DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.
DMD Fast Forward. Published on April 28, 2008 as doi:10.1124/dmd.107.019828

ROLE OF ORGANIC ANION TRANSPORTERS IN THE PHARMACOKINETICS OF ZONAMPANEL, AN α-AMINO-3-HYDROXY-5-METHYLISOXAZOLE-4-PROPIONATE RECEPTOR ANTAGONIST, IN RATS

Tsuyoshi Minematsu, Tadashi Hashimoto, Toshiko Aoki, Takashi Usui, Hidetaka Kamimura

Drug Metabolism Research Laboratories, Astellas Pharma Inc. (T.M., T.H., T.U., H.K.); and Astellas Research Technologies Co.,Ltd. (T.A.), Tokyo, Japan

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

Copyright 2008 by the American Society for Pharmacology and Experimental Therapeutics.

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

a) Running title: Organic anion transporters in pharmacokinetics of zonampanel

b) Correspondence

Tsuyoshi Minematsu Ph.D., Drug Metabolism Research Laboratories, Astellas Pharma Inc., 1-8, Azusawa 1-chome, Itabashi-ku, Tokyo 174-8511, Japan. Phone, +81-3-5916-2163. Fax, +81-3-3960-6220. Email, tsuyoshi.minematsu@jp.astellas.com

c) Number of:

text pages: 37

tables: 4

figures: 6

references: 25

words in abstract: 254

words in introduction: 518

words in discussion: 1550

d) List of non-standard abbreviations used in this paper

A_e, cumulative urinary excretion ratio

AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

AUC, area under the time-concentration curve based on plasma concentration

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

C_0, estimated plasma concentration of zonampanel at time zero

$CL_{bile,int,all}$, overall intrinsic clearance of biliary excretion

$CL_{nonrenal,b}$, non-renal clearance based on blood concentration

$CL_{renal,b}$, renal clearance based on blood concentration

$CL_{renal,sr,b}$, renal secretion clearance based on blood concentration

$CL_{renal,sr,int}$, intrinsic renal secretion clearance

$CL_{tot,b}$ total body clearance based on blood concentration

$CL_{tot,p}$, total body clearance based on plasma concentration

CL_{uptake}, uptake clearance in the absence of inhibitor

$CL_{uptake,+inh}$, uptake clearance in the presence of inhibitor

f_p, unbound fraction in plasma

GFR, glomerular filtration rate

HPLC-UV, high performance liquid chromatography with ultraviolet detection

[I], inhibitor concentration

K_i, inhibition constant

K_m, Michaelis-Menten constant

LLOQ, lower limit of quantification

MOPS, 3-[N-morpholino]propanesulfonic acid

Mrp, multi-drug resistance associated protein

OAT, organic anion transporter

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Oatp, organic anion transporting polypeptide

P_{dif}, uptake clearance of nonsaturable component

Q_{renal}, renal blood flow

$Q_{hepatic}$, hepatic blood flow

R_b, blood-to-plasma concentration ratio

[S], substrate concentration in the medium

$t_{1/2}$, half-life in the terminal phase

Tris, tris[hydroxymethyl]aminomethane

V, uptake velocity of the substrate

V_{max}, maximum uptake velocity

V_{ss}, distribution volume in the steady state

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Zonampanel monohydrate (YM872) is a novel α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor antagonist. In humans, almost all administered zonampanel is excreted in the urine unchanged. Further, zonampanel is transported by human organic anion transporter 1 (OAT1), OAT3 and OAT4, but not by OAT2, suggesting the contribution of OATs to renal excretion. In rats also, zonampanel is predominantly eliminated *via* urine but partly also *via* bile as the unchanged form. In this study, the molecular mechanism of the excretion of zonampanel was elucidated using cells expressing rat Oat1, Oat2, and Oat3. Furthermore, zonampanel (15 mg/kg) was given intravenously to rats with or without probenecid (50 mg/kg) or cimetidine (40 mg/kg), and pharmacokinetic parameters were compared. Zonampanel inhibited the uptake of typical substrates by Oat1, Oat2, and Oat3 with inhibition constant (K_i) values of 7.02 to 10.4 μM. A time- and saturable concentration-dependent increase in [^{14}C]zonampanel uptake was observed in these cells (Michaelis-Menten constant (K_m) values: 13.4 to 53.6 μM). Probenecid and cimetidine inhibited [^{14}C]zonampanel uptake by Oats. In *in vivo* experiments, probenecid and cimetidine decreased intrinsic clearance for both the renal secretion and biliary excretion of zonampanel. Considering the tissue distribution and localization of each transporter, these results suggest that, in rats, zonampanel is taken up from the blood into proximal tubular cells *via* Oat1 and Oat3 and, unlike the case in humans, also into hepatocytes *via* Oat2 and Oat3. The inter-species differences in the excretion of zonampanel between rats and humans may thus be explained by those in the substrate selectivity and tissue distribution of OATs.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Zonampanel monohydrate (YM872), [2,3-dioxo-7-(1*H*-imidazol-1-yl)-6-nitro-1,2,3,4-tetrahydro-1-quinoxalinyl] acetic acid monohydrate (Fig. 1), is a selective antagonist of the glutamate receptor subtype, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor and has been expected as a drug for the treatment of cerebral vascular disorders such as cerebral ischemia. Intravenous infusion of zonampanel has been shown to reduce the volume of ischemic damage in rats (Kawasaki-Yatsugi et al., 1998) and cats (Takahashi et al., 1998).

The major elimination route of zonampanel is renal excretion in humans (Minematsu et al., 2005). Potential mechanisms by which intravenously administered zonampanel is excreted unchanged into the urine in humans have been identified. The high renal clearance of this drug, about 30-fold greater than the product of the glomerular filtration rate (GFR), and the unbound fraction in plasma (f_p) suggests that renal tubular secretion contributes to excretion in the urine (Minematsu et al., 2005). Moreover, zonampanel, which exists as an anion under physiological conditions (pH around 7.0 to 7.4), is transported by human organic anion transporter 1 (OAT1) and OAT3, but not by OAT2 (Hashimoto et al., 2004). A recent review article described the expression of transporters in the human proximal renal tubule, including OAT1 and OAT3 on the basolateral membrane (Shitara et al., 2005). Human OAT1 and OAT3 on the basolateral membrane transport zonampanel from the blood into renal proximal tubular cells. Renal tubular secretion is accomplished by membrane transport in two steps: 1. uptake from blood through the basolateral membrane to the proximal tubular cells; and 2. excretion from cells to the tubular lumen

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

through the apical membrane. It is therefore likely that zonampanel is rapidly secreted from the blood into the urine *via* OAT1 and OAT3.

Although renal excretion is also the major elimination route of zonampanel in animal species, inter-species differences in the renal/non-renal excretion ratio have been observed. After intravenous administration of [^{14}C]zonampanel to humans, urinary excretion of radioactivity and unchanged zonampanel accounted for 94.9% and 90.6% of the dose, respectively, whereas fecal excretion of radioactivity was only 0.5% (Minematsu et al., 2005). After intravenous administration of [^{14}C]zonampanel to rats, in contrast, about 30% of administered radioactivity was excreted in the feces and no metabolite was found in the bile (unpublished data), indicating that about 30% of administered zonampanel is excreted unchanged *via* bile into the feces in rats. In addition, urinary excretion of radioactivity and unchanged zonampanel were 75.6% and 71.5% of the dose, respectively (Sohda et al., 2004). The molecular mechanisms of the excretion of zonampanel in rats as well as the reasons for the inter-species differences remain unknown.

Here, we investigated the interaction of zonampanel with rat Oats using cell lines stably expressing these transporters. Transport of zonampanel was also investigated using rat organic anion transporting polypeptide 1 (oatp1)-, oatp2- and oatp4-expessing Xenopus laevis oocytes, and multi-drug resistance associated protein 2 (Mrp2)-expressing membrane vesicles. We also evaluated the effects of organic anion transporter inhibitors on the pharmacokinetics of zonampanel in rats using the inhibitors probenecid and cimetidine (Burckhardt and Burckhardt, 2003). While

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

cimetidine is a well-known inhibitor of organic cation transporters, it is also known as an Oat3 inhibitor (Burckhardt and Burckhardt, 2003).

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Materials.

Zonampanel monohydrate (molecular weight 349.26) and its internal standard, YM-53362, 3-[7-(1*H*-imidazol-1-yl)-6-nitro-2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-1-yl] propionic acid monohydrochloride 0.7 hydrate, were synthesized at Astellas Pharma Inc. [^{14}C]zonampanel (specific radioactivity 3.50 MBq/mg; radiochemical purity 99.2%) was synthesized by Amersham Pharmacia Biotech (Buckinghamshire, UK). [^{3}H]*p*-aminohippuric acid, [^{14}C]salicylic acid [^{3}H]estrone-3-sulfate, [^{3}H]estradiol 17β-D-glucuronide and [^{3}H]taurocholic acid (138.8, 1.74, 2120, 1735 and 185 GBq/mmol, respectively) were purchased from Perkin-Elmer (Boston, MA). All other chemicals and reagents used were commercially available and of guaranteed purity. Dose levels and concentrations of zonampanel were expressed as zonampanel monohydrate. Water-injected and rat oatp1, oatp2 and oatp4-expressing Xenopus laevis oocytes were purchased from BD Gentest (Woburn, MA, USA). Membrane vesicles prepared from rat Mrp2-expressing and mock Sf9 insect cells were purchased from Genomembrane (Kanagawa, Japan).

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

Establishment of Transfectants and Cell Culture.

