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Change in OATP-mediated hepatic uptake of SN-38 in renal failure

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ABSTRACT

Purpose: Clinical study has previously revealed that plasma concentration of 7-ethyl-10-hydroxycamptothecin (SN-38), an active metabolite of irinotecan, was higher in patients with end-stage renal failure than those with normal kidney function although SN-38 is mainly eliminated in the liver. Here, we focused on inhibition by uremic toxins of hepatic SN-38 uptake and down-regulation of uptake transporter(s) by uremic plasma in humans.

Methods: We evaluated SN-38 uptake and its inhibition by uremic toxins, 3-carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF), indoxyl sulfate (Indox), hippuric acid (HA) and indole acetate (IA), with cryopreserved human hepatocytes and HEK293 cells stably expressing hepatic uptake transporters, organic anion transporting polypeptides (OATPs). We also collected plasma samples from patients with severe renal failure to examine their effects on mRNA level of OATPs in primary cultured human hepatocytes.

Results: SN-38 was taken up by hepatocytes, which showed biphasic saturation patterns. The SN-38 uptake by hepatocytes was significantly inhibited by a uremic toxin mixture including clinically relevant concentrations of CMPF, Indox, HA and IA.

Kinetic analyses for OATP-mediated transport revealed that the uptake of SN-38 by OATP1B1 was the highest, followed by OATP1B3. Among the uremic toxins, CMPF exhibited most potent inhibition of OATP1B1-mediated SN-38 uptake and directly inhibited the uptake of SN-38 also in hepatocytes. In addition, gene expression of OATP1B1 and OATP1B3 in hepatocytes was significantly down-regulated by the treatment with the uremic plasma.

Conclusions: OATP1B1-mediated hepatic uptake of SN-38 was inhibited by uremic toxins, and gene expression of OATP1B1 was down-regulated by uremic plasma.

Key words: SN-38; human hepatocytes; organic anion transporting polypeptide; uremic toxins; renal failure

ABBREVIATIONS

ABCB1, ATP-binding cassette, sub-family B, member 1; BCRP, breast cancer resistance protein; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionate; CL_{cr}, creatinine clearance; $E_2$17βG, estradiol 17-β-D-glucuronide; eGFR, estimated glomerular filtration rate; HA, hippuric acid; HPLC, high-performance liquid chromatography; IA, indole acetate; Indox, indoxyl sulfate; MRP2, multidrug resistance-associated protein 2; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; RIF, rifampicin; RT-PCR, real-time polymerase chain reaction**;** SEM, standard error of mean; SN-38, 7-ethyl-10-hydroxycamptothecin; UGT, UDP-glucuronosyltransferase (UGT);

INTRODUCTION

Irinotecan hydrochloride has been widely used in cancer chemotherapy. 7-Ethyl-10-hydroxycamptothecin (SN-38) is an active metabolite of irinotecan that is extensively produced in the liver by carboxylesterase. It is subsequently detoxified, predominantly by UDP-glucuronosyltransferase (UGT) 1A1 in the liver, to form SN-38

glucuronide (chemical structures; http://www.pharmgkb.org/views/pathway/PA2001-metablites.html). Transporters expressed in the liver are implicated in various aspects of SN-38 pharmacokinetics. A primary active transport system is involved in permeation of SN-38 across canalicular membranes in both humans and rats, and multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP) mediate its biliary excretion (1-3) (http://www.pharmgkb.org/do/serve?objId=PA2001&objCls=Pathway). A part of SN-38 produced in the liver is considered to be transported to systemic circulation across sinusoidal membranes by the unidentified transporter(s) because of the fact that SN-38 is detectable in plasma immediately after the irinotecan injection in patients with cancer. SN-38 is a substrate of organic anion transporting polypeptides (OATPs), which are localized on sinusoidal membranes in humans (4, 5), and playing roles in the uptake of a variety of therapeutic drugs into hepatocytes, although their contribution to overall hepatic uptake of SN-38 has not yet been established.

