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Title

In vivo responsiveness to ezetimibe correlates with NPC1L1 binding affinity:

Comparison of multiple species NPC1L1 orthologs

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Running Title

Running Title: Responsiveness to ezetimibe is dependent on NPC1L1 binding

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Words in Introduction	672
Words in Discussion	1209

List of nonstandard abbreviations:

NPC1L1 - Niemann-Pick C1 Like-1

SCH – Schering Plough compound number

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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. Following oral administration, ezetimibe is glucuronidated. Both ezetimibe and the glucuronide lower plasma cholesterol, however the glucuronide exhibits greater potency. Recent identification of 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 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.

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Introduction

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-hydroxymethyl-glutaryl coenzyme A (HMG-CoA) 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, the first in class representative of the 2-azetidinones, blocks both dietary and biliary cholesterol absorption in the proximal jejunum in hamsters (Salisbury et al., 1995). Ezetimibe administration also prevents development of atherosclerosis in ApoE knockout mice (Davis et al., 2000a; Davis et al., 2000b; Davis et al., 2001a). In human studies, treatment with ezetimibe (10 mg/day) produces a decrease in cholesterol absorption of greater then 50% (Jeu and Cheng 2003) and a consequent decrease in total blood cholesterol levels by 15-20% (Lipka et al., 2000; Bays et al., 2001; Dujovne et al., 2002; Knopp et al., 2003). Furthermore, co-administration of statins and ezetimibe produces dual pathway inhibition resulting in an additive effect on plasma cholesterol reduction (Gagne et al., 2002; Davidson et al., 2002; Melani et al., 2003; Kerzner et al., 2003; Ballantyne et al., 2003). This therapeutic strategy is particularly effective since the two drug classes decrease cholesterol by distinct mechanisms, inhibition of cholesterol synthesis (statins) and inhibition of cholesterol absorption (ezetimibe).

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Pre-clinical studies show that ezetimibe selectively lowers cholesterol absorption in hamster, mouse, rat, dog, and monkey (van Heek et al., 1997; van Heek et al., 2001a; van Heek et al., 2001b; Davis et al., 2001b; van Heek 2001c, Davis 2001). Ezetimibe efficacy is species dependent and a rank order as been previously proposed (monkey > dog > hamster and rat >> mouse) based upon comparison of cholesterol-fed animal models (Burnett, 2004). Animal studies have also revealed that ezetimibe is glucuronidated following 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 (Sudhop et al., 2002; Ballantyne, 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 likely 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 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 to 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.

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Materials and Methods

Materials

The [^{3}H]ezetimibe glucuronide (EZE-gluc) [1-([2,6-3H]-4-fluorophenyl)-(3R)-[3-(4-fluorophenyl)-(3S)-hydroxypropyl]-(4S)-[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. All other compounds were synthesized by Discovery Chemistry, Schering-Plough Research Institute and are >99.9% pure. Compounds used in this study include SCH60663(1-O-[4-[trans-(2S,3R)-1-(4-fluorophenyl)-4-oxo-3-[3(S)-hydroxy-3-(4-fluorophenyl)propyl]-2-azetidinyl]phenyl]-beta-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-(2S,3R)-1-(4- fluorophenyl)-4-oxo-3-[3(S)-hydroxy-3-(4- fluorophenyl)propyl]-2- azetidinyl]phenylL]-3-O-(beta-D-glucopyranosyl)-beta-D-glucopyranose), SCH604813 ((R)-[3-(4- fluorophenyl)-3(S)- hydroxy propyl]-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-(3R,4S)-1-(4-methoxyphenyl)-2-oxo-3-(3-phenylpropyl)-4-azetidinyl]phenyl]-2,3,4,6-tetra-O-(phenylmethyl)-beta-D- glucopyranose), SCH50032 (rel-(3R,4S)-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-3a,4a-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]-beta-D-glucopyranuronic acid), SCH610396 (1-O-[4-[1-[4-[3-[[3-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-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]-beta-D-glucopyranuronic acid, methyl ester).

