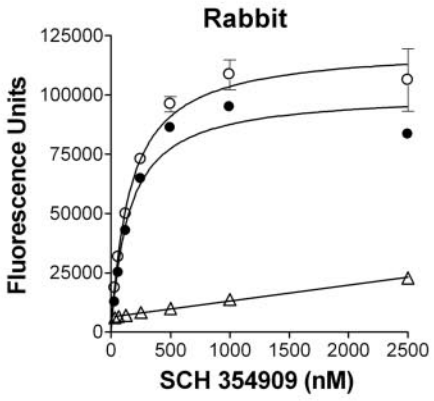


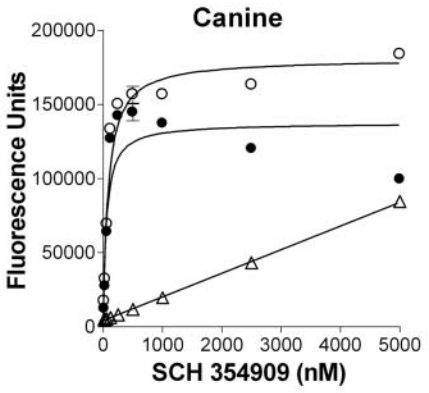


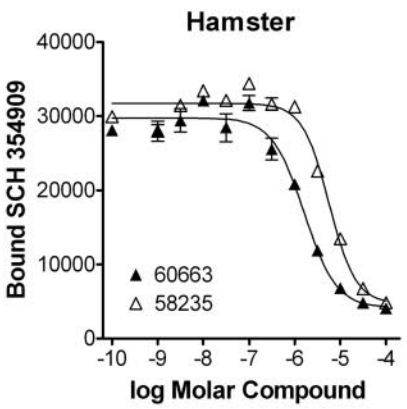


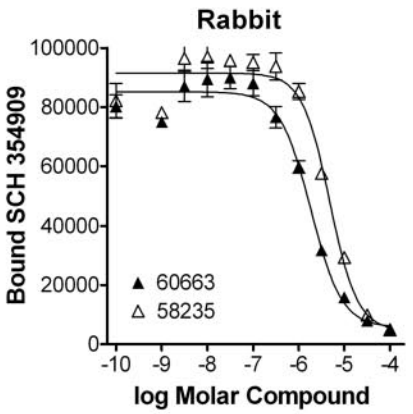


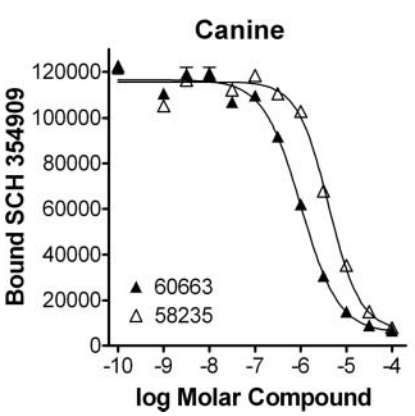












were immediately extracted with Tri-reagent, and the total RNA was isolated by following the manufacturer's instructions (Molecular Research Center Inc., Cincinnati, OH) mRNA was isolated using FastTrack 2.0 (Invitrogen, Carlsbad, CA), and cDNA was prepared using Superscript Choice System (Invitrogen, Carlsbad, CA) after oligo-(dT)-primed first-strand synthesis. NPC1L1-specific oligonucleotide primers corresponding to highly conserved regions in the human, mouse, and rat were used in varied combinations to polymerase chain reaction (PCR) amplify each cDNA sample. PCR products were sequenced to determine species-specific NPC1L1 sequence. To obtain species-specific gene sequences from the 5′-start codon region and the 3′-stop codon region, 5′ and 3′ RACE PCR analyses were performed using Marathon-Ready cDNA Amplification Kit or Smart RACE cDNA Amplification Kit according to the manufacturer's instructions (Clontech, Mountain View, CA). The species-specific oligonucleotide primers for 5′ and 3′ RACE PCR were designed according to available species-specific *NPC1L1* gene sequences. In some cases, oligonucleotide primers based on consensus gene sequences among species were also used in the 5′ and 3′ RACE PCR reaction. Sequence analysis of RACE PCR products identified coding sequence for the start and stop of the protein open reading frame. Preparation of the final NPC1L1 cDNA was carried out by PCR amplification of the complete open reading frame using species-specific forward and reverse primers encompassing the start and stop codons, respectively. Sequencing of multiple clones from independent PCR reactions resulted in cDNA sequences free from nucleotide errors introduced by *Taq* polymerase.