We previously reported that the plasma concentration profile of SN-38, but not irinotecan or SN-38 glucuronide, was significantly delayed in patients with severe

renal failure who underwent hemodialysis, as compared with that in patients without renal failure (terminal elimination rate constant, 0.0084 ± 0.0037 vs. 0.081 ± 0.034 h^{-1} [mean ± standard deviation]) (6), even though SN-38 is mainly eliminated by the liver via glucuronidation and biliary excretion. Mean area under the concentration-time curve of SN-38 calculated from time zero to 24 h in the patients with severe renal failure was 1.6-fold greater than that in the patients without renal failure (1.3 ± 0.7 vs. 0.83 ± 0.55 μM·h) (6). The mechanism(s) underlying the delayed elimination of SN-38 in these patients remains unclear. It should be noted that all the patients with severe renal failure suffered from grade 2 to 4 neutropenia, even though dialyses were performed (6). The prolonged neutropenia seen in these patients resulted in delay of the second irinotecan treatment (6). Since SN-38 concentrations has been reported to be still remained detectable even at 500 hr after drug administration in patients with normal renal function (7), it could be postulated that the long period of exposure to relatively high concentration of SN-38 was one of causes of the prolonged neutropenia in these cancer patients with severe renal failure. Thus, it is important to clarify the mechanism(s) of delayed elimination of SN-38 in order to establish an appropriate irinotecan

chemotherapy protocol for renal failure patients, without increased risk of severe adverse reactions.

Several lines of evidence have recently suggested that renal failure differentially affects drug uptake/efflux transporters and drug-metabolizing enzymes in the liver. Therefore, even drugs that are predominantly eliminated by hepatic transport and/or metabolism can be affected by severe renal failure, leading to unexpected consequences, such as atypical pharmacokinetics and an increased risk of adverse drug reactions. High levels of uremic toxins in such patients are at least partially implicated in these effects of renal failure (8). Indeed, in our previous study, plasma concentrations of organic anionic uremic toxins, such as 3-carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF), indoxyl sulfate (Indox), indole acetate (IA) and hippuric acid (HA), increased in parallel with the degree of renal impairment (6). These four uremic toxins were selected for study since they are organic anions that might interact with organic anion transporters in the liver, and there is a sufficient body of literature on their properties (6). It has been reported that CMPF and Indox directly inhibit OATP1B1-mediated uptake of a typical substrate,

estrone-3-sulfate (E3S), in a mammalian cell line exogenously transfected with OATP1B1 cDNA (9). We hypothesized that the elevation of SN-38 plasma concentration in patients with severe renal failure was caused by the inhibition of hepatic uptake of SN-38 by uremic toxins. So far, information on the characteristics of inhibition of hepatic uptake transporters by uremic toxins is still quite limited (9). In addition, there has been no report directly concerning the interaction of uremic toxins with hepatic transporters responsible for SN-38 uptake in human hepatocytes. Another hypothesis was that reduced gene expression of uptake transporters in patients with severe renal failure (8).

Therefore, in the present study, we set out to examine the inhibition of SN-38 uptake in human liver by organic anionic uremic toxins mentioned above. To clarify the interaction of the SN-38 with hepatic uptake transporters, cryopreserved human hepatocytes and HEK293 cells stably expressing human OATPs were used. We further investigated the inhibition of SN-38 uptake by the uremic toxins in the human hepatocytes and the HEK293 cells in order to estimate direct effects of the toxins in humans. Additionally, to clarify the possible long-term effects of uremic plasma on

hepatic uptake transporters in humans, we examined effects of human plasma obtained from patients with severe renal failure (uremic plasma) on gene expression of OATPs in cultured human hepatocytes.

MATERIALS AND METHODS

Materials.

[^{3}H]E3S (57.3 Ci/mmol) and [^{14}C]inulin (2.8 mCi/g) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). [^{3}H]Estradiol 17β-glucuronide ([^{3}H]$E_2$17βG) (41.8 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Camptothecin, IA, HA and rifampicin (RIF) were obtained from Wako Pure Chemical (Osaka, Japan), and SN-38 and Indox from Sigma-Aldrich Japan (Tokyo, Japan). SN-38 was first dissolved in 50 mM phosphate buffer at pH 9.0 and kept overnight to convert the compound to its carboxylate form (>99%) (2). CMPF was purchased from Cayman Chemical Company (Ann Arbor, MI). Cryopreserved human hepatocytes (H1000.H15T, Lot#694, 29-year-old male Caucasian and H1500.H15T, Lot#706, 53-year-old female Caucasian; [XenoTech, Lenexa, KS])

were obtained from Sekisui Medical (Tokyo, Japan). Plateable cryopreserved human hepatocytes (Cat#454541, Lot#276, 59-year-old female Hispanic; and Cat#HMCPUS, Lot#Hu1314, 25-year-old female Caucasian) were purchased from BD Biosciences (San Jose, CA) and Life Technologies (Carlsbad, CA), respectively. All other chemicals and reagents were of analytical grade and obtained from commercial sources.