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cDNA cloning

Cloning and sequencing of NPC1L1 from human (Genbank AY437865), rat (Genbank AY437867) and mouse (Genbank AY437865) have been reported (Altmann et al., 2004). Jejunal enterocytes were isolated as previously described (Altmann et al., 2002) from freshly isolated tissue samples from rhesus monkey, cynomologus monkey, hamster, rabbit and beagle dog. Isolated enterocytes were immediately extracted with Tri-reagent and the total RNA isolated following manufacturer's instructions (Molecular Research Center Inc.) Messenger RNA was isolated using FastTrack 2.0 (Invitrogen) and cDNA prepared using superscript Choice System (Life Technologies) following oligo-(dT) primed first strand synthesis. NPC1L1 specific oligo 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 were performed using Marathon–Ready cDNA Amplification Kit, or Smart RACE cDNA Amplification Kit according to the manufacturer's instructions (BD Biosciences Clontech). The species-specific oligonucleotide primers for 5' and 3' RACE PCR were designed according to available species-specific NPC1L1 gene sequences. In some cases, oligo primers based upon 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 ORF using species specific forward and reverse primers encompassing the start and stop codons respectively.

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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 DMEM supplemented with 10% FBS, 100 U/ml pen/strep, and 500 μg/ml geneticin at 37C, 5% CO_2. All cell culture reagents were obtained from Invitrogen Life Technologies, (Carlsbad, CA). Cell membranes were prepared by lysing cells in 5 mM HEPES with protease inhibitors (Complete™ Protease Inhibitor Cocktail Tablets; Roche Diagnostics Corp., Indianapolis, IN) for 15 min at 4C. A membrane pellet was obtained by centrifuging the cell lysates at 12,000xg for 25 min. The membranes were resuspended in 5 mM HEPES with protease inhibitors and triturated with a 21G needle.

NPC1L1 Binding Assays

Fluorescence

Cells were plated into 384-well black/clear plates (BD Biosciences, Bedford MA) for and incubated overnight to enable attachment of the cells to the plates. The growth media was then replaced with growth media (20 μl) containing 250 nM BODIPY-labeled glucuronidated ezetimibe (SCH354909) (Burnett et al., 2002). Media (20μl) containing the indicated concentration of compound was then added to the wells. Unlabeled

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glucuronidated ezetimibe (SCH60663; 100 μM) was used to determine nonspecific binding. The binding reaction was allowed to proceed for 4 hours at 37C. Subsequently the media was aspirated and the cells washed once with PBS. 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. Subsequently, [^{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 hours at 37C. Samples were transferred to Unifilter-96 GF/C plates (Perkin Elmer, Wellesley, MA) and filtered using a Brandel harvester (Gaithersburg MD). The plates were washed several times with cold wash buffer (120 mM NaCl, 0.1% sodium cholate, 20 mM MES pH 6.7) and dried. Liquid scintillant (50 μl; Microscint-20, Perkin Elmer, Wellesley, MA) was added and the bound radioactivity was measured using a microplate scintillation counter.

Acute cholesterol absorption assay

^{14}C-cholesterol absorption was determined acutely in rats using conditions previously described (van Heek et al., 1997). ^{14}C-cholesterol was obtained from NEN (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%). Following a 30 min incubation, a cholesterol emulsion containing 3 mg cholesterol and 2 μCi ^{14}C-cholesterol (3 ml) was delivered to each rat as a bolus via intestinal catheter, followed by 1 ml saline rinse. Animals were sacrificed 90 min later and ^{14}C-cholesterol levels in plasma, liver, intestinal contents, and intestinal wall were determined.

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Results

The effective dose of ezetimibe that inhibits cholesterol absorption varies among several species that have been studied. Since NPC1L1 has been identified as the direct proximal target of ezetimibe, we cloned NPC1L1 from jejunal enterocytes of rhesus and cynomolgus monkey (Genbank DQ_897677 and Genbank DQ_897678), canine (Genbank DQ_897676), hamster (Genbank DQ_897680), and rabbit (Genbank 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 XM_519072) and bovine (Genbank XM_588051) are shown in supplemental Figure 1. At the sequence level, the positions of the Cys residues, of which there are ~40, are highly conserved across all species and suggestive of a highly constrained structure. Several Cys 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 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 (SSD) (Carstea et al., 1997) and residues highlighted in red identify non-conserved positions between human and monkey NPC1L1.