Cell Culture and Membrane Preparation. Each plasmid pCR3.1 harboring NPC1L1 was prepared using standard molecular biology protocols. Stable cell lines expressing human, rhesus monkey, mouse, rat, hamster, canine, or rabbit NPC1L1 were generated using Lipofectamine 2000 transfection reagent in HEK-293 cells according to the manufacturer's protocol. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, and 500 μg/ml Geneticin at 37°C in 5% CO_2. All cell culture reagents were obtained from Invitrogen. Cell membranes were prepared by lysing cells in 5 mM HEPES with protease inhibitors (Complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics Corporation, Indianapolis, IN) for 15 min at 4°C. A membrane pellet was obtained by centrifuging the cell lysates at 12,000*g* for 25 min. The membranes were resuspended in 5 mM HEPES with protease inhibitors and triturated with a 21-gauge needle.

NPC1L1 Binding Assays

Fluorescence. Cells were plated into 384-well black/clear plates (BD Biosciences Discovery Labware, Bedford, MA) and incubated overnight to enable attachment of the cells to the plates. The growth medium was then replaced with growth media (20 μl) containing 250 nM BODIPY-labeled glucuronidated ezetimibe (SCH354909) (Burnett et al., 2002). Media (20 μl) containing the indicated concentration of compound was then added to the wells. Unlabeled glucuronidated ezetimibe (SCH60663; 100 μM) was used to determine nonspecific binding. The binding reaction was allowed to proceed for 4 h at 37°C. Thereafter, the media were aspirated, and the cells were washed once with phosphate-buffered saline. The remaining SCH354909 bound to the cells was quantified using a FlexStation plate reader (Molecular Devices, Sunnyvale, CA).

Radioligand. Binding of [^{3}H]SCH60663 to membranes from cells expressing NPC1L1 was measured using a filtration binding assay (Garcia-Calvo et al., 2005). Reactions were performed in binding buffer (5 mM HEPES, 5.5 mM glucose, 117 mM NaCl, and 5.4 mM KCl, pH 7.4) Cell membranes (50 μg in 20 μl) were added to each well. Thereafter,[^{3}H]SCH60663 (20 nM, 20 μl) was added to each well. Compounds (20 μl) were then added to the wells as indicated in the figure legends. Nonspecific binding was determined by including unlabeled SCH60663 (100 μM) in the binding reaction. Binding reactions were incubated for 2 h at 37°C. Samples were transferred to Unifilter-96 GF/C plates (PerkinElmer Life and Analytical Sciences, Boston, MA) and filtered using a Brandel harvester (Gaithersburg, MD). The plates were washed several times with cold (4°C) wash buffer (120 mM NaCl, 0.1% sodium cholate, and 20 mM MES, pH 6.7) and dried. Liquid scintillant (50 μl; Microscint-20, PerkinElmer) was added, and the bound radioactivity was measured using a microplate scintillation counter.

Short-Term Cholesterol Absorption Assay. [^{14}C]cholesterol absorption was determined immediately in rats using conditions described previously (van Heek et al., 1997). [^{14}C]cholesterol was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA; 40–60 mCi/mmol). Compounds were dissolved in rat bile and delivered (1.0 ml) intraduodenally by bolus injection via an intestinal catheter, followed by 1.0 ml saline rinse (0.9%). After a 30-min incubation, a cholesterol emulsion containing 3 mg of cholesterol and 2 μCi [^{14}C]cholesterol (3 ml) was delivered to each rat as a bolus via intestinal catheter followed by 1 ml of saline rinse. Animals were killed 90 min later, and [^{14}C]cholesterol levels in plasma, liver, intestinal contents, and intestinal wall were determined.

Results

The effective dose of ezetimibe that inhibits cholesterol absorption varies among several species that have been studied. Because NPC1L1 has been identified as the direct proximal target of ezetimibe, we cloned NPC1L1 from jejunal enterocytes of rhesus and cynomolgus monkey (GenBank accession numbers DQ_897677 and DQ_897678), canine (GenBank accession number DQ_897676), hamster (GenBank accession number DQ_897680), and rabbit (GenBank accession number DQ_897679). Comparison of the amino acid sequences of NPC1L1 in those species along with previously published amino acid sequences of human, rat, and mouse NPC1L1 (Altmann et al., 2004) and predicted sequences from chimpanzee (GenBank accession number XM_519072) and bovine (GenBank accession number XM_588051) are shown in supplemental Fig. S1. At the sequence level, the positions of the cysteine residues, of which there are ~40, are highly conserved across all species and are suggestive of a highly constrained structure. Several cysteine residues are located within predicted transmembrane helices 1, 6, and 9 with the potential of fixing these transmembrane helices in close proximity. The proposed protein topology defined by the predicted transmembrane helices is consistent with the location of the putative N-linked glycosylation sites, which reside in three large extracellular loops exposed to the