Transport Studies in HEK293 Cells Stably Transfected with OATP1B1, OATP1B3 or OATP2B1.

HEK293 cells were transfected with pcDNA3 constructs containing full-length OATP1B1, OATP1B3 or OATP2B1 according to the calcium phosphate precipitation method. Cells were then selected by adding G418 (1 mg/mL, Wako Pure Chemical) to the culture medium to obtain HEK293/OATP1B1, HEK293/OATP1B3 and HEK293/OATP2B1 cells. These cells were routinely grown in D-MEM without L-glutamine and phenol red (Wako Pure Chemical) containing 10% fetal bovine serum, penicillin, streptomycin and 1 mg/mL G418 in a humidified incubator at 37°C and 5% CO_2. After the cells had reached confluence, they were harvested and suspended in the transport buffer containing 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM

$CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM HEPES (adjusted to pH 7.4 with 1 N NaOH). The cell suspension and the transport buffer containing SN-38 or [^{3}H]E3S were mixed to initiate the transport reaction. At the designated times, the mixture was diluted 6-fold with ice-cold transport buffer and then centrifuged for 1 min, and the supernatant was removed. The cells were washed three times with ice-cold buffer, and then disrupted in distilled water with a sonicator. The concentrations of SN-38 in the supernatant and cell lysates were measured by high-performance liquid chromatography (HPLC) as described below. [^{3}H]E3S was measured as radioactivity using Clearsol I (Nacalai Tesque, Kyoto, Japan) as the scintillation fluid. The radioactivity was determined using a liquid scintillation counter (LSC-5100; Aloka, Tokyo, Japan).

Transport Studies Using Cryopreserved Human Hepatocytes.

Cryopreserved human hepatocytes were thawed and purified using a hepatocyte isolation kit (K2000, XenoTech) according to the manufacturer's instruction. The cells were resuspended in the transport buffer (pH 7.4) to give a final concentration of 2 mg protein/mL. The number of viable cells was determined by trypan blue staining. The uptake experiment was then performed according to the silicone oil layer method

(10) with some modifications. In brief, 20 μL of the cell suspension was preincubated for 5 min at 37˚C, then the reaction was started by mixing the suspension with 80 μL of prewarmed transport buffer containing SN-38 with or without inhibitors. The reaction was stopped by addition of 600 μL of ice-cold transport buffer, and the mixture was then quickly centrifuged through a silicone oil layer (density, 1.015) to separate the cells from the medium. This dilution process was also expected to reduce the amount of cell-associated SN-38 in adhering water. Hepatocytes were then lysed overnight with alkali (3 N KOH), and the lysed hepatocytes and medium were stored at -20~-30˚C until HPLC analysis. The hepatocellular protein content was determined according to the method of Bradford using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard. The amounts taken up by the hepatocytes were expressed as the intracellular distribution volume (intracellular content divided by the SN-38 concentration in the medium) and corrected for the adherent water volume (1.94 μ L/mg protein) which was determined as apparent distribution volume of extracellular marker [^{14}C]inulin.

Measurement of SN-38 by HPLC.

Total (lactone and carboxylate) concentration of SN-38 was analyzed by a modified reverse-phase HPLC method (11). A 40-μL sample was mixed with 60 μL of methanol, 10% perchloric acid (50:50, v/v) and 10 μL of camptothecin (internal standard) in a vortex mixer. The mixture was centrifuged at 15,000 rpm for 10 min, and the supernatant was injected into an HPLC system (Shimadzu LC-10Avp series; Shimadzu, Kyoto, Japan), equipped with a TSK-gel ODS-120T analytical column (4.6 × 250 mm, 4 μm; TOSOH, Tokyo, Japan). HPLC was carried out at 40°C at a flow rate of 1.0 mL/min. SN-38 was determined fluorometrically (excitation 355 nm; emission 515 nm). The mobile phase consisted of 75 mM ammonium acetate (pH 4.75)/acetonitrile (86:14) for solvent A, and 75 mM ammonium acetate (pH 4.75)/acetonitrile (60:40) for solvent B; a 25-min run was carried out with a linear gradient of 100-0 % solvent A.