Construction of stable transfectants expressing rat Oat1, Oat2 and Oat3 was carried out as follows. The full coding region of rat Oat1, Oat2 and Oat3 was amplified from rat liver or kidney cDNA by reverse transcription-polymerase chain reaction following the reported sequences given by accession numbers NM_017224, AB017446, and NM_053537, respectively. Full-length rat Oat1,

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Oat2 and Oat3 were subcloned into mammalian expression vector pcDNA3.1/zeo(+) (Invitrogen, Carlsbad, CA). The vector constructs of rat Oat1 and Oat3, as well as the empty vector were introduced into parental HEK293 cells (rat Oat1-HEK293, rat Oat3-HEK293, and mock-HEK293, respectively) with Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer's protocol. Stably transfected cells were selected by adding zeocin (Invitrogen) to the culture medium. Rat Oat1-HEK293, rat Oat3-HEK293, and mock-HEK293 were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml zeocin at 37 °C with 5% CO_2 under humidified conditions on the bottom of a dish. When the vector construct of rat Oat2 was introduced into parental HEK293 cells, no rat Oat2 function (the uptake of [^{3}H]salicylic acid) was observed. Therefore, the vector construct of rat Oat2 and the empty vector were subsequently introduced into parental LLC-PK1 cells (rat Oat2-LLC-PK1 and mock-LLC-PK1, respectively) with Lipofectamine 2000 transfection reagent. Stably transfected cells were selected by adding zeocin to the culture medium. Rat Oat2-LLC-PK1 and mock-LLC-PK1 were grown in medium 199 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/ml zeocin at 37 °C with 5% CO_2 under humidified conditions on the bottom of a dish. The cells were seeded in poly-D-lysine-coated 12-well plates (BD Biosciences, Franklin Lakes, NJ) at a density of 2.0×10^5 cells/well. Cell culture medium was replaced with culture medium supplemented with approximately 5 mM sodium butyrate one day before transport studies to induce the expression of proteins.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Transport Studies Using Rat Oat-expressing Cells.

Uptake was initiated by adding Dulbecco's phosphate-buffered saline (DPBS, Sigma, St Louis, MO) containing a radiolabeled ligand at a designated concentration after cells had been washed twice and preincubated with DPBS. The DPBS consisted of 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.10 mM Na_2HPO_4, 0.904 mM $CaCl_2$, and 0.492 mM $MgCl_2$ (pH 7.4). Uptake was terminated at the designated time by the addition of ice-cold DPBS after removal of the incubation buffer. Cells were then washed with 1 ml of ice-cold DPBS. To determine the uptake of radiolabeled ligands, cells were dissolved in 500 µl of 1 M NaOH and, after cell lysis, neutralized with 250 µl of 2 M HCl. Aliquots (600 µl) were transferred to vials and the scintillation cocktail Hionic-Fluor (Perkin-Elmer) was added. Radioactivity was measured using liquid scintillation counters Tricarb-2900 and -3100 (Perkin-Elmer). Aliquots (40 µl) were used to determine protein concentrations by the method of Lowry using a DC protein assay kit (Biorad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

Transport Studies Using Rat Oatp1, Oatp2 and Oatp4-expressing Oocytes.

Upon receipt, purchased oocytes were kept at 16°C until use in the transport studies on the same day. The uptake experiment was performed at room temperature and initiated by pipetting oocytes into 2 ml of Na^+ buffer containing a radiolabeled ligand at a designated concentration after oocytes had been washed with the buffer . The Na^+ buffer consisted of 100 mM NaCl, 2 mM KCl,

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4). Uptake was terminated at the designated time by pipetting the oocytes into ice-cold Na^+ buffer (2 ml). Oocytes were then washed by transferring the oocytes into 2 ml of fresh ice-cold Na^+ buffer (repeated 3 times). To determine the uptake of radiolabeled ligands, each oocyte was transferred to a scintillation vial and dissolved using 10%(w/v) sodium dodecyl sulfate solution. For water-injected and transporter-expressing oocytes, 10 and 15 oocytes, respectively, were used. For the uptake of [^{14}C]zonampanel, five of oocytes were pooled to obtain a radioactivity level above the lower limit of detection (consequently two and three samples of pooled oocytes for water-injected and transporter-expressing oocytes, respectively, were prepared). Subsequently, Hionic-Fluor scintillation cocktail was added to vials, and the radioactivity was measured using liquid scintillation counters Tricarb-3100.

Vesicular Transport Assays Using Rat Mrp2-expressing Membrane Vesicles.

Aliquots (65 µl) of reaction mixture (50 mM 3-[N-morpholino]propanesulfonic acid (MOPS), 21 mM tris[hydroxymethyl]aminomethane (Tris), 70 mM KCl, 12.1 mM $MgCl_2$, 2.3 mM glutathione and either 4.62 mM ATP or AMP, pH 7.0) containing radiolabeled compound was pre-warmed for 5 min at 37 ºC. Uptake was initiated by adding 10 µl of membrane vesicle solution (5 mg protein/ml in 50 mM Tris-HCl, 50 mM mannitol, 2 mM ethylene glycol bis(beta-aminoethylether)-N,N,N,N-tetraacetic acid, 8 µg/ml aprotinin, 10 µg/ml leupeptin, and 2 mM dithiothreitol) to the reaction mixture. After incubation at 37 ºC for designated time, the

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

uptake was terminated by diluting the reaction mixture with 1 ml of ice-cold washing buffer (40 mM MOPS, 17 mM Tris, and 70 mM KCl, pH 7.0). Diluted samples were rapidly filtered through 0.7-μm-pore glass microfibre filter [GF/F, Whatman, Maidstone, UK; pre-soaked in saline containing 10% (w/v) bovine serum albumin], followed by two washings with 5 ml of ice-cold washing buffer. Subsequently, the radioactivity on the filter was measured by liquid scintillation counting using Tricarb-3100TR, after adding the liquid scintillation cocktail Filtercount (Perkin-Elmer) to the filter. The "sidedness" (inside-out / right side-out ratio) of the membrane vesicles was not determined (ATP-dependent uptake can only occur in inside-out vesicles).

Kinetic Analysis.

Model fitting was performed by WinNonlin (Pharsight Corporation, Mountain View, CA) using of the Gauss-Newton algorithms with Levenberg and Hartley modification. Kinetic parameters were obtained by simultaneous fitting of the data in transporter-expressing cells and mock cells with the weighting factor $1/y^2$ to the following equations: $V = V_{max} \cdot [S]/ (K_m + [S]) + P_{dif} \cdot [S]$ for transporter-expressing cells; $V = P_{dif} \cdot [S]$ for mock cells, where V is uptake velocity of the substrate (pmol/min/mg protein); [S] is substrate concentration in the medium (μM); K_m is the Michaelis-Menten constant (μM); V_{max} is maximum uptake velocity (pmol/min/mg protein); and P_{dif} is uptake clearance of the nonsaturable component (μl/min/mg protein). Inhibition constant (K_i) values were calculated by simultaneous fitting of the data in transporter-expressing cells and mock cells without weighting factor to the following equations, assuming competitive inhibition:

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

$CL_{uptake,+inh} = CL_{uptake}/(1 + [I]/K_i) + P_{dif}$ for transporter-expressing cells; and $CL_{uptake,+inh}$ (= $CL_{uptake,}$) = P_{dif} for mock cells, where CL_{uptake} is uptake clearance in the absence of inhibitor and $CL_{uptake,+inh}$ is that in the presence of inhibitor; and [I] is the inhibitor concentration. Substrate concentration was low compared with K_m value in the inhibition study.

***In Vivo* Study in Rats.**

Seven-week-old male Sprague-Dawley rats were purchased from Japan SLC (Hamamatsu, Japan) and used at eight weeks after acclimatization for at least one week. During acclimatization, the rats were kept in an air-conditioned room with temperature and humidity controlled at 22.9 to 23.3 °C and 50% to 78%, and lit for 12 h 30 min from 7:30 to 20:00. They were given free access to solid food (CE-2; CLEA Japan Inc; Tokyo, Japan) and water until just before drug administration. This animal experiment was approved by the Animal Care Committee of Astellas Pharma Inc. Zonampanel (15 mg/kg) was administered at a single bolus dose into the rat tail vein with or without probenecid (50 mg/kg) or cimetidine (40 mg/kg). At 5, 15, and 30 min, and 1, 2, 3, 4, and 6 h after dosing, blood was sampled under ether anesthesia *via* the inferior vena cava using a heparinized syringe and immediately stored on ice using 4 rats each per sampling time point per administration group (total 128 rats for 8 sampling time points and 4 administration groups). Plasma was obtained by centrifugation at 4 °C, 1,870 × *g* for 15 min and kept frozen at –20 °C until assay as described below. Rats for plasma sampling at 3 and 6 h after administration were housed in metabolic cages after administration, and spontaneously excreted urine was

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

collected and the cages were washed using water. Regarding specific gravity as 1, urine volume (including the cage-wash water) was calculated according to differences in the weight of the sampling tube before and after sampling. Urine samples were kept frozen at –20 °C until assay as described below. All plasma and urine samples were protected from light throughout the sampling, storage and assay procedures.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

Analytical Methods.