Fig. 3. Characterization of NPC1L1 binding in multiple species. HEK 293 cells expressing human (A), monkey (B), rat (C), hamster (D), rabbit (E), or canine (F) NPC1L1 were exposed to the indicated concentration of SCH354909 for 4 h. The amount of fluorescence bound to the cells was quantified as total binding (○). Addition of 100 μM SCH60663 was used to determine nonspecific binding (△). Specific binding (●) was determined by subtraction of nonspecific from total binding. The K_d values were calculated using Prism software and are the mean of at least three separate experiments. Competition binding of SCH58235 and SCH60663 to NPC1L1 is also shown. Binding of SCH354909 to HEK 293 cells expressing each species NPC1L1 in the presence of the indicated concentration of SCH 58235 (○) or SCH60663 (●) was determined. The data shown are averages ± S.D. (n = 4) from one representative experiment. Where error bars are not visible, error is smaller than the symbol size. Averaged K_i values were calculated from at least three separate experiments.

intestinal lumen. Figure 1 presents a ball model of the predicted membrane topology of human NPC1L1 (Iyer et al., 2005). Residues in black constitute the sterol sensing domain (Carstea et al., 1997), and residues highlighted in red identify nonconserved positions between human and monkey NPC1L1.

Relative NPC1L1 sequence homology among species is shown in Fig. 2. NPC1L1 is most highly conserved among the primates with human, chimpanzee, and monkey exhibiting >95% amino acid identity. Nucleotide sequences in rhesus and cynomolgus monkey coding regions show only nine substitutions, none of which result in amino acid differences (data not shown). Human and monkey NPC1L1 amino acid sequences are highly homologous being less than 5% divergent. Of the 53 amino acid substitutions in monkey, 28 reside in the extracellular domains, and 17 are located within the cytoplasmic domains. The remaining eight changes occur in the transmembrane domains, two of which are located in the sterol sensing domain.

The rodent family consisting of sequences from hamster, rat, and mouse also exhibit nearly 90% identity in amino acid sequences. In contrast, primates and rodents share only 77 to 78% amino acid sequence identity with each other. The homology of canine NPC1L1 compared with the other species is less (74–81%), as is bovine (75–81%). Likewise, rabbit NPC1L1 exhibits only 75 to 79% homology to the other species examined. A phylogenetic tree representing the homology of NPC1L1 in the various species is shown in Fig. 2B. As expected, canine and rabbit NPC1L1 are more divergent compared with both primate and rodent families.

To understand the physiological impact of the NPC1L1 sequence diversity among species, binding characteristics of ezetimibe (SCH58235) and its glucuronidated metabolite (SCH60663) to the NPC1L1 orthologs of several species are examined in this study. Stable HEK-293 cell lines expressing human, rhesus monkey, canine, rat, hamster, rabbit, or mouse NPC1L1 cDNA were derived and used in subsequent experiments. The saturation binding curves of a fluorescently labeled (BODIPY) ezetimibe glucuronide (SCH354909) to each species NPC1L1 ortholog (except mouse) are shown in Fig. 3. The calculated K_d values are as follows: monkey, 46 nM; hamster, 49 nM, canine, 52 nM; rat, 58 nM; human, 61 nM; and rabbit, 151 nM. SCH354909 binding to mouse NPC1L1 could not be detected despite demonstrable expression of mouse NPC1L1 in HEK-293 cells by Western blot analysis (data not shown).

In an effort to detect binding to mouse NPC1L1, several related ezetimibe analogs were examined as possible alternatives to SCH354909 in the binding assay. The compound SCH610396, which is a fluorescently labeled synthetic precursor for SCH354909 (Burnett et al., 2002), was identified as a viable option for detection of mouse NPC1L1 binding. SCH610396 contains a methyl ester substitution for the carboxylic acid on the glucuronide portion of the molecule (compound structures shown in Fig. 4A). Saturation binding anal-