Kinetic Analyses.

Kinetic parameters for transport activity were estimated by nonlinear least-squares fitting of the data to the following equations using the MULTI program (12):

$$V = V_{max} \cdot S/(K_m + S) \quad (1)$$

$$V = V_{max} \cdot S/(K_m + S) + P_{dif} \cdot S \quad (2)$$

$$V = V_{max,1} \cdot S/(K_{m,1} + S) + V_{max,2} \cdot S/(K_{m,2} + S) \quad (3)$$

where V, S, K_m, V_{max} and P_{dif} represent the initial uptake velocity, substrate concentration, Michaelis constant, maximum uptake velocity and clearance for non-saturable uptake, respectively. The selection of equation was based on the goodness of fit, considering Akaike's information criteria. Concentration-dependent inhibition of the uptake by an inhibitor was fitted to the following equation:

$$R = IC_{50}^{n}/(IC_{50}^{n} + I^{n}) \quad (4)$$

where R, I, IC_{50} and n are the uptake normalized by the control (without inhibitor) value, the inhibitor concentration, the concentration for half-maximal inhibition, and Hill's coefficient, respectively.

Human Plasma Samples.

Plasma samples were obtained from patients with normal renal function (estimated glomerular filtration rate (eGFR) of equal to or higher than 60 mL/min) or those with severe renal failure (<15 mL/min eGFR) (uremic plasma). All patients

enrolled did not receive dialysis, signed a written informed consent form, granting permission for their peripheral blood samples and medical information to be used for research purposes. The study protocol was approved by the Institutional Review Board of Saitama Medical University.

Culture of Plateable Cryopreserved Human Hepatocytes.

Plateable cryopreserved human hepatocytes were thawed and purified using a hepatocyte isolation kit (BD Gentest High Viability CryoHepatocyte Recovery kit, BD Biosciences) with 10% fetal calf serum according to the manufacturer's instruction. Cell suspensions with viability rates of >95% as assessed by trypan blue dye exclusion were used for the experiments. Hepatocytes were plated on BD BioCoat collagen I-coated plates (BD Biosciences). Seeding density was 40,000 cells per well of 24-well plate. Williams' E medium supplemented with BD ITS Universal Culture Supplements, 4 mM L-glutamine, 0.1 μM dexamethasone and 100 U/mL penicilline-streptomycin was used as culture medium. After an incubation period of 4 hr at 37˚C, 5% CO_2, and 95% humidity, nonattached cells were removed, and culture medium without serum was supplied. Twenty four hr after plating, medium was removed, and hepatocytes were

overlaid with 0.5 mL per well of culture medium containing 0.25 mg/mL BD Matrigel Matrix (BD Biosciences) and incubated for 24 hr at 37˚C, 5% CO_2, and 95% humidity to obtain the sandwich cultures. Hepatocytes were then incubated for 48 hr with 10% human plasma obtained from patients with normal renal function (normal plasma) or uremic plasma diluted with culture medium. Hepatocytes were harvested by scraping in QIAzol® Lysis Reagent (Qiagen, Mississauga, ON) for mRNA analysis.

Determination of mRNA by Real-time Polymerase Chain Reaction (RT-PCR).

Total RNA was extracted by the miRNeasy Mini Kit (Qiagen), and cDNA was synthesized using oligo $(dT)_{12-18}$ primer, deoxynucleotide triphosphate mix, RT buffer and MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA). Quantification of mRNA for OATP1B1, OATP1B3 and ATP-binding cassette, sub-family B, member 1 (ABCB1) was performed using Mx3005P (Agilent Technologies, Santa Clara, CA). The sequences of the primers were as follows: OATP1B1 forward, GTT GCC GGA CTA ACC ATG AC and reverse, TCC ACA GAC TGG TTC CCA TT; OATP1B3 forward, CGG CCT AAC CTT GAC CTA TG and reverse, CAC AGA CTG GTT CCC ACT GA; ABCB1 forward, GCC CTT GTT AGA

CAG CCT CA and reverse, CCT TCT CTG GCT TTG TCC AG; 36B4 forward, GAA GTG CTT GAT ATC ACA GAG GAA and reverse, TGA TGC AAC AGT TGG GTA GC. The PCR conditions were as follows: initial denaturation at 95˚C for 15 min, followed by 40 cycles of denaturation at 95˚C for 10 sec (followed by an immediate change to the next temperature) and combined annealing extension at 60˚C for 30 sec.