Relative NPC1L1 sequence homology among species is shown in Figure 2. NPC1L1 is most highly conserved among the primates with human, chimpanzee and monkey exhibiting > 95% amino acid identity. Nucleotide sequences in rhesus and cynomologous monkey coding regions show only 9 substitutions, none of which result in

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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 8 changes occur in the transmembrane domains, 2 of which are located in the SSD.

The rodent family consisting of sequences from hamster, rat, and mouse also exhibit close to 90% identity in amino acid sequences. By contrast, primates and rodents share only 77-78% amino acid sequence identity with each other. The homology of canine NPC1L1 compared to the other species is less (74-81%) as is bovine (75-81%). Likewise, rabbit NPC1L1 exhibits only 75-79% homology to the other species examined. A phylogenetic tree representing the homology of NPC1L1 in the various species is shown in Figure 2B. As expected, canine and rabbit NPC1L1 are more divergent compared to 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, mouse, 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 Figure 3. The calculated K_d values are: monkey 46 nM; hamster 49 nM, canine 52 nM; rat 58 nM; human 61 nM, 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).

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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 Figure 4A). Saturation binding analysis with SCH610396 (Figure 4B) demonstrates binding to mouse NPC1L1 with a K_d of 118 nM.

Binding affinities at each species NPC1L1 ortholog were determined for both SCH58235 and SCH60663 (Figures 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 column 1 versus column 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. By comparison, the rank order of *in vivo* potency of ezetimibe among species is monkey > dog > (rat and hamster) >> mouse. It should be noted that following 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.

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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) as well as 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 following 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.

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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 of [^{3}H]SCH60663 was determined for both human and monkey NPC1L1 in saturation binding assays (Figures 5A & 5B). A K_d of 206 nM for human and 102 nM for monkey are consistent with previously reported values of 220 nM 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 of SCH354909 at human NPC1L1 is calculated to be 455 nM and 272 nM at monkey NPC1L1. By comparison, the K_i of SCH610396 is calculated to be 107 nM 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 (over 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.

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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 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 LDL cholesterol. The observed variations in ezetimibe efficacy among species (van Heek et al., 1997; van Heek et al., 2001a; van Heek et al., 2001b; Davis et al., 2001b; van Heek 2001c, 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). Recently, NPC1L1, an intestinally expressed protein critical to the absorption of sterols was 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 inter-species 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 utilized 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.

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Intraduodenal delivery of SCH58235 leads to significant levels of the compound detected in portal plasma of which >95% is the glucuronide SCH60663 following 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). This data indicates 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 likely 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 therapy *in vivo* (e.g. monkey). Recently, 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-azetidinones is the interaction of associated proteins with the compounds and NPC1L1. We have previously shown that SCH354909 and SCH58235 bind to scavenger receptor class B, type I (SR-BI) with

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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. Further, SCH58235 blocks SR-BI mediated cholesterol uptake in CHO 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 since 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.

Clearly, changes in compound structure affect NPC1L1 binding ability (Table2). Glucuronidation of SCH58235 following oral administration (forming SCH60663) enhances NPC1L1 binding and improves *in vivo* potency. By contrast, 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 previously been reported that hydroxylation of the 3-phenylpropyl side chain improves *in vivo* potency of this class of compounds (Burnett, 2004; Clader et al., 1996). Consistent with that conclusion, compounds that lack the hydroxyl group at the 3-phenylpropyl side chain exhibit decreased (SCH58832) or total loss (SCH60179, SCH50032) of NPC1L1 binding activity.

Even small alterations in compound structure or NPC1L1 can influence NPC1L1 binding. In Figure 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 (Figure 4A) (Burnett, 2004). Consistent with other ezetimibe

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analogs, both SCH354909 and SCH610396 exhibit stronger affinity for monkey NPC1L1 compared to 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 on 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 as well as 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 utilizing 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 non-responsive individuals

The data in this study demonstrate that the ability of ezetimibe analogs to bind to NPC1L1 critically factors into 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

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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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Footnotes

Ezetimibe is manufactured and marketed by Merck/Schering-Plough under the trademark name Zetia.

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Legends for Figures

Figure 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.

Figure 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 percent identity and percent divergence. (B) Phylogenetic tree representation of amino acid sequence alignment using Treeview (Page, 1996).

Figure 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 (open circles). Addition of 100 uM SCH60663 was used to determine nonspecific binding (open triangles). Specific binding (filled circles) was determined by subtraction of nonspecific from total binding. The Kd 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 (open circles) or SCH60663 (filled circles) was determined. The data shown are averages ±

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standard deviation (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.