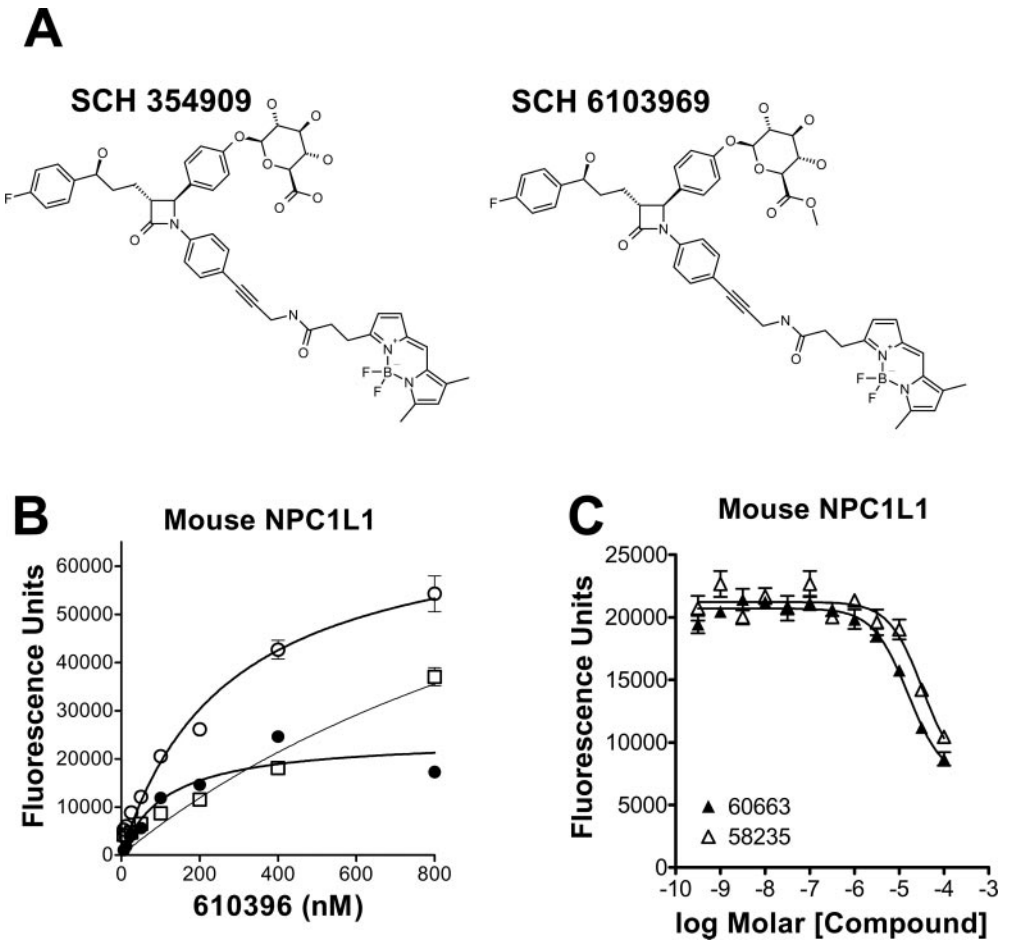


Fig. 4. Binding of SCH610396 to mouse NPC1L1 and generation of K_i values for SCH58235 and SCH60663. A, structure of SCH354909 and SCH610396. B, saturation binding of SCH610396 to membranes derived from HEK 293 cells expressing mouse NPC1L1. Bound SCH610396 was determined in the absence (total, ●) or presence (nonspecific, ○) of 100 μM concentration of unlabeled SCH 60663. Specific binding (▲) was determined by subtracting nonspecific from total binding. K_d values were determined using Prism software. C, determination of K_i values of SCH60663 and SCH58235 in competition binding studies with SCH610396 using membranes from HEK cells expressing mouse NPC1L1. The data shown are averages ± S.D. (n = 4) from one representative experiment. Where error bars are not visible, error is smaller than the symbol size. Averaged K_i values from at least three separate experiments and are listed in Table 1.

ysis with SCH610396 (Fig. 4B) demonstrates binding to mouse NPC1L1 with a K_d value of 118 nM.