Determination of plasma zonampanel concentrations was conducted using a validated high performance liquid chromatography assay with ultraviolet detection (HPLC-UV) (Noguchi et al., 2008). Briefly, 0.1 ml portions of internal standard solution (10 µg/ml YM-53362 solution in 0.01 M HCl) were added to 1 ml of plasma, followed by the addition of 0.1 M tris-HCl buffer (pH 7). The mixture was vortexed for about 5 s and centrifuged at 830 × *g* for 5 min at 4 °C, and the supernatant was applied to a Sep-Pak cartridge (Waters, Milford, MA) preconditioned using methanol and water. After the cartridge was washed twice using 2.5 ml each of 0.1 M tris-HCl buffer (pH 7), zonampanel and internal standard were eluted using 75% methanol. After addition of 0.1 ml of 0.5 M HCl, the eluate was vortexed for about 5 sec. Then, after addition of 6 ml of tri-n-butyl phosphate with 5% water, the sample was shaken for 10 min (200 stroke/min) and centrifuged at 830×*g* for 5 min at 4°C, and the organic phase was aspirated off (performed twice. An aliquot (0.1 ml) of aqueous phase was injected into the HPLC system. Chromatographic separation was done with a TSKgel ODS-80Ts 4.6 mm I.D. × 250 mm (Tosoh Corporation, Tokyo,

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Japan) and a TSKguardgel ODS-80Ts 3.2 mm I.D. × 15 mm guard column (Tosoh Corporation) at 35 °C. The mobile phase consisted of 0.5 M phosphoric acid, acetonitrile and water (100:20:880, v/v/v), pumped at a rate of 1.0 ml/min. The detection absorbance was set at 333 nm, and calibration ranged from 10 to 5000 ng/ml. Accuracy and precision at concentrations including the lower limit of quantification (LLOQ) were 0.78% to 5.36% and 0.70% to 4.51%, respectively. When the concentration of a sample was expected to exceed the upper limit of quantification, the sample was diluted using blank plasma. For the calculation of means and standard deviations, measured concentrations below the LLOQ (10 ng/ml) were regarded as 0.00 ng/ml.

Determination of urinary zonampanel concentrations was conducted using a validated HPLC-UV method, which was modified from the method for plasma. Briefly, 0.1-ml portions of internal standard solution (10 μg/ml YM-53362 solution in 0.01 M HCl) were added to 0.2 ml of the urine sample diluted 100-fold with water. After mixing, 0.1 ml of aqueous phase was injected into the HPLC system under the same conditions as above. Calibration ranged from 10 to 5000 μg/ml. Accuracy and precision at concentrations including the LLOQ were –2.37% to –1.00% and 0.62% to 2.36%, respectively. Cumulative urinary excretion ratio of zonampanel (A_e, % of dose) was calculated using dose, urine volume and urinary concentration.

Pharmacokinetic Analysis.

Time profiles of the mean plasma concentration of zonampanel were applied to a non-compartmental model (linear trapezoidal method) using the WinNonlin program and the

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

following parameters were calculated: estimated plasma concentration of zonampanel at time zero (C_0), area under the time-concentration curve based on plasma concentration (AUC), half-life at the terminal phase ($t_{1/2}$), distribution volume at the steady state (V_{ss}), and total body clearance based on plasma concentration ($CL_{tot,p}$). Using calculated values of these parameters, and the hypotheses and equations (3) to (8) below, the following pharmacokinetic parameters were calculated: total body clearance based on blood concentration ($CL_{tot,b}$), renal clearance based on blood concentration ($CL_{renal,b}$), non-renal clearance based on blood concentration ($CL_{nonrenal,b}$), renal secretion clearance based on blood concentration ($CL_{renal,sr,b}$), intrinsic renal secretion clearance ($CL_{renal,sr,nt}$), and overall intrinsic clearance of biliary excretion ($CL_{bile,int,all}$).

Hypothesis for renal clearance

1. It was hypothesized that resorption is negligible since $CL_{renal,b}$ is much larger than $(f_p/R_b) \cdot GFR$.
2. It has been reported that the parallel tube model is more suitable for the description of renal tubular secretion than the well-stirred model (Janku, 1993; Janku and Zvara, 1993). It was thus hypothesized that $CL_{renal,sr,b}$ can be described according to the parallel tube model using the following equation (1):

$$CL_{renal,sr,b} = (Q_{renal} - (f_p/R_b) \cdot GFR) \cdot [1 - \exp\{-(f_p/R_b) \cdot CL_{renal,sr,int} / (Q_{renal} - GFR)\}] \quad (1)$$

where R_b is blood-to-plasma concentration ratio and Q_{renal} is renal blood flow.

Hypothesis for biliary excretion clearance

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

1. Nonrenal clearance represents biliary clearance ($CL_{bile,b}$), because zonampanel is hardly metabolized (Sohda et al., 2004).

2. Nonrenal clearance can be described by the following equation (Shitara et al., 2005):

$$CL_{nonrenal,b} = CL_{bile,b} = Q_{hepatic} \cdot (f_p/R_b) \cdot CL_{bile,int,all} / \{Q_{hepatic} + (f_p/R_b) \cdot CL_{bile,int,all}\} \quad (2)$$

where $Q_{hepatic}$ is hepatic blood flow.

<u>Equations for parameters</u>

$$CL_{tot,b} = CL_{tot,p} / R_b \quad (3)$$

$$CL_{renal,b} = (A_{e0\rightarrow 6h} / 100) \cdot CL_{tot,b} \quad (4)$$

$$CL_{nonrenal,b} = CL_{tot,b} - CL_{renal,b} \quad (5)$$

$$CL_{renal,sr,b} = CL_{renal,b} - (f_p/R_b) \cdot GFR \quad (6)$$

Equation (1) gives

$$CL_{renal,sr,int} = -(Q_{renal} - GFR)/(f_p/R_b) \cdot \ln[1 - CL_{renal,sr,b} / \{Q_{renal} - (f_p/R_b) \cdot GFR\}] \quad (7)$$

Equation (2) gives

$$CL_{bile,int,all} = Q_{hepatic} \cdot CL_{nonrenal,b} / \{(f_p/R_b) \cdot (Q_{hepatic} - CL_{nonrenal,b})\} \quad (8)$$

where $A_{e0\rightarrow 6h}$ is urinary excretion of zonampanel from 0 to 6 h after dosing.

Values of R_b, f_p, GFR, Q_{renal}, and $Q_{hepatic}$ were fixed at 0.64 for R_b (unpublished data), 0.125 for f_p (unpublished data), 11.4 ml/min/kg for GFR (Fischer et al., 2000), 40.71 ml/min/kg for Q_{renal} (Delp et al., 1991), and 55.2 ml/min/kg for $Q_{hepatic}$ (Davies and Morris, 1993) for the calculation of parameters $CL_{tot,b}$, $CL_{renal,b}$, $CL_{nonrenal,b}$, $CL_{renal,sr,b}$, $CL_{renal,sr,int}$, and $CL_{bile,int,all}$. Two studies

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

reported that probenecid does not affect GFR in rats (Darling and Morris, 1991; Foote and Halstenson, 1998), while another noted that, given that it has no effect on blood flow in the renal vein in dogs (Barza et al., 1975), probenecid is unlikely to change renal blood flow in rats. Cimetidine did not affect GFR or renal blood flow rate in rats (Foote and Halstenson, 1998; Rothwell NJ et al., 1984). The change in the $CL_{bile,int,all}$ should be carefully considered (the decrease in $CL_{bile,int,all}$ may be underestimated), given probenecid increases blood flow in the portal vein of dogs (Barza et al., 1975).

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Functional Verification of Cells Stably Expressing Rat Oat1, Oat2 and Oat3 using Their Prototypical Substrates and Inhibitors.

Time- and concentration-dependent uptake of [^{3}H]*p*-aminohippuric acid, [^{14}C]salicylic acid, and [^{3}H]estrone-3-sulfate was observed for rat Oat1, Oat2 and Oat3, respectively (data not shown). Respective K_m, V_{max}, and P_{dif} values were 32.5 ± 3.0 μM, 4320 ± 310 pmol/min/mg protein, and 4.21 ± 0.88 μl/min/mg protein for rat Oat1-mediated uptake of [^{3}H]*p*-aminohippuric acid (1 min); 122 ± 23 μM, 2120 ± 370 pmol/min/mg protein, and 3.86 ± 0.46 μl/min/mg protein for rat Oat2-mediated uptake of [^{14}C]salicylic acid (2 min); and 7.13 ± 0.92 μM, 642 ± 67 pmol/min/mg protein, and 5.80 ± 0.96 μl/min/mg protein for rat Oat3-mediated uptake of [^{3}H]estrone-3-sulfate (1 min) (estimate ± standard error). The K_i values of probenecid on rat Oat1-mediated uptake of [^{3}H]*p*-aminohippuric acid (1 μM, 1 min), rat Oat2-mediated uptake of [^{14}C]salicylic acid (5 μM, 2 min), and rat Oat3-mediated uptake of [^{3}H]estrone-3-sulfate (1 μM, 1 min) were 6.16 ± 0.96, 372 ± 69, and 0.920 ± 0.053 μM, respectively, and those of cimetidine were >1000, >1000, and 33.7 ± 12.8 μM, respectively (estimate ± standard error). In this study, about 25% of the rat Oat2-mediated uptake activity for [^{14}C]salicylic acid remained in the presence of 1 mM probenecid, which was comparable with the results of the previous study (Morita et al., 2001). Values for the kinetic parameters K_m and K_i were comparable with those in the literature (Burckhardt and Burckhardt, 2003). Therefore, functional verification of cells stably expressing rat Oat1, Oat2, and Oat3 using their prototypical substrates and inhibitors was successfully performed.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Inhibitory Effects of Zonampanel on Rat Oat1, Oat2 and Oat3-mediated Uptake of Their Prototypical Substrates.

Zonampanel inhibited rat Oat1, Oat2 and Oat3-mediated uptake of [^{3}H]*p*-aminohippuric acid, [^{14}C]salicylic acid, and [^{3}H]estrone-3-sulfate, respectively, in a concentration-dependent manner (Fig. 2, Table 1). Estimated K_i values were similar among rat Oats, ranging from 7.02 to 10.4 μM.

Zonampanel Uptake Mediated by Rat Oat1, Oat2 and Oat3.

Uptake of [^{14}C]zonampanel by the three tested transporters, rat Oat1, Oat2 and Oat3, was time- and concentration-dependent (Fig. 3). The Eadie-Hofstee plots indicated there was one saturable component. Kinetic parameters are listed in Table 2. Estimated K_m values were similar among rat Oats, ranging from 13.4 to 53.6 μM, and relatively comparable with the K_i values (Tables 1 and 2).