Statistical Analyses.

Statistical analyses were performed by using Student's t test. A difference between means was considered to be significant when the two-tailed p-value was less than 0.05.

RESULTS

Hepatic Uptake of SN-38.

SN-38 is mainly eliminated by the liver through glucuronidation and biliary excretion, but no information is available on the characteristics of SN-38 uptake by human hepatocytes. Therefore, we directly examined hepatic SN-38 uptake using cryopreserved human hepatocytes. Both SN-38 and [^{3}H]E3S were taken up by human

hepatocytes in a time-dependent manner (Fig. 1A, 1B). In both cases, uptake was higher in hepatocytes of Lot#706 than in those of Lot#694 (Fig. 1A, 1B). Kinetic analyses were then performed for SN-38 uptake by Lot#706 and Lot#694, which showed biphasic saturation patterns (Fig. 1C, 1D). The value of Akaike's information criteria obtained by fitting of the data shown in Fig. 1C to Eqs. (1), (2) and (3) (-13.3, -24.0 and -22.7, respectively) was the lowest for Eq. (2), which yielded K_m, V_{max} and P_{dif} values of 9.29 μM, 63.5 pmol/min/mg protein and 3.77 μL/min/mg protein, respectively. Thus, the saturable portion of the uptake accounts for 60-70% of the overall uptake in Lot#706 hepatocytes. The value of Akaike's information criteria for the data in Fig. 1D to three equations was the lowest for Eq. (3) (-23.0). $K_{m,1}$, $V_{max,1}$, $K_{m,2}$ and $V_{max,2}$ were 0.22 μM, 0.78 pmol/min/mg protein, 40.9 μM and 334 pmol/min/mg protein, respectively.

Inhibition by Uremic Toxin Mixtures of Hepatic SN-38 Uptake.

Next, we examined the effects of the uremic toxins, CMPF, Indox, HA and IA, on the hepatic uptake of SN-38 in order to elucidate whether clinically relevant concentrations of these toxins inhibit the SN-38 uptake, which could be associated with

the increased plasma SN-38 concentration in patients with severe renal failure (6). Mix1 consisted of a mixture of the toxins at the concentrations reported in our patients (6), while Mix2 was a mixture of the toxins at the highest concentrations previously reported in patients (13). When the human hepatocytes were incubated with SN-38 and the Mix1 (100 μM CMPF, 100 μM Indox, 100 μM HA and 10 μM IA), SN-38 uptake in Lot#706 was significantly reduced (Fig. 2A). The SN-38 uptake in Lot#694 tended to be inhibited by the addition of Mix1 to the similar extent to Lot#706 (Fig. 2B). When the hepatocytes were incubated with the Mix2 (300 μM CMPF, 1,000 μM Indox, 3,000 μM HA and 100 μM IA), the SN-38 uptake was significantly reduced to the same extent as 100 μM RIF (Fig. 2A, 2B).

Contribution of OATPs in the Hepatic Uptake of SN-38.

We estimated the contribution of OATP1B1 and another OATP uptake transporter, OATP1B3, to hepatic uptake of SN-38 in humans, since SN-38 has been reported to be a substrate of these OATP uptake transporters (4, 5). For this purpose, we added OATP inhibitors, E3S (10 μM), RIF (100 μM) or E3S (10 μM) plus RIF (100 μM), to incubation mixture of human hepatocytes, since E3S can inhibit OATP1B1, but