Binding affinities at each species NPC1L1 ortholog were determined for both SCH58235 and SCH60663 (Figs. 3 and 4C). The calculated K_i values are listed in Table 1 (columns 1 and 2) and are compared with in vivo ED_{50} values derived for each species tested (column 3). Divergence in the affinities of SCH58235 and SCH60663 for NPC1L1 is consistently observed across species. For all species tested, the affinity of SCH60663 for NPC1L1 is greater than that of SCH58235 (compare columns 1 and 2). Against monkey NPC1L1, the difference in affinity of these two compounds is most obvious at nearly 10-fold, whereas the difference in affinity is less than 3-fold against rabbit or mouse NPC1L1. Rank-order species affinity for SCH58235 is (monkey, dog, rat) > hamster > (human and rabbit) ≫ mouse. The rank-order species affinity for SCH60663 is slightly modified with monkey > dog > (rat and hamster) > (human and rabbit) ≫ mouse. In comparison, the rank order of in vivo potency of ezetimibe among species is monkey > dog > (rat and hamster) ≫ mouse. It should be noted that after oral administration, 90% of ezetimibe is glucuronidated, thereby converting SCH58235 to SCH60663. Therefore, the predominant form of ezetimibe present at the site of action in vivo (NPC1L1 in the jejunum) is the glucuronide SCH60663.

Expanding the study to several other ezetimibe analogs supports the observation that NPC1L1 binding correlates with in vivo cholesterol-lowering activity. Ezetimibe analogs that exhibit in vivo cholesterol-lowering activity (SCH61159, SCH60481, and SCH58832) and analogs that display no in vivo cholesterol-lowering activity (SCH60179 and SCH50032) were evaluated for binding to NPC1L1 orthologs of multiple species. The compound structures and the K_i values at each species NPC1L1 are listed in Table 2. In vivo data measuring the ability of each compound to lower cholesterol levels in plasma and liver in hamster are also provided for comparison in Table 2. The three active compounds exhibit variable affinity when evaluated against each species of NPC1L1 with the rank order of affinity among species similar to that of SCH58235 and SCH60663. Higher affinity is observed at monkey, dog, and rat NPC1L1 and lower affinity at human and rabbit NPC1L1 with affinity for hamster NPC1L1 somewhat intermediate. In comparison, the affinities of the compounds are markedly lower at mouse NPC1L1. Compounds that lack in vivo efficacy exhibit no detectable binding to NPC1L1 orthologs from any of the species tested. These data demonstrate that compound binding to NPC1L1 correlates with in vivo activity. Prediction of the extent of in vivo potency is confounded by metabolic parameters after oral administration. Glucuronidation of SCH58235 produces a metabolite (SCH60663) with higher affinity for NPC1L1. Similar metabolism may affect related compounds. The ability to generate metabolites with high affinity for NPC1L1 will affect overall in vivo responsiveness. The key determinant of in vivo efficacy is the ability of the predominant compound metabolite to bind to NPC1L1. Minor changes in compound structure or NPC1L1 amino acid sequence can profoundly affect binding affinity and consequently in vivo efficacy.

An example of the effects of small modifications on the binding affinity of related compounds for NPC1L1 is provided by comparison of the binding characteristics of SCH610396 and SCH354909. The K_d value of [^{3}H]SCH60663 was determined for both human and monkey NPC1L1 in saturation binding assays (Fig. 5, A and B). K_d values of 206 nM for human and 102 nM for monkey are consistent with previously reported values of 220 and 41 nM, respectively (Garcia-Calvo et al., 2005). Competition binding studies using [^{3}H]SCH60663 were performed to derive K_i values for SCH354909 and SCH610396 at both human NPC1L1 (Fig. 5C) and monkey NPC1L1 (Fig. 5D). The K_i value of SCH354909 at human NPC1L1 is calculated to be 455 and 272 nM at monkey NPC1L1. In comparison, the K_i value of SCH610396 is calculated to be 107 and 65 nM at human and monkey NPC1L1, respectively. The results demonstrate that the small modification of substituting the methyl ester for the carboxylic acid on the glucuronide increases the affinity for human NPC1L1 (more than 4-fold). This further illustrates that small variations in ezetimibe-related compounds can cause diverse binding interactions. Likewise, diversity in NPC1L1 also affects the binding interaction, as demonstrated by the differences in binding among species orthologs. Given the data demonstrating the correlation between NPC1L1 binding affinity and in vivo efficacy, the binding interaction between compounds and NPC1L1 is a major determinant regulating in vivo responsiveness.

Discussion

Understanding drug in vivo efficacy is complicated by a host of considerations including the metabolism and pharmacokinetic properties of each particular compound. Although compounds may share the same mechanism of action and even exhibit similar in vitro binding characteristics, the in

TABLE 1

Comparison of in vitro binding affinity of SCH58235 and SCH60663 with *in vivo* potency of SCH58235 among several species

The K_i values of SCH58235 and its glucuronide metabolite SCH60663 are shown. Available in vivo potency data from previous studies (van Heek et al., 1997, 2001a,b,c; Davis, 2001; Davis et al., 2001b) is also displayed for comparison.