Inhibitory Effects of Probenecid and Cimetidine on Rat Oat1, Oat2 and Oat3-mediated Uptake of Zonampanel.

Probenecid inhibited rat Oat1- and Oat3-mediated uptake of [^{14}C]zonampanel, but had only a weak inhibitory effect on rat Oat2-mediated uptake (Fig. 4, Table 3). In contrast to the results for rat Oat2-mediated uptake of [^{14}C]salicylic acid, more than 50% of the activity remained for

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Oat2-mediated transport of [^{14}C]zonampanel in the presence of probenecid (1 mM) (Fig. 4) (concentration of zonampanle was 2 μM, which was less than its Km value 13.4 μM). This difference in the inhibitory effects of probenecid indicates that there may be substrate differences. Cimetidine inhibited rat Oat3-mediated uptake of [^{14}C]zonampanel with K_i value of 8.74 μM, but had only weak inhibitory effects on rat Oat1- and Oat2-mediated uptake (Fig. 4, Table 3)

Zonampanel Uptake Mediated by Rat Oatp1,Oatp2, Oatp4, and Mrp2.

Marked uptake of [^{14}C]zonampanel (10 μM) by rat oatp1, oatp2, oatp4 and Mrp2 (Fig. 5B and 5C) was not observed, but marked transporter-mediated transport was observed for the prototypical substrates of each transporters ([^{3}H]estrone-3-sulfate for oatp1 and oatp4, [^{3}H]taurocholic acid for oatp2, and [^{3}H]estradiol 17β-D-glucuronide for Mrp2) (Figs. 5A and 5D). In addition, zonampanel (1 mM) did not affect the Mrp2-mediated transport of [^{3}H]estradiol 17β-D-glucuronide. The uptake of [^{14}C]zonampanel into oatp4-expressing oocytes was slightly higher (about 1.8-fold) than those into water-injected oocytes.

Effects of Probenecid and Cimetidine on the Pharmacokinetics of Zonampanel in Rats

Time profiles of plasma zonampanel concentrations after intravenous administration of zonampanel to rats with or without probenecid or cimetidine are shown in Fig. 6. Concentrations were constantly higher at all sampling points in the inhibitor-coadministered group than in the control group. Zonampanel concentration decreased below the LLOQ (10 ng/ml) from 4 h.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Pharmacokinetic parameters are listed in Table 4. Urinary excretion was almost completed within 3 h, and no significant effect of probenecid or cimetidine was seen on A_e at 6 h after administration (Student's unpaired t-test). Compared with the control group, V_{ss}, $CL_{tot,b}$, $CL_{renal,b}$, $CL_{nonrenal,b}$, $CL_{renal,sr,int}$, and $CL_{bile,int,all}$ were markedly reduced in the inhibitor-coadministered group (Table 4).

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Although zonampanel is metabolized only slightly in both rats and humans (Sohda et al., 2004; Minematsu et al., 2005), fecal excretion of [^{14}C]zonampanel is much greater in rats (30% of dose, unpublished data) than in humans (0.5% of dose) (Minematsu et al., 2005). Here, we investigated the molecular mechanisms of zonampanel excretion in rats and the reason for the inter-species differences in excretion between rats and humans using cell lines stably expressing rat Oat1, Oat2, and Oat3. In addition, we also tested the effects of the organic anion transporter inhibitors probenecid and cimetidine on the pharmacokinetics of zonampanel in rats. Species-differences in the substrate selectivity and distribution/localization of OATs well explained the difference in the excretion.

Zonampanel is a substrate of rat Oat1, Oat2, and Oat3 (Fig. 3), and human OAT1 and OAT3, but is not transported by human OAT2 (Hashimoto et al., 2004). Expression levels of OAT1 mRNA in the liver are much lower than those in the kidney in both rats and humans (Race et al., 1999; Buist et al., 2002). In both species, OAT2 is expressed on the basolatelaral membranes of the hepatocytes (Shitara et al., 2005). In contrast, although the mRNA of OAT3 is expressed in rat liver (Kusuhara et al., 1999; Buist et al., 2002), it is not detected in human liver (Race et al., 1999). In rats, OAT2 and OAT3 may accelerate the uptake of zonampanel from the blood into the liver, resulting in greater fecal excretion *via* bile than in humans. Regarding renal excretion, a recent review article described the expression of OAT1 and OAT3 on the basolateral membrane of rat and human proximal renal tubule (Shitara et al., 2005). It is therefore likely that zonampanel is

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

rapidly secreted from blood into the urine *via* OAT1 and OAT3 in both rats and humans. OAT2 is localized in different regions of the kidney in rats and humans. For rats, it is found on the apical membrane of the tubules in the medullary thick ascending limb of Henle's loop and collecting ducts for rats; while for humans, it is found on the basolateral membrane of the tubule for humans (Kojima et al., 2002; Shitara et al., 2005). Therefore, rat Oat2 may mediate zonampanel reabsorption rather than its secretion into the urine.

In this study, after intravenous administration of zonampanel (15 mg/kg) without probenecid or cimetidine to rats, plasma concentration of zonampanel was about 30 μg/ml (86 μM) at the first sampling time (5 min), which rapidly decreased to around 0.1 μg/ml within 120 min. Because the unbound fraction of zonampanel in rat plasma was 0.125, when total plasma concentration of zonampanel is 30 μg/ml, the unbound plasma concentration is 11 μM, which is lower than the K_m values of zonampanel for rat Oat1, Oat2, and Oat3. Similar plasma concentrations were obtained in a preliminary study (unpublished results). In a clinical study, using intravenous constant infusion, the plasma concentration of zonampanel reached 2.93 μg/ml (8.4 μM; 0.84 μM as unbound) as previously reported in our report (Minematsu et al., 2005). Intravenous bolus adminsitration of zonampanel 15 mg/kg to rats can result in this concentration. Therefore, the dose of zonampanel was chosen as 15 mg/kg. While C_0 ranged from 57.8-102 μg/ml (165-291 μM) (Table 4), the unbound concentrations were about 21-36 μM, which are still around the K_m values, suggesting only slight saturation for kinetics just after administration. In this study, zonampanel was excreted unchanged in the urine, accounting for 76.2% to 79.3% of the dose

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

within 6 h in rats (Table 4). In addition, renal clearance of zonampanel was higher than the product of f_p and GFR, indicating that tubular secretion plays an important role in the renal excretion of this drug. In combination with probenecid, renal clearance and intrinsic renal secretion clearance decreased to 33.8% and 17.4%, respectively, compared with the control group (Table 4), suggesting a transporter inhibition-induced decrease in tubular secretion.

Non-renal and intrinsic non-renal clearance of zonampanel in the probenecid-coadministered group also decreased to 36.9% and 32.9%, respectively, of that in the control (Table 4). Probenecid has been reported to inhibit the transporter-mediated biliary excretion of NBQX, 6-nitro-7-sulfamoyl-benzo(f)quinoxaline-2,3-dione, which has a similar chemical structure to zonampanel, in rats (Hansen, 1995). On this basis, probenecid was considered to inhibit transporter-mediated biliary excretion (in this case the uptake into the hepatocytes) of zonampanel. In a rat study, radioactivity after intravenous administration of [^{14}C]zonampanel was mainly distributed in the kidney and liver (unpublished data). The reduced V_{ss} (Table 4) agrees with decreased cellular uptake due to the inhibition of transporters by probenecid in the basolateral membrane of the proximal renal tubule and of hepatocytes. When probenecid was given to rats at 50 mg/kg, unbound plasma concentrations were approximately 100 μg/ml (350 μM) at 2.5 min, > 10 μg/ml (35 μM) at 1 h and > 2 μg/ml (7 μM) at 2 h after dosing (Emanuelsson and Paalzow, 1988), which were higher than the K_i values for probenecid on [^{14}C]zonampanel uptake by rat Oat1 and Oat3 (1.44 and 1.13 μM, respectively, Table 3). Considering the K_i value for the effect of probenecid on the rat Oat2-mediated zonampanel transport (>1000 μM), it is unlikely that

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

probenecid inhibited Oat2 in the *in vivo* experiments. The lack of complete inhibition of the renal and non-renal clearance of zonampanel could also be due to lack of Oat2 inhibition, slight saturation for kinetics just after administration as described above, and/or involvement of the other transporters.

Similar to the case with probenecid, the renal and intrinsic renal secretion clearance of zonampanel in the cimetidine-coadministered group was decreased to 64.9% and 49.6%, respectively, of that in the control group (Table 4), suggesting the inhibition of Oat3. The non-renal and intrinsic non-renal clearance of zonampanel in the cimetidine-coadministered group was also decreased to 57.2% and 53.5%, respectively, of that in the control group (Table 4), also suggesting the inhibition of Oat3. Further, the V_{ss} of zonampanel also decreased in the cimetidine-coadministered group (Table 4). When cimetidine was given to rats at 40 mg/kg, the plasma cimetidine concentration was approximately 20 µg/ml (80 µM) at about 5-10 min, 10 µg/ml (40 µM) at 0.5 h, > 3 µg/ml (12 µM) at 1 h and > 1 µg/ml (4.0 µM) at 2 h after administration, and f_p was 0.78 to 0.83 (5 to 50 µg/ml) in rats (Adedoyin et al., 1987), suggesting an unbound plasma cimetidine concentration of approximately 64 µM at about 5-10 min, 32 µM at 0.5 h, > 9.5 µM at 1 h and > 3.2 µM at 2 h (K_i values for cimetidine on [^{14}C]zonampanel uptake by rat Oat3, 8.74 µM; Table 3). Considering the K_i values for cimetidine on the rat Oat1- and Oat2-mediated zonampanel transport (>1000 µM), it was unlikely that cimetidine inhibited Oat1 and Oat2 in the *in vivo* experiments. Although cimetidine did not completely inhibit renal and non-renal clearance

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

of zonampanel, this may be due to lack of Oat1/Oat2 inhibition, slight saturation for kinetics just after administration as described above, and/or involvement of the other transporters.