RIF can inhibit both OATP1B1 and OATP1B3 when appropriate concentrations of E3S and RIF are used (9, 14). Uptake of SN-38 in Lot#706 of human hepatocytes was significantly inhibited by 10 μM E3S or 100 μM RIF (Fig. 2A). Inhibition of the hepatic uptake of SN-38 by E3S was tended to be less potent than that by RIF or a mixture of E3S and RIF (Fig. 2A). If E3S and RIF equally inhibit the SN-38 uptake, the uptake could be mainly mediated by OATP1B1, whereas, if E3S does not inhibit the SN-38 uptake but RIF does, the uptake could be predominantly mediated by OATP1B3. The present results thus indicate the greater contribution of OATP1B1 to the hepatic uptake of SN-38 than OATP1B3. Significant inhibition of the SN-38 uptake by E3S, RIF or the mixture was also observed in human hepatocytes of Lot#694 (Fig. 2B). [^{3}H]E3S is known to be taken up into human hepatocytes by OATPs (15). As shown in figure 2C, the uptake of [^{3}H]E3S by human hepatocytes was sufficiently inhibited by E3S, RIF and the mixture of E3S and RIF.

Characterization of OATP1B1-, OATP1B3- or OATP2B1-mediated Uptake of SN-38.

Next, we characterized the OATP1B1-, OATP1B3- or OATP2B1-mediated

transport of SN-38. The uptake of SN-38 by HEK293/OATP1B1 and HEK/OATP1B3 cells increased in a time-dependent manner and was higher than that in HEK293/mock cells (Fig. 3A, 3B), whereas the uptake by HEK293/OATP2B1 cells was almost comparable with that in HEK293/mock cells (Fig. 3C). OATP1B1- or OATP1B3-mediated uptake, which was obtained by subtracting the uptake in HEK293/mock cells from that in HEK293/OATP1B1 or HEK293/OATP1B3 cells, respectively, linearly increased up to 80 min (Fig. 3A, 3B). In positive control studies, OATP1B1-, OATP1B3-, and OATP2B1-mediated uptake of typical substrates were measured. OATP1B1-mediated [^{3}H]E3S uptake, OATP1B3-mediated [^{3}H]$E_2$17βG uptake, and OATP2B1-mediated [^{3}H]E3S uptake were 113 ± 4 μL/mg protein/3 min, 17.5 ± 2.9 μL/mg protein/10 min, and 207 ± 4 μL/mg protein/5 min, respectively. Kinetic analyses of the initial phase of OATP1B1- and OATP1B3-mediated SN-38 uptake were performed. Clearance (V/S) for the OATP1B1-mediated SN-38 uptake was much higher than that for OATP1B3-mediated uptake (Fig. 3D, 3E), and this was consistent with higher value for the time course of OATP1B1-mediated uptake as compared with OATP1B3-mediated one (Fig. 3A, 3B). The data of OATP1B1-mediated

SN-38 uptake (Fig. 3D) was well fitted to Eq. (1) with the Km and Vmax of 4.97 μM and 1.71 pmol/min/mg protein, respectively. The SN-38 uptake by OATP1B3 (Fig. 3E) was well fitted to Eq. (3) with $K_{m,1}$ and $K_{m,2}$ of 0.0177 and 23.2 μM, respectively and $V_{max,1}$ and $V_{max,2}$ of 0.00371 and 0.561 pmol/min/mg protein, respectively.

We further characterized the uptake of SN-38 by OATP1B1 and OATP1B3 with the use of OATP inhibitors, E3S and RIF. OATP1B1-mediated uptake of SN-38 was almost completely blocked in the presence of E3S (10-30 μM) and RIF (30-100 μM) (Fig. 4A). OATP1B3-mediated uptake of SN-38 was minimally inhibited by 10 μM E3S ($p > 0.05$), but was almost completely blocked in the presence of 100 μM RIF (Fig. 4B). The differential inhibition of the two OATP clones by E3S is consistent with the effects observed in pitavastatin uptake (14). Thus, 10 μM E3S is sufficient to inhibit OATP1B1, while 100 μM RIF is sufficient to inhibit both OATP1B1 and OATP1B3.

Inhibition of OATP1B1- or OATP1B3-mediated SN-38 Uptake by Organic Anion Uremic Toxins.