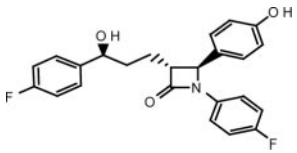
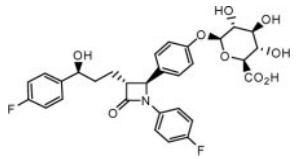
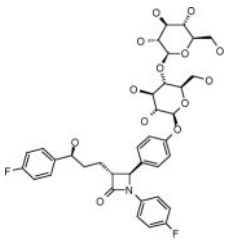
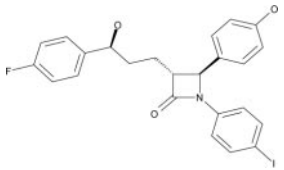
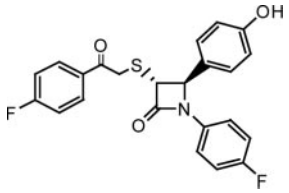
Species	K_i		SCH 58235 ED_{50}
	SCH 58235	SCH 60663	
	nM	*nM*	*μg/kg*
Human	1590 ± 200	660 ± 190	N.D.
Monkey	900 ± 200	92 ± 10	0.5
Hamster	1530 ± 350	370 ± 80	40
Canine	770 ± 160	192 ± 40	7
Rat	970 ± 280	352 ± 110	30
Rabbit	2350 ± 210	830 ± 160	N.D.
Mouse	9000 ± 1600	5400 ± 970	700

N.D., not determined.

TABLE 2

Comparison of species binding potency of SCH58235 analogs

The K_i values of compounds at several species NPC1L1 orthologs are shown. The effects of oral administration of compounds on cholesterol levels in liver and plasma in hamsters are also displayed. Values are mean ± S.D. from at least three determinations. In vivo data for SCH 58235 and SCH 60663 are taken from previously reported studies (Vaccaro and Davis, 1998).

Compound	Structure	K_i							Cholesterol Decrease	
		Human	Monkey	Mouse	Hamster	Rat	Dog	Rabbit	Liver	Plasma
					μM				%	
SCH 58235		1.59 ± 0.2	0.90 ± 0.2	9.0 ± 1.6	1.53 ± 0.35	0.97 ± 0.28	0.77 ± 0.16	2.35 ± 0.21	93	43
SCH 60663		0.66 ± 0.19	0.092 ± 0.01	5.4 ± 1.97	0.37 ± 0.08	0.35 ± 0.11	0.19 ± 0.04	0.83 ± 0.16	92	48
SCH 61159		2.15 ± 0.23	0.37 ± 0.05	12.8 ± 3.4	1.2 ± 0.29	0.54 ± 0.05	1.28 ± 0.33	1.89 ± 0.29	86 ± 7	36 ± 4
SCH 60481		4.59 ± 2.18	1.6 ± 0.17	16.4 ± 2.8	2.34 ± 1.25	1.46 ± 0.48	1.43 ± 0.83	5.00 ± 0.62	63 ± 10	22 ± 10
SCH 58832		3.23 ± 1.68	3.23 ± 0.81	21.6 ± 4.46	4.28 ± 0.99	2.11 ± 0.50	2.30 ± 0.90	5.60 ± 1.35	96 ± 1	47 ± 2

vivo efficacy can be quite variable. When the protein target is unknown or compound/target interaction cannot be measured, determination of the cause of these differences becomes highly speculative. Such was the case for ezetimibe (SCH58235), a first in class approved cholesterol absorption inhibitor marketed as Zetia for lowering of low-density lipoprotein cholesterol. The observed variations in ezetimibe efficacy among species (van Heek et al., 1997, 2001a,b,c; Davis et al., 2001b; Davis, 2001) is obscured further by recycling of the compound between two active metabolic forms at the site of action (glucuronidated and nonglucuronidated) (van Heek et al., 2000). NPC1L1, an intestinally expressed protein critical to the absorption of sterols, has been identified as the molecular target of ezetimibe (Altmann et al., 2004; Davis et al., 2004; Garcia-Calvo et al., 2005). Discovery of the drug target enabled in vitro analysis of drug binding and experimental opportunities to explore the interspecies variability in ezetimibe potency and efficacy. Here, we describe the cloning and expression of NPC1L1 in multiple species for studies comparing target interaction of SCH58235 and the active in vivo glucuronidated metabolite SCH60663. A novel fluorescent compound binding assay is used to assess the binding properties of several ezetimibe-related compounds at the NPC1L1 orthologs of multiple species, enabling structure-activity relationships to be developed and the interaction of ezetimibe and NPC1L1 to be better understood.