In rats, organic anion transporters other than Oats, such as oatp1, oatp2 and oatp4 are localized on the basolateral membrane of hepatocytes: oatp1 is on the apical membrane of the renal tubules, and Mrp2 is on the apical membranes of the hepatocytes and renal tubules (Shitara et al., 2005). The importance of these transporters in the pharmacokinetics of zonampanel may be low, but oatp4 might be partly involved in the uptake of zonampanel into hepatocytes (Fig. 5; any statistical significance in the difference between the uptake into water-injected and oatp4-expressing oocytes could not be tested). A future detailed study may reveal the involvement of oatp4 in more quantitative manner. Because zonampanel is not a good substrate of rat Mrp2, we could not find the transporters involved in the secretion across the apical membrane of hepatocytes and renal tubules. For the accumulation of zonampanel in the liver and kidney, the tissue-to-plasma radioacivity concentration ratio was about 1.6 for the liver and 7 for the kidney after the intravenous administration of [^{14}C]zonampanel to rats (unpublished data; radioactivity represents the unchanged zonampanl, because zonampanel is metabolized only slightly as described above). Therefore, the uptake process on the basolateral membrane is likely dominant to the efflux process on the apical membrane.

In conclusion, zonampanel was transported by rat Oat1, Oat2, and Oat3 in a concentration-dependent manner with K_m values of 13.4, 13.4, and 53.6 μM, respectively. The Oats inhibitor probenecid and Oat3 inhibitor cimetidine decreased the intrinsic clearance for renal

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

secretion and biliary excretion of zonampanel in rats, as calculated using a physiological model. Considering the tissue distribution and localization of each transporter, these results suggest that, in rats, zonampanel is taken up *via* Oat1 and Oat3 from the blood into proximal tubular cells and, unlike in humans, *via* Oat2 and Oat3 from the blood into hepatocytes. The inter-species differences in the excretion of zonampanel between rats and humans may be explained by these differences in the substrate selectivity and tissue distribution/localization of OATs. The approaches considering inter-species-differences in transporters using *in vitro* transporter-expressing systems and *in vivo* chemical inhibition experiments would be useful in a drug development, especially in cases of extrapolation from animals to humans. It should be kept in mind that localization of the transporters requires careful attention when inter-species differences are discussed.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgement

We would like to thank Dr. Ken-ichi Umehara for his technical instruction during the molecular biology experiments.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

References

Adedoyin A, Aarons L and Houston JB (1987) Dose-dependent pharmacokinetics of cimetidine in the rat. *Xenobiotica* **17:**595-604.

Barza M, Brusch J, Bergeron MG, Kemmotsu O and Weinstein L (1975) Extraction of antibiotics from the circulation by liver and kidney: effect of probenecid. *J Infect Dis* **131 Suppl:**S86-97.

Buist SC, Cherrington NJ, Choudhuri S, Hartley DP and Klaassen CD (2002) Gender-specific and developmental influences on the expression of rat organic anion transporters. *J Pharmacol Exp Ther* **301:**145-151.

Burckhardt BC and Burckhardt G (2003) Transport of organic anions across the basolateral membrane of proximal tubule cells. *Rev Physiol Biochem Pharmacol* **146:**95-158.

Darling IM and Morris ME (1991) Evaluation of "true" creatinine clearance in rats reveals extensive renal secretion. *Pharm Res* **8:**1318-1322.

Davies B and Morris T (1993) Physiological parameters in laboratory animals and humans. *Pharm Res* **10:**1093-1095.

Delp MD, Manning RO, Bruckner JV and Armstrong RB (1991) Distribution of cardiac output during diurnal changes of activity in rats. *Am J Physiol* **261:**H1487-1493.

Emanuelsson BM and Paalzow LK (1988) Dose-dependent pharmacokinetics of probenecid in the rat. *Biopharm Drug Dispos* **9:**59-70.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Fischer PA, Bogoliuk CB, Ramirez AJ, Sanchez RA and Masnatta LD (2000) A new procedure for evaluation of renal function without urine collection in rat. *Kidney Int* **58:**1336-1341.

Foote EF and Halstenson CE (1998) Effects of probenecid and cimetidine on renal disposition of ofloxacin in rats. *Antimicrob Agents Chemother* **42:**456-458.

Hansen KT (1995) Hepatic uptake and biliary excretion of the neuroprotectant 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo-(F)-quinoxaline in rat liver. Involvement of an organic anion transport system. *Biochem Pharmacol* **49:**489-493.

Hashimoto T, Narikawa S, Huang XL, Minematsu T, Usui T, Kamimura H and Endou H (2004) Characterization of the renal tubular transport of zonampanel, a novel α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor antagonist, by human organic anion transporters. *Drug Metab Dispos* **32:**1096-1102.

Janku I (1993) Physiological modelling of renal drug clearance. *Eur J Clin Pharmacol* **44:**513-519.

Janku I and Zvara K (1993) Quantitative analysis of drug handling by the kidney using a physiological model of renal drug clearance. *Eur J Clin Pharmacol* **44:**521-524.

Kawasaki-Yatsugi S, Yatsugi S, Takahashi M, Toya T, Ichiki C, Shimizu-Sasamata M, Yamaguchi T and Minematsu K (1998) A novel AMPA receptor antagonist, YM872, reduces infarct size after middle cerebral artery occlusion in rats. *Brain Res* **793:**39-46.

Kojima R, Sekine T, Kawachi M, Cha SH, Suzuki Y, Endou H (2002) Immunolocalization of multispecific organic anion transporters, OAT1, OAT2, and OAT3, in rat kidney. *J Am Soc Nephrol* **13:** 848-857.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Kusuhara H, Sekine T, Utsunomiya-Tate N, Tsuda M, Kojima R, Cha SH, Sugiyama Y, Kanai Y and Endou H (1999) Molecular cloning and characterization of a new multispecific organic anion transporter from rat brain. *J Biol Chem* **274:**13675-13680.

Minematsu T, Sohda KY, Hashimoto T, Imai H, Usui T and Kamimura H (2005) Identification of metabolites of [^{14}C]zonampanel, an α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor antagonist, following intravenous infusion in healthy volunteers. *Xenobiotica* **35:**359-371.

Morita N, Kusuhara H, Sekine T, Endou H (2001) Sugiyama Y, Functional characterization of rat organic anion transporter 2 in LLC-PK1 cells. *J Pharmacol Exp Ther* **298:**1179-1184.

Noguchi K, Miyashita A, Hashimoto T, Aoki T, Soeishi Y and Kamimura H (2008) Solid-phase extraction followed by purification with tri-n-butylphosphate as a simple and useful rat plasma clean-up method for HPLC-UV determination of zonampanel monohydrate (YM872), a highly water-soluble α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor antagonist. *J Liq Chromatogr Related Technol* **31:**1072-1080.

Race JE, Grassl SM, Williams WJ and Holtzman EJ (1999) Molecular cloning and characterization of two novel human renal organic anion transporters (hOAT1 and hOAT3). *Biochem Biophys Res Commun* **255:**508-514.

Rothwell NJ, Stock MJ, Wyllie MG (1984) Effects of histamine antagonists on noradrenaline-stimulated blood flow and oxygen consumption of brown adipose tissue in the rat. *Pfulgers Arch* **402:**325-329.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Shitara Y, Sato H and Sugiyama Y (2005) Evaluation of drug-drug interaction in the hepatobiliary and renal transport of drugs. *Annu Rev Pharmacol Toxicol* **45:**689-723.

Sohda KY, Minematsu T, Hashimoto T, Suzumura K, Funatsu M, Suzuki K, Imai H, Usui T and Kamimura H (2004) Application of LC-NMR for characterization of rat urinary metabolites of zonampanel monohydrate (YM872). *Chem Pharm Bull (Tokyo)* **52:**1322-1325.

Takahashi M, Ni JW, Kawasaki-Yatsugi S, Toya T, Yatsugi SI, Shimizu-Sasamata M, Koshiya K, Shishikura JI, Sakamoto S and Yamaguchi T (1998) YM872, a novel selective alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor antagonist, reduces brain damage after permanent focal cerebral ischemia in cats. *J Pharmacol Exp Ther* **284:**467-473.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

FIG. 1. Chemical structure of zonampanel monohydrate. An asterisk indicates the position of [*U*-ring-^{14}C] radiolabel.

FIG. 2. Inhibitory effects of zonampanel on the rat Oat1- Oat2, and Oat3-mediated uptake of [^{3}H]*p*-aminohippuric acid, [^{14}C]salicylic acid, and [^{3}H]estrone-3-sulfate, respectively. Rat Oat1-, Oat3-, and mock-HEK293 cells, and rat Oat2- and mock-LLC-PK1 cells were incubated in DPBS containing designated concentrations of zonampanel in the presence of [^{3}H]p-aminohippuric acid (1 μM) for Oat1, [^{14}C]salicylic acid (5 μM) for Oat2, and [^{3}H]estrone-3-sulfate (1 μM) for Oat3 at 37 °C for 1, 2, and 1 min, respectively. Data are expressed as the mean ± S.D. of three samples. A broken line represents the best-fit line drawn using the equations described in the text.