Figure 4

Binding of SCH 610396 to mouse NPC1L1 and generation of Ki values for SCH58235 and SCH606636. (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, filled circles) or presence (nonspecific, open circles) of 100 μM unlabeled SCH 60663. Specific binding (filled triangles) was determined by subtracting nonspecific from total binding. K_d values were determined using Prism software. (C) Determination of Ki values of SCH60663 and SCH58235 in competition binding studies with SCH610396 using membranes from HEK-cells expressing mouse NPC1L1. The data shown are averages ± standard deviation (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.

Figure 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 Ki 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 ± standard deviation (n=4) from one representative experiment. Where error bars are not

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visible, error is smaller than the symbol size. Averaged K_i values are the mean of three separate experiments.

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Tables

Table 1

Comparison of *in vitro* binding affinity of SCH58235 and SCH60663 with *in vivo* potency of SCH58235 among several species. The Ki values of SCH58235 and its glucuronide metabolite SCH60663 are shown. Available *in vivo* potency data from previous studies (van Heek et al., 1997; van Heek et al., 2001a; van Heek et al., 2001b; Davis et al., 2001b; van Heek 2001c, Davis 2001) is also displayed for comparison.

	SCH 58235 Ki (nM)	SCH 60663 Ki (nM)	SCH 58235 ED50 (ug/kg)
Human	1590 ± 200	660 ± 190	ND
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	ND
Mouse	9000 ± 1600	5400 ± 1970	700

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Table 2

Comparison of species binding potency of SCH58235 analogs. The Ki 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 3 determinations. *In vivo* data for SCH 58235 and SCH 60663 are taken from previously reported studies (Vaccaro and Davis .)1998).

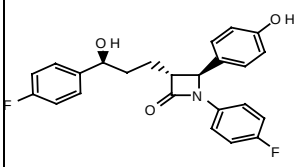
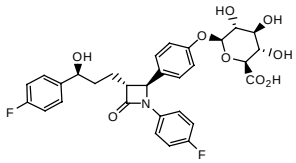
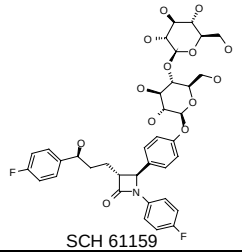
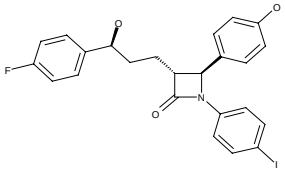
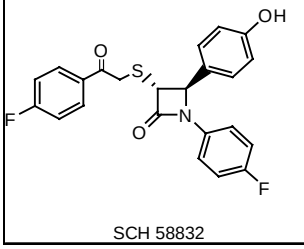
Compound	Human Ki (μM)	Monkey Ki (μM)	Mouse Ki (μM)	Hamster Ki (μM)	Rat Ki (μM)	Dog Ki (μM)	Rabbit Ki (μM)	Liver cholesterol % decrease	Plasma cholesterol % decrease
 SCH 58235	1.59 ± 0.2	0.90 ± 0.2	9.0 ± 1.6	1.53 ± 0.35	0.97 ± 0.28	0.77 ± .16	2.35 ± 0.21	93	43
 SCH 60663	0.66 ± 0.19	0.092 ± 0.01	5.4 ± 1.97	0.37 ± 0.8	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 ± 1.26	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