Intraduodenal delivery of SCH58235 leads to significant levels of the compound detected in portal plasma of which >95% is the glucuronide SCH60663 after first-pass metabolism in the intestine. Traveling from portal plasma to the liver and back to the intestine via bile, SCH60663 is redelivered to the site of action where it accumulates in the intestinal lumen (van Heek et al., 2000). Although both SCH58235 and SCH60663 bind to NPC1L1, the binding affinity of SCH60663 is greater than that of SCH58235 in all species examined, consistent with the stronger potency of SCH60663 observed in in vivo efficacy studies (van Heek et al., 2000). The compounds differ in affinity by as much as 10-fold in monkey and as little as 2-fold in mouse (Table 1), but the rank order of potency is similar for both compounds (monkey, rat, dog, and hamster > human and rabbit > mouse) and correlates well with animal efficacy studies (Table 1). These data indicate that compound potency is primarily dictated by the binding affinity of the compound for NPC1L1 of a particular species. However, the rate and efficiency of glucuronidation in each species also probably contribute to the diversity in species responsiveness to oral administration of ezetimibe given the binding differential between SCH58235 and SCH60663. Indeed, compound metabolism may be a critical factor in determination of ezetimibe potency in species that exhibit the highest degree of separation between SCH58235 and SCH60663 binding affinities and that are particularly responsive to ezetimibe treatment in vivo (e.g., monkey). The UDP-glucuronosyltransferase enzyme(s) responsible for glucuronidating SCH58235 in humans was identified (Ghosal, 2004); however, little comparative information is available for this enzyme or related enzymes across multiple species.

Another factor that may affect in vivo activity of 2-azetidi-

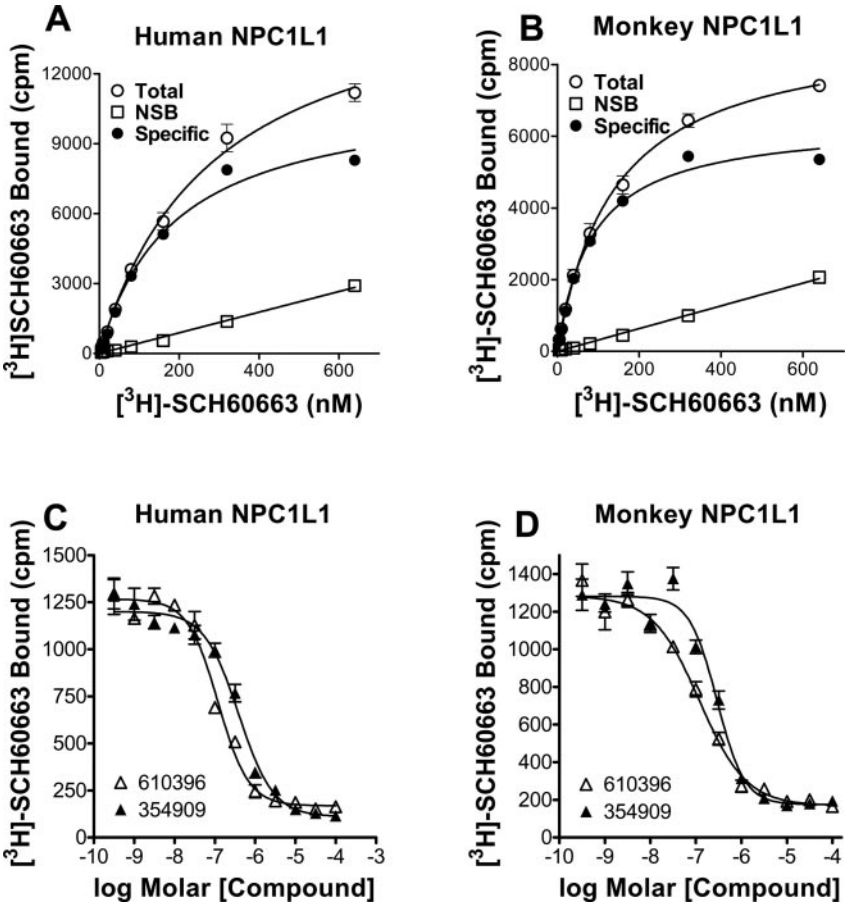


Fig. 5. Comparison of SCH354909 and SCH610396 binding to human and monkey NPC1L1. Saturation binding of [^{3}H]SCH60663 to human (A) and monkey (B) NPC1L1 was performed to determine K_d values. The K_i values of SCH354909 (triangles) and SCH610396 (circles) at human (C) and monkey (D) NPC1L1 were then determined in competition binding assays with [^{3}H]SCH60663. The data shown are averages ± S.D. (n = 4) from one representative experiment. Where error bars are not visible, error is smaller than the symbol size. Averaged K_i values are the means of three separate experiments.