FIG. 3. Time profiles and concentration dependency (Eadie-Hofstee plots) for the rat Oat1- Oat2, and Oat3-mediated uptake of [^{14}C]zonampanel. Rat Oat1-, Oat3-, and mock-HEK293 cells, and rat Oat2- and mock-LLC-PK1 cells were incubated in DPBS containing [^{14}C]zonampanel (3 μM for time profiles; various concentrations for concentration dependency) at 37 °C for the designated incubation time (time profiles) and for 5 min (concentration dependency). Data are expressed as the mean ± S.D. of three samples. A broken line represents the best-fit line drawn using the parameters in Table 2.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

FIG. 4. Inhibitory effects of probenecid and cimetidine on the rat Oat1- Oat2-, and Oat3-mediated uptake of [^{14}C]zonampanel. Rat Oat1-, Oat3-, and mock-HEK293 cells, and rat Oat2- and mock-LLC-PK1 cells were incubated in DPBS containing [^{14}C]zonampanel (1 μM for Oat1, 2 μM for Oat2, and 5 μM for Oat3) in the presence or absence of designated concentrations of probenecid or cimetidine at 37 °C for 5 min. Data are expressed as the mean ± S.D. of three samples. A broken line represents the best-fit line drawn using the equations described in the text.

FIG. 5. A. The uptake of prototypical substrates (2 μM; [^{3}H]estrone-3-sulfate for rat oatp1 and oatp4, and [^{3}H]taurocholic acid for rat oatp2) into water-injected, and rat oatp1-, oatp2-, and oatp4-expressing oocytes for 60 min. Data are expressed as the mean ± S.D. of 10 or 15 oocytes. **B.** The uptake of [^{14}C]zonampanel (10 μM) into water-injected oocytes, and rat oatp1, oatp2, and oatp4-expressing oocytes for 60 and 120 min. Data are expressed as the mean (a) or the mean ± S.D. of 2 or 3 samples of pooled oocytes, respectively. **C.** Time courses of the uptake of [^{14}C]zonampanel (10 μM) into membrane vesicles prepared from mock- and rat Mrp2-expressing Sf-9 insect cells in the presence and absence of ATP. Data are expressed as the mean of two samples. **D.** Effects of zonampanel (1 mM) on the 5-min uptake of [^{3}H]estradiol 17β-D-glucuronide (2.2 μM) into membrane vesicles prepared from rat Mrp2-expressing Sf-9 insect cells in the presence and absence of ATP. Data are expressed as the mean of two samples.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

FIG. 6. Time profiles of plasma zonampanel concentrations after intravenous bolus administration of zonampanel (15 mg/kg) with or without probenecid (50 mg/kg) or cimetidine (40 mg/kg) to rats. Data are expressed as the mean ± S.D. of four rats.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 1

K_i values for zonampanel in OAT1, OAT2, and OAT3-mediated uptake of their prototypical substrates in rats and humans

	Rat		Human[b]	
	Substrate	K_i (μM)[a]	Substrate	K_i (μM)
OAT1	*p*-Aminohippuric acid	7.02 ± 0.21	*p*-Aminohippuric acid	1.4
OAT2	Salicylic acid	9.51 ± 2.24	Prostaglandin $F_{2\alpha}$	>1000
OAT3	Estrone-3-sulfate	10.4 ± 2.0	Estrone-3-sulfate	11.8

a: Parameters were calculated from the data in Fig. 2 (estimate ± standard error).

b: Cited from our previous study (Hashimoto et al., 2004).

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 2

Kinetic parameters for the OAT1, OAT2, and OAT3-mediated uptake of [^{14}C]zonampanel in rats and humans.

	Rat				Human[b]
	K_m (μM)[a]	V_{max} (pmol/min/mg protein)[a]	P_{dif} (μl/min/mg protein)[a]	V_{max}/K_m (μl/min/mg protein)	K_m (μM)
OAT1	13.4 ± 1.6	454 ± 46	1.12 ± 0.44	33.9	1.4
OAT2	13.4 ± 1.9	118 ± 14	0.508 ± 0.098	8.81	NT
OAT3	53.6 ± 9.8	226 ± 36	0.467 ± 0.043	4.22	7.7

a: Parameters were calculated from the data in Fig. 3 (estimate ± standard error).

b: Cited from our previous study (Hashimoto et al., 2004).

NT: not transported

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 3

K_i values (μM) for probenecid and cimetidine in OAT1, OAT2, and OAT3-mediated uptake of [^{14}C]zonampanel in rats and humans

	Probenecid		Cimetidine	
	Rat[a]	Human[b]	Rat[a]	Human[b]
OAT1	1.44 ± 0.04	5.1	>1000	ND
OAT2	>1000	ND	>1000	ND
OAT3	1.13 ± 0.12	2.6	8.74 ± 5.62	42.9

a: Parameters were calculated from the data in Fig. 4 (estimate ± standard error).

b: Cited from our previous study (Hashimoto et al., 2004).

ND: not determined

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 4

Pharmacokinetic parameters of zonampanel after intravenous bolus administration of zonampanel (15 mg/kg) with or without probenecid (50 mg/kg) or cimetidine (40 mg/kg) to rats

Parameter		Probenecid			Cimetidine		
		Control group	Coadministered group		Control group	Coadministered group	
$A_{e0\rightarrow 3h}$[a]	% of dose	77.7 ± 2.6	85.5 ± 3.0		67.4 ± 12.1	75.7 ± 4.0	
$A_{e0\rightarrow 6h}$[a]	% of dose	77.8 ± 3.9	76.2 ± 6.0		77.2 ± 3.1	79.3 ± 2.0	
C_0	μg/ml	57.8	95.1	(165%)	73.5	102	(139%)
AUC_{last}	μg·min/ml	584	1691	(290%)	686	1090	(158%)
AUC_{inf}	μg·min/ml	584	1690	(290%)	686	1090	(158%)
$t_{1/2}$	min	13.0	14.1	(108.7%)	16.6	14.1	(84.8%)
V_{ss}	ml/kg	236	151	(63.8%)	211	159	(75.6%)
$CL_{tot,p}$	ml/min/kg	25.7	8.9	(34.5%)	21.9	13.8	(63.2%)
$CL_{tot,b}$	ml/min/kg	40.1	13.9	(34.5%)	34.2	21.6	(63.2%)
$CL_{renal,b}$	ml/min/kg	31.2	10.6	(33.8%)	26.4	17.1	(64.9%)
$CL_{nonrenal,b}$	ml/min/kg	8.92	3.29	(36.9%)	7.79	4.46	(57.2%)
$CL_{renal,sr,b}$	ml/min/kg	29.0	8.3	(28.8%)	24.1	14.9	(61.7%)
$CL_{renal,sr,int}$	ml/min/kg	210	36.6	(17.4%)	148	73.4	(49.6%)
$CL_{bile,int,all}$	ml/min/kg	54.4	17.9	(32.9%)	46.5	24.8	(53.5%)

a: mean ± S.D. of four individual rats

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Values in parentheses represent the ratio to the respective control group.

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

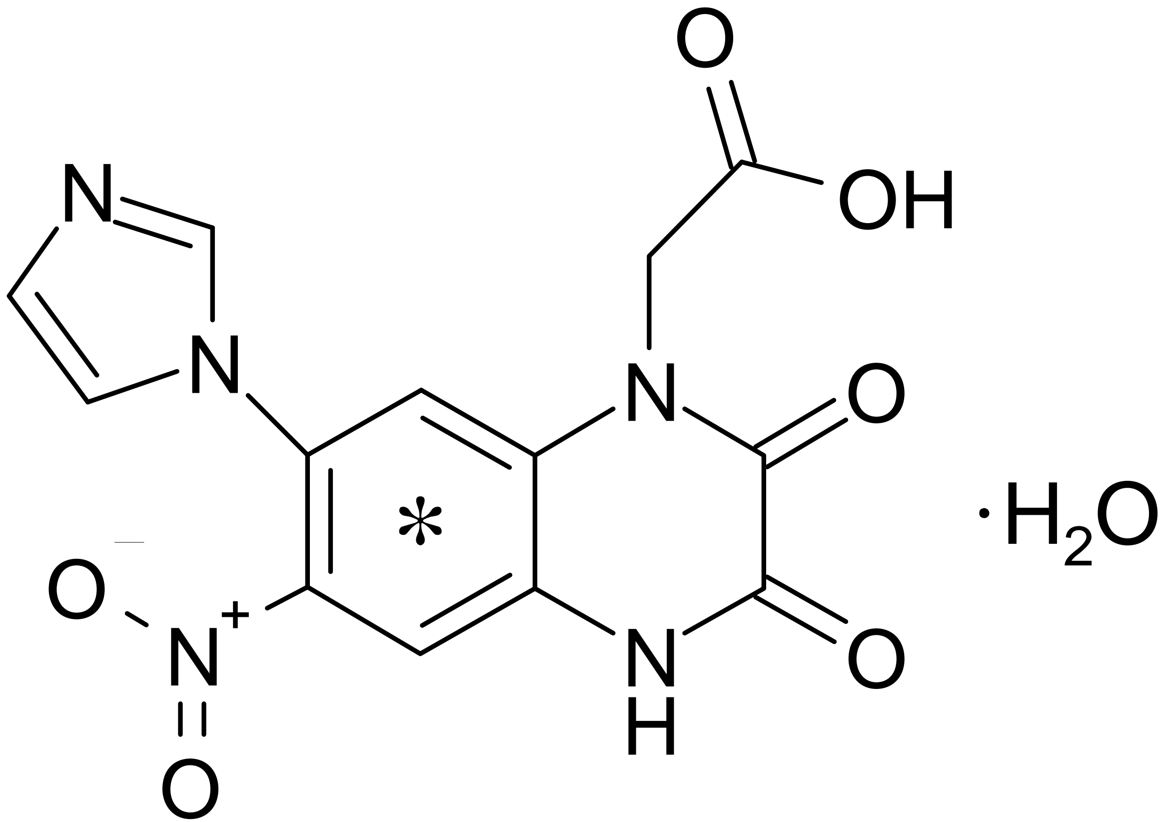
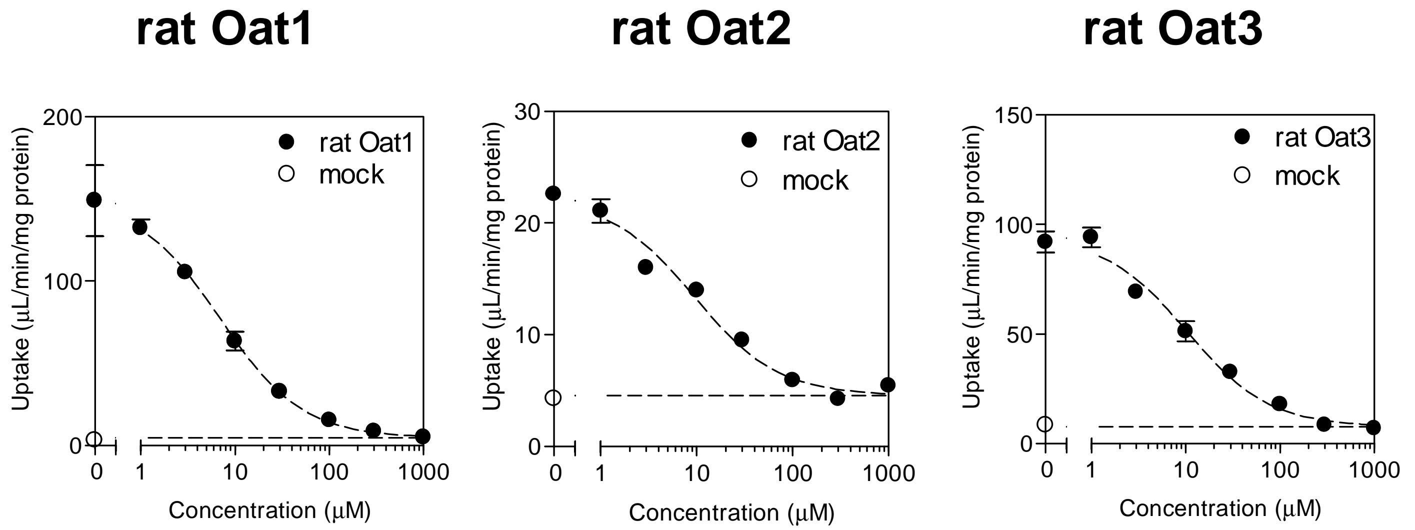