Figure 1

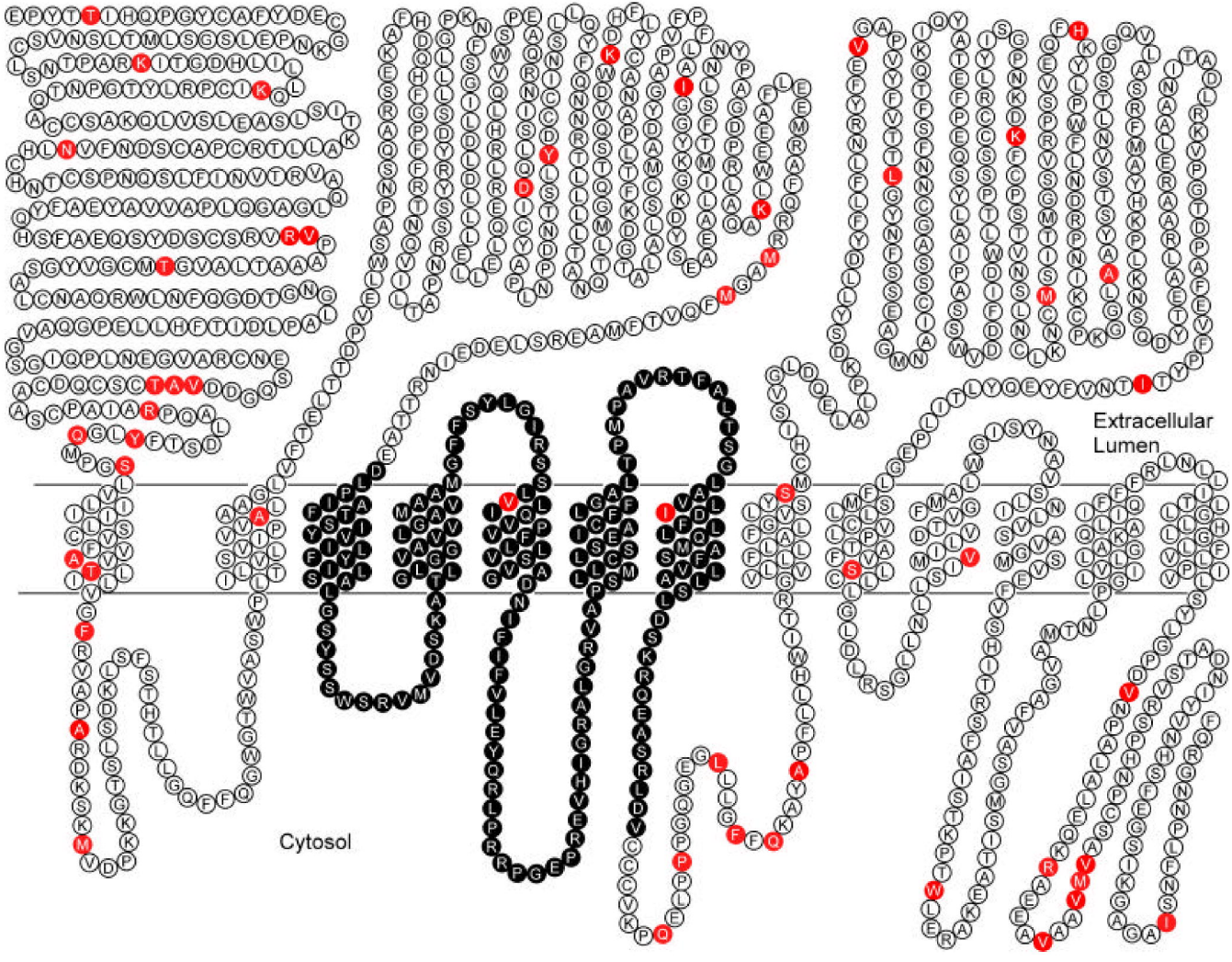


Figure 2

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

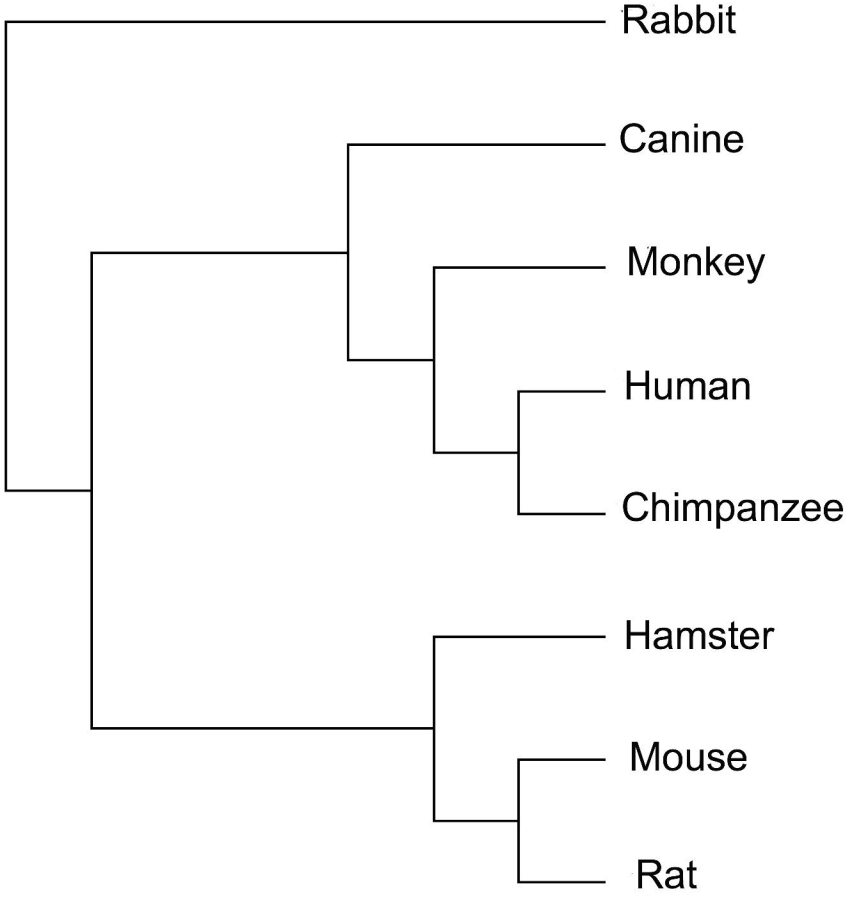


Figure 3

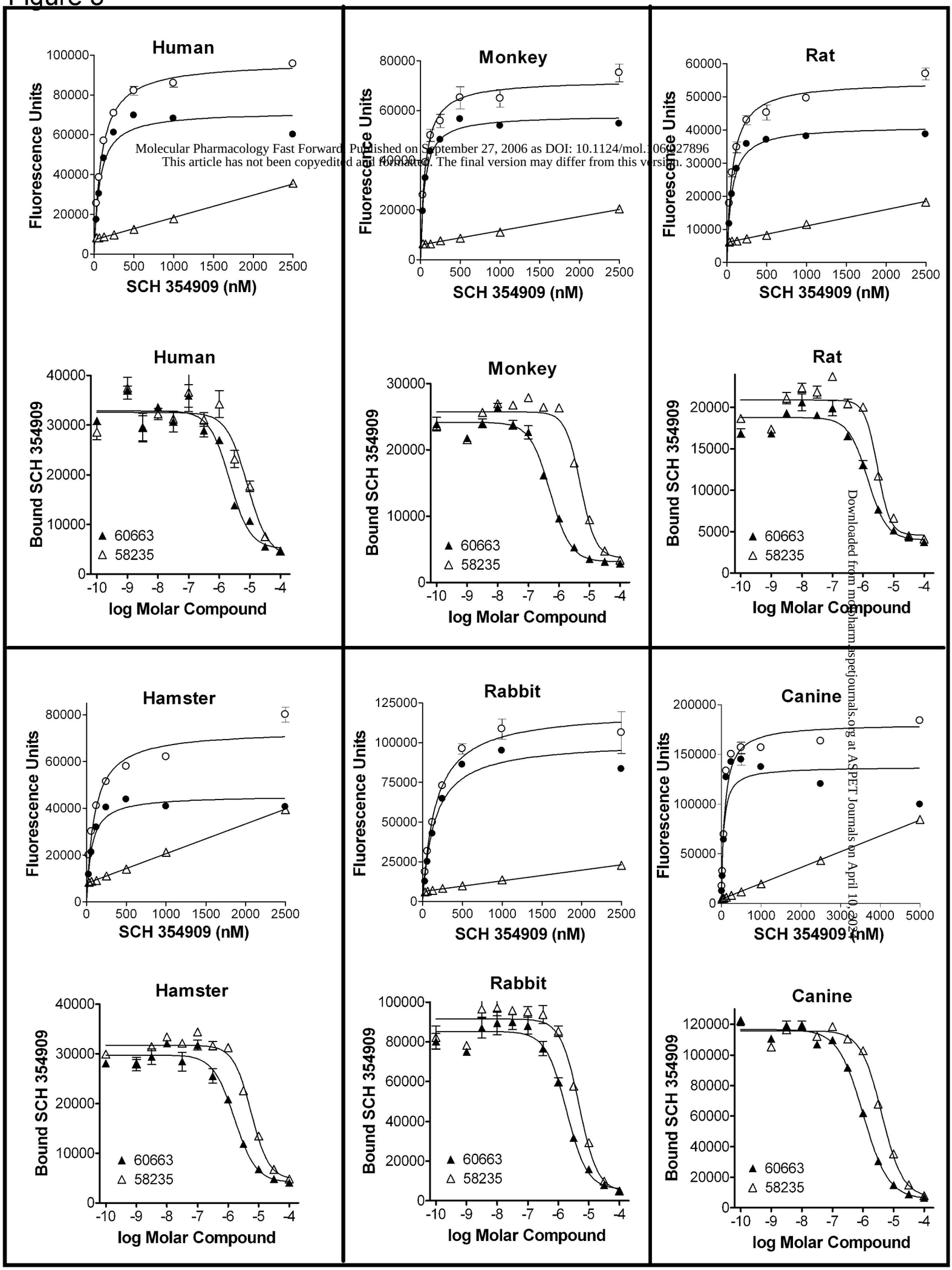


Figure 4