nones is the interaction of associated proteins with the compounds and NPC1L1. We have shown previously that SCH354909 and SCH58235 bind to scavenger receptor class B, type I (SR-BI) with relatively high affinity (Altmann et al., 2002). SR-BI has been suggested to play a role in cholesterol transport (Hauser et al., 1998), and its expression in the intestine is restricted to enterocytes similar to NPC1L1. Furthermore, SCH58235 blocks SR-BI-mediated cholesterol uptake in Chinese hamster ovary cells. However, studies in SR-BI knockout mice clearly demonstrate SCH58235 is still efficacious in vivo despite no SR-BI expression. Although NPC1L1 has been established as the molecular target of ezetimibe, SR-BI may also have limited effects on the in vivo efficacy among various species because SR-BI may be associated with cholesterol transport and can bind to 2-azetidinones. Analysis of the ability of species orthologs of SR-BI to bind various 2-azetidinones is beyond the scope of the present study.

Changes in compound structure clearly affect NPC1L1 binding ability (Table 2). Glucuronidation of SCH58235 after oral administration (forming SCH60663) enhances NPC1L1 binding and improves in vivo potency. In contrast, the addition of a protective second glucuronide group to the first glucuronide moiety (SCH61159) causes the K_i value to revert to that observed for the nonglucuronidated form. It has been reported previously that hydroxylation of the 3-phenylpropyl side chain improves in vivo potency of this class of compounds (Clader et al., 1996; Burnett, 2004). Consistent with that conclusion, compounds that lack the hydroxyl group at the 3-phenylpropyl side chain exhibit decreased (SCH58832) or total loss (SCH60179 and SCH50032) of NPC1L1 binding activity.

Even small alterations in compound structure or NPC1L1 can influence NPC1L1 binding. In Fig. 5, binding of SCH354909 and SCH610396 to human and monkey NPC1L1 are compared. SCH354909 is BODIPY-labeled SCH60663 and differs from SCH610396 only by a substitution of a methyl ester for the carboxylic acid on the glucuronide moiety (Fig. 4A) (Burnett, 2004). Consistent with other ezetimibe analogs, both SCH354909 and SCH610396 exhibit stronger affinity for monkey NPC1L1 compared with human NPC1L1. The substitution of the methyl ester on the glucuronide in SCH610396 confers higher affinity (>4-fold) to both human and monkey NPC1L1.

Noting that the substitution of the methyl ester (SCH610396) for the carboxylic acid (SCH354909) causes a functional shift from acidic to neutral pH, it is tempting to speculate which amino acids in human and monkey NPC1L1 are responsible for mediating the observed diversity in responsiveness to these compounds. Residues located in the transmembrane helices and extracellular loops have often been associated with drug binding and make for probable candidates (Strader et al., 1995). Comparison of monkey and human NPC1L1 yields 36 residues within these regions that differ between the two species. Amino acid substitutions leading to qualitative changes from lipophilic to basic and hydrophilic are predicted to be of particular interest. Studies using chimeric proteins or point mutations will shed light on this issue. Analysis of coding single nucleotide polymorphisms may provide additional understanding of critical amino acids within the NPC1L1 protein. Intestinal cholesterol absorption studies in humans have shown wide individual variation in both cholesterol absorption and responsiveness to ezetimibe (Sudhop et al., 2002). Defining the ezetimibe binding pocket in NPC1L1 will aid in the development of the next-generation cholesterol absorption inhibitors and provide some rationale for nonresponsive individuals.

The data in this study demonstrate that the ability of ezetimibe analogs to bind to NPC1L1 critically factors into the determination of in vivo efficacy. There is wide diversity in responsiveness to ezetimibe among several species that have been tested. Comparison of the binding affinities of several ezetimibe analogs demonstrates that there is a strong correlation between NPC1L1 binding affinity and the ability of compounds to affect cholesterol absorption in vivo. Other factors such as variations in bioavailability and the pharmacokinetic/pharmacodynamic properties of compounds within specific species also affect the overall responsiveness to ezetimibe therapy. The ability of the predominant compound metabolite present at the jejunal site of action to bind to NPC1L1 is the major determinant of in vivo responsiveness.

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