Fig. 1

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

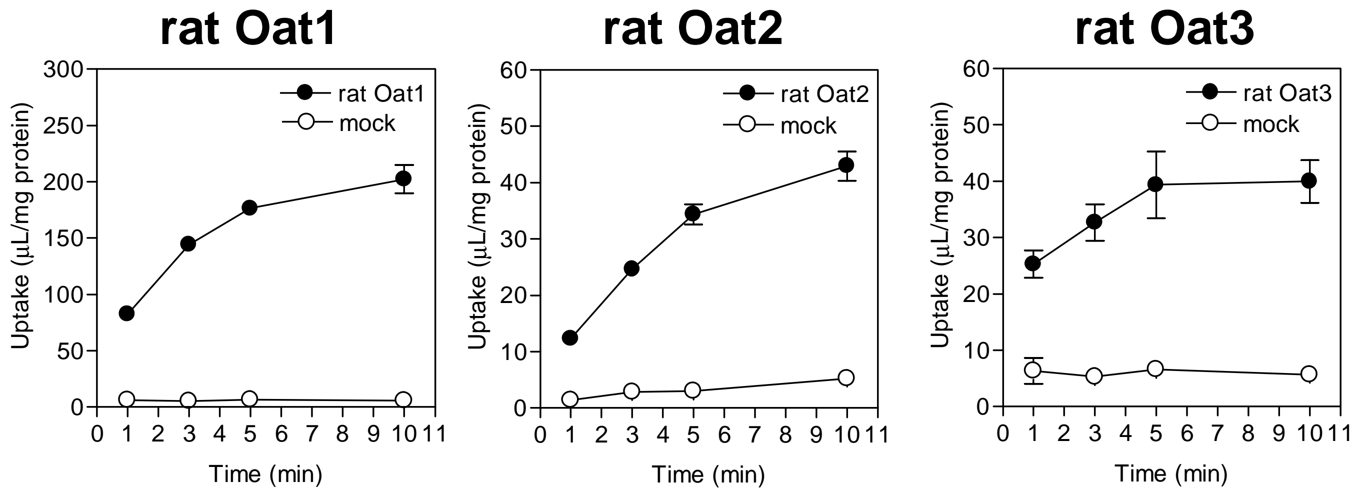


Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

Fig. 2

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

Time courses



Eadie-Hofstee Plots

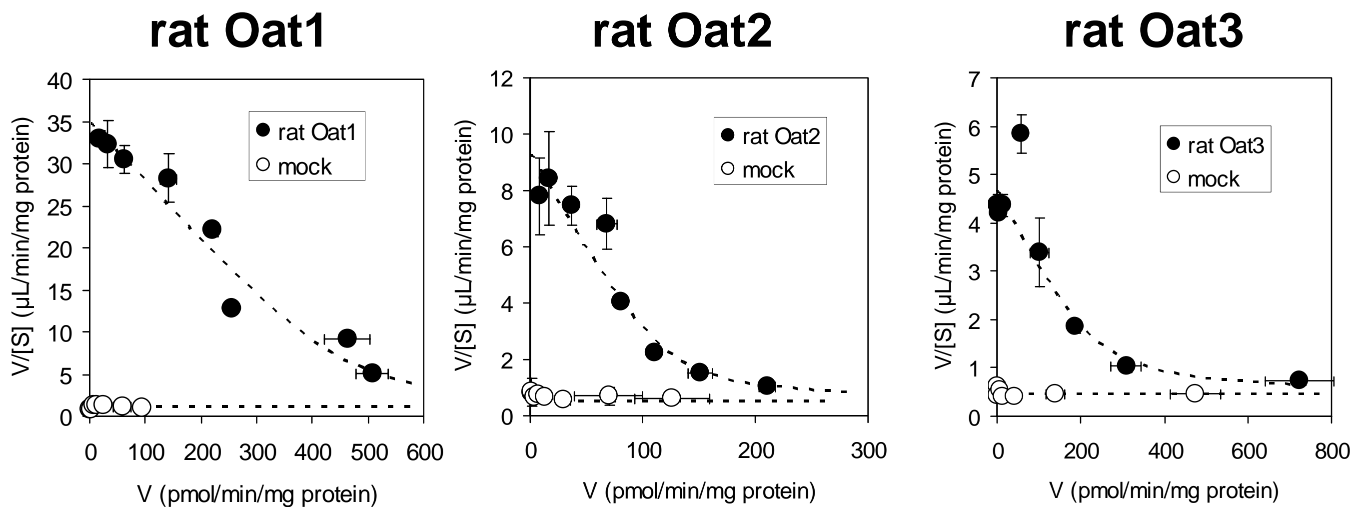
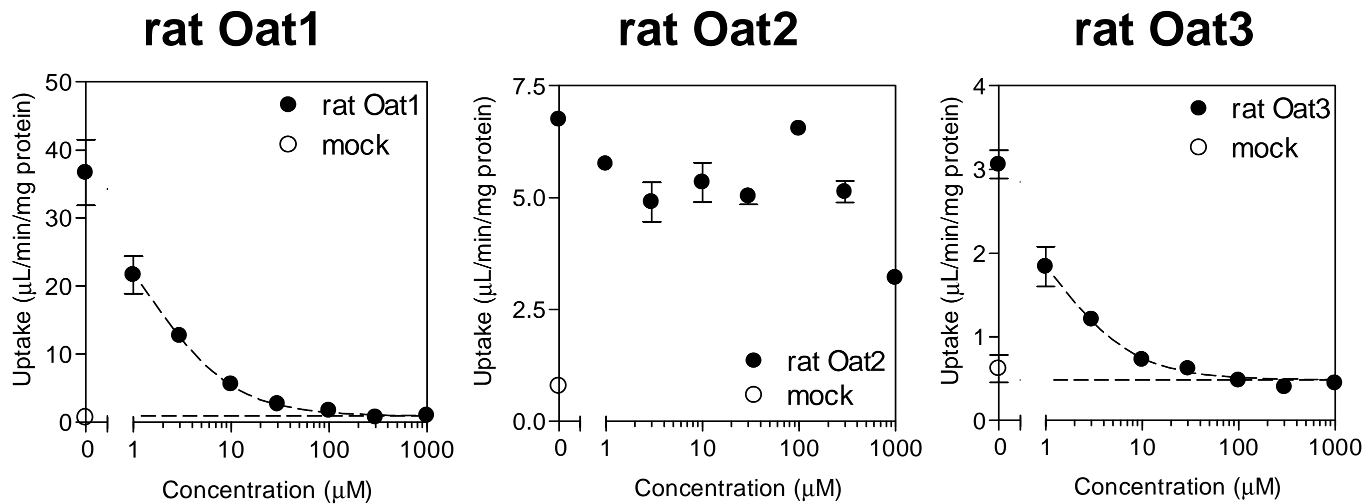


Fig. 3

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.