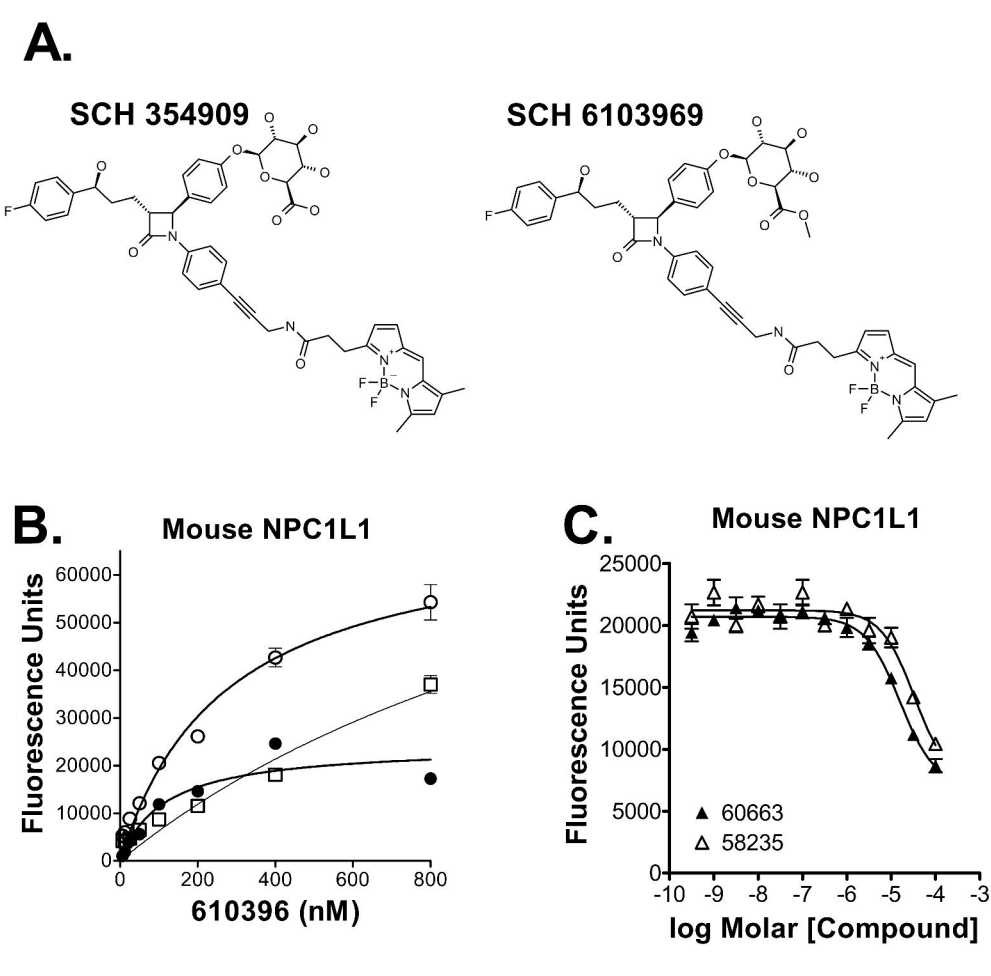
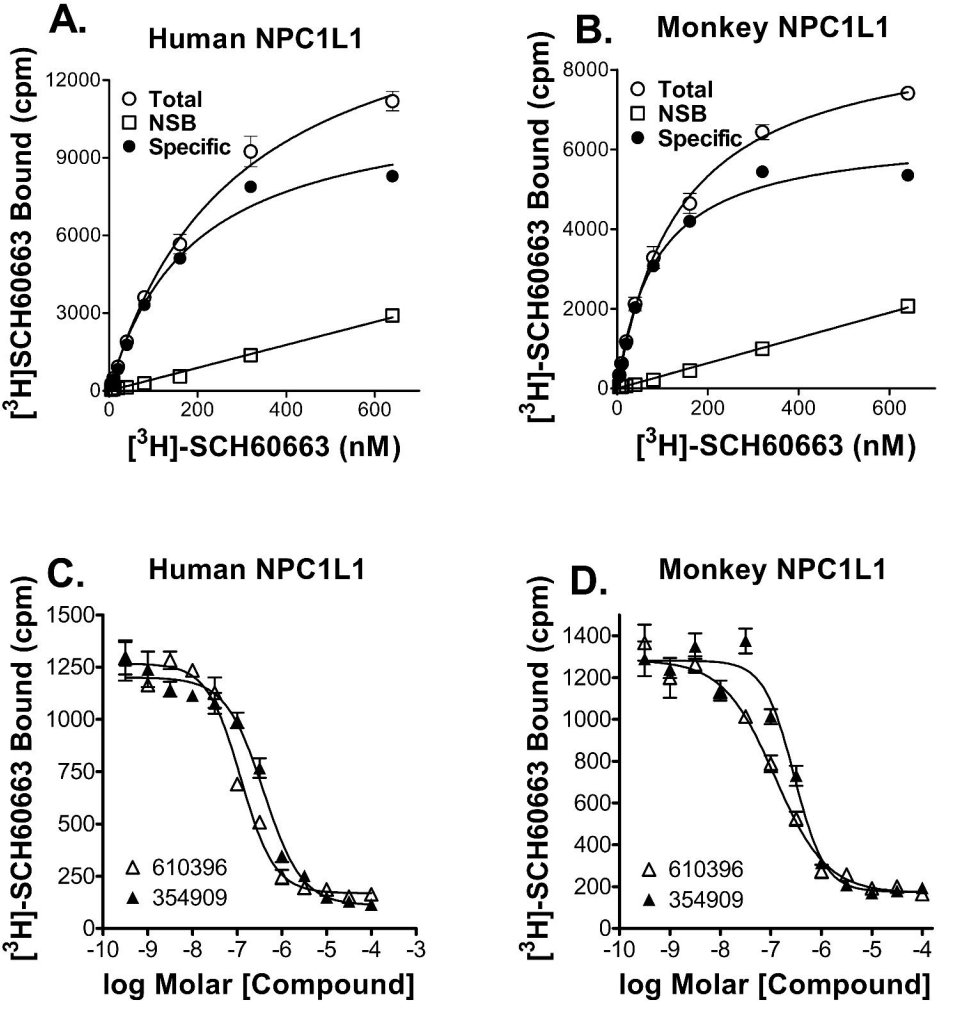

A.
SCH 354909
SCH 6103969
B.
Mouse NPC1L1
Fluorescence Units
610396 (nM)
C.
Mouse NPC1L1
Fluorescence Units
60663
58235
log Molar [Compound]

Figure 5


A.
Human NPC1L1
[3H]SCH60663 Bound (cpm)
12000
9000
6000
3000
0
Total
NSB
Specific
0
200
400
600
[3H]-SCH60663 (nM)
B.
Monkey NPC1L1
[3H]-SCH60663 Bound (cpm)
8000
6000
4000
2000
0
Total
NSB
Specific
0
200
400
600
[3H]-SCH60663 (nM)
C.
Human NPC1L1
[3H]-SCH60663 Bound (cpm)
1500
1250
1000
750
500
250
0
610396
354909
-10
-9
-8
-7
-6
-5
-4
-3
log Molar [Compound]
D.
Monkey NPC1L1
[3H]-SCH60663 Bound (cpm)
1400
1200
1000
800
600
400
200
0
610396
354909
-10
-9
-8
-7
-6
-5
-4
log Molar [Compound]