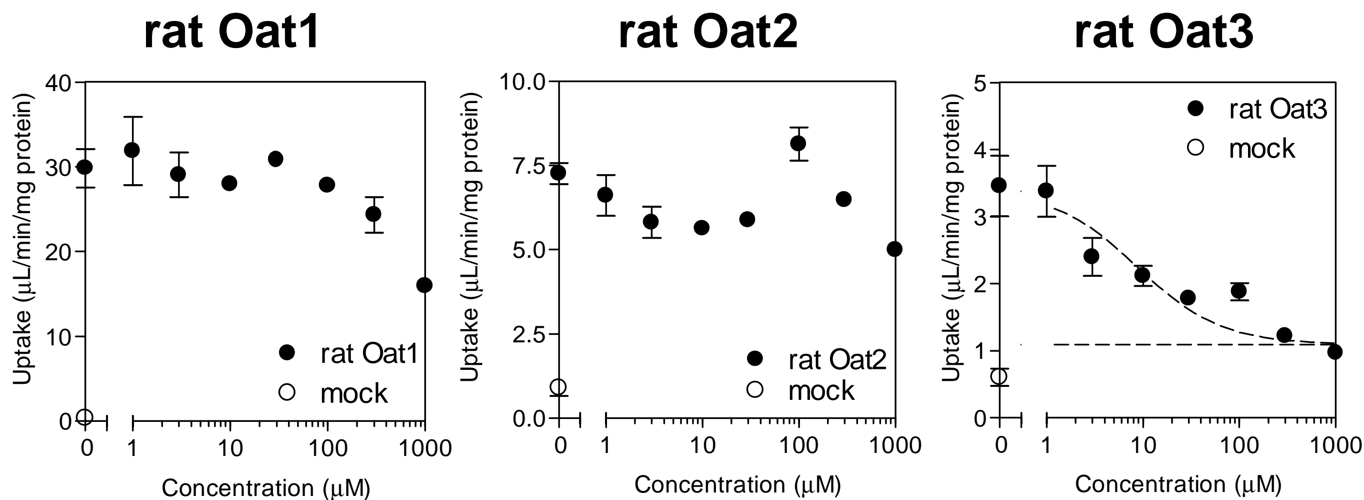


Fig. 4

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

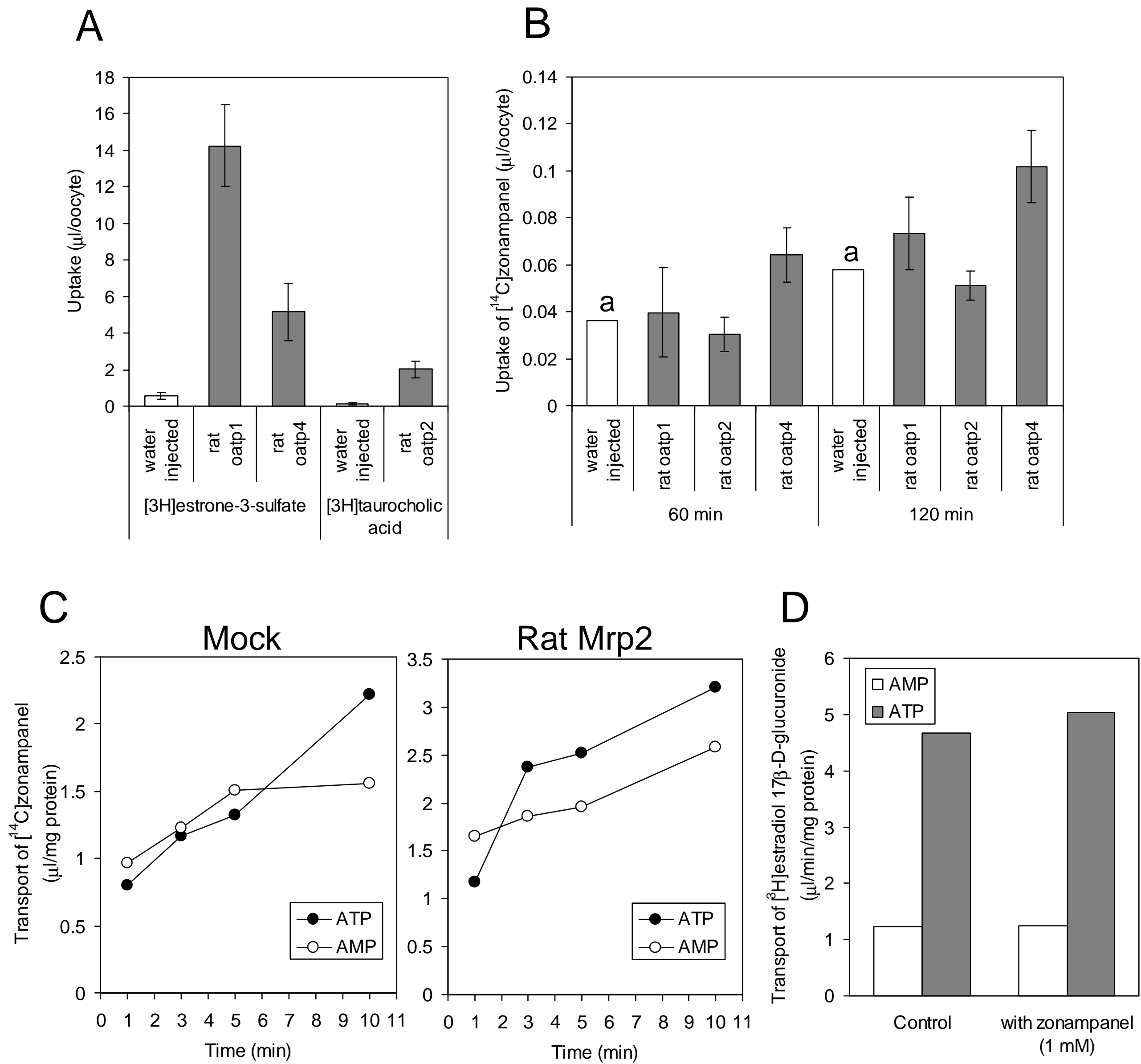
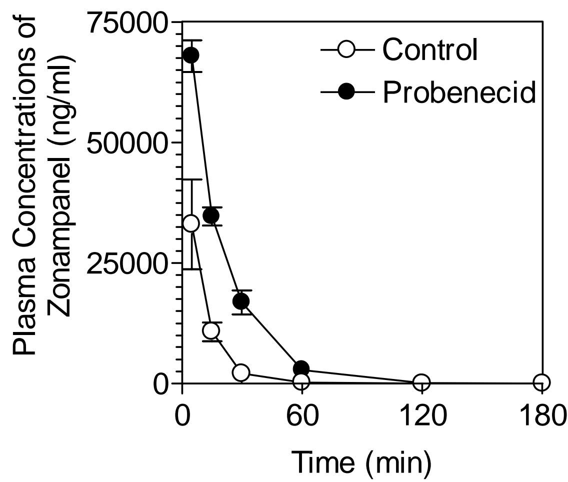


Fig. 5

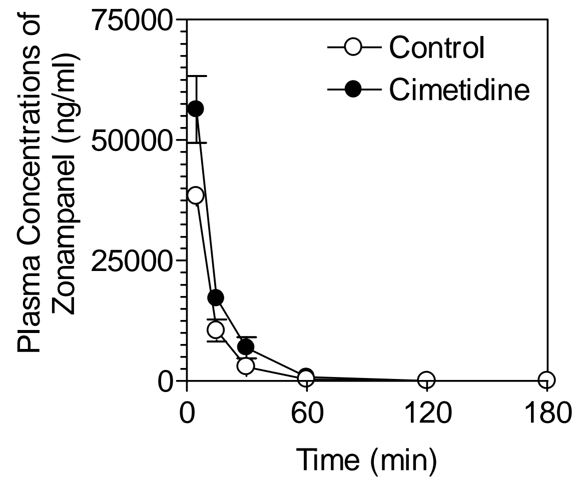
Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021

DMD Fast Forward. Published on April 28, 2008 as DOI: 10.1124/dmd.107.019828
This article has not been copyedited and formatted. The final version may differ from this version.

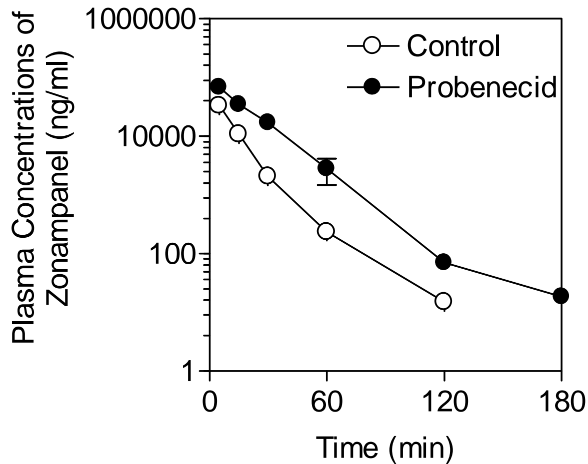
Probenecid



Cimetidine



Probenecid (semi-logarithmic scale)



Cimetidine (semi-logarithmic scale)

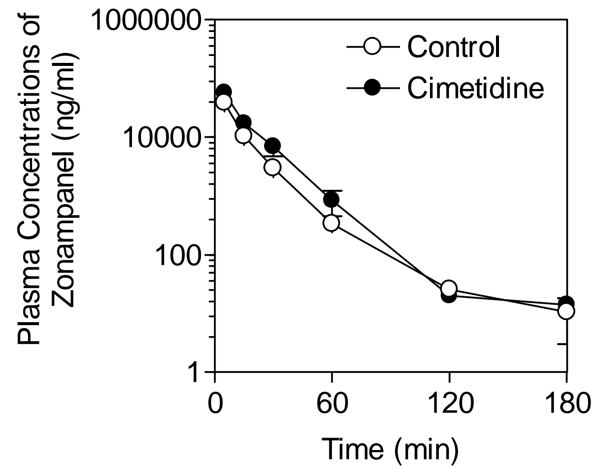


Fig. 6

Downloaded from dmd.aspetjournals.org at ASPET Journals on June 23, 2021