Since no information has been reported on the inhibitory potency of uremic toxins towards SN-38 transport mediated by OATP1B1 or OATP1B3, we examined the

effects of uremic toxin mixtures on OATP1B1- or OATP1B3-mediated uptake of SN-38. Both Mix1 and Mix2 significantly inhibited OATP1B1-mediated SN-38 uptake (Fig. 4A). The mixtures also significantly inhibited OATP1B3-mediated SN-38 uptake (Fig. 4B). Since clearance for the SN-38 uptake by OATP1B1 was higher than that by OATP1B3 (Fig. 3), we next examined the effect of each uremic toxin, CMPF, Indox, HA and IA on OATP1B1-mediated SN-38 uptake. CMPF, Indox and HA each inhibited OATP1B1-mediated uptake of both SN-38 and [^{3}H]E3S in a concentration-dependent manner (Fig. 5A, 5B, 5C), whereas IA was less inhibitory. CMPF showed the lowest IC_{50} value among the four uremic toxins (Table 1).

CMPF, Indox and HA showed greater inhibitory effects on OATP1B1-mediated uptake of SN-38 than on that of [^{3}H]E3S (Fig. 5A, 5B, 5C). The IC_{50} values of CMPF and Indox for SN-38 uptake were lower than those for [^{3}H]E3S uptake (Table 1). The inhibition profile of CMPF for SN-38 uptake was gradual, compared with those of the other uremic toxins, and it was also more gradual than the inhibition profile of CMPF for [^{3}H]E3S uptake (Fig. 5A). The inhibitions were examined within the uremic toxin concentrations where these chemicals could be

dissolved in the incubation mixture.

To further characterize the inhibition by CMPF, uptake of SN-38 was examined at various concentrations of both SN-38 and CMPF. The Lineweaver-Burk plots exhibited mixed-type inhibition (Fig. 6). Thus, the inhibitory effect of CMPF is substrate-dependent and shows unusual kinetics, especially for SN-38 uptake.

Inhibition by CMPF of Hepatic SN-38 Uptake.

CMPF was demonstrated to relatively strongly inhibit OATP1B1-mediated uptake of SN-38 comparing to other uremic toxins (Fig. 5). Thus, the inhibition by CMPF of hepatic uptake of SN-38 and [^{3}H]E3S was examined in human hepatocytes. Both of SN-38 and [^{3}H]E3S uptakes were significantly inhibited by CMPF at higher concentrations than 100 and 30 μM, respectively (Fig. 7A, 7B). The reduction was at most ~40% in the higher concentration range of CMPF (Fig. 7A, 7B).

Down-regulation of Gene Expression for OATP1B1 and OATP1B3 in the Presence of Uremic Plasma.

Finally, we examined the possible suppression of hepatic OATP1B1 and OATP1B3 mRNA expressions in patients with severe renal failure. For this purpose,

plateable human hepatocytes were incubated for 48 hr with 10% normal human plasma or uremic human plasma. The mRNA levels of OATP1B1, OATP1B3 and ABCB1 observed in hepatocytes treated with normal or uremic plasma are shown in figure 8A, 8B, 8C, respectively. Then, we combined the respective 3 data to compare the average mRNA levels of the transporters between hepatocytes incubated with normal and uremic plasma. The mRNA levels of OATP1B1 and OATP1B3 in hepatocytes treated with uremic plasma were much lower than those with normal plasma, whereas the mRNA level of ABCB1 was not different (Fig. 8D).

DISCUSSION

It seems paradoxical that plasma SN-38 concentration is elevated in patients with severe renal failure (6), given that SN-38 is mainly eliminated in the liver. In patients with severe renal dysfunction, plasma concentrations of various uremic toxins are elevated. In our patients with end-stage renal failure, concentrations of CMPF, Indox and HA were 81.1, 90.3 and 80.5 μM, respectively (6). While another report described the maximal plasma concentrations of 400 μM for CMPF, 940 μM for Indox and 2.6

mM for HA (13). SN-38 in the systemic circulation is taken up into hepatocytes, followed by metabolism or excretion into the bile. Therefore, we first hypothesized that, if a transporter(s) is involved in the uptake of SN-38 into hepatocytes, the elevated level of uremic toxins in plasma of the patients might inhibit the uptake process of SN-38 to increase the plasma concentration. Our present results first demonstrated that SN-38 was taken up into cryopreserved human hepatocytes (Fig. 1). We next showed that the uremic toxin mixtures did indeed significantly, but partially, inhibit the hepatic uptake of SN-38 (Fig. 2A, 2B). SN-38 uptake was found to be significantly inhibited by CMPF in a concentration range of 100-300 μM (Fig. 7A). These results indicate that the hepatic uptake of SN-38 is inhibited by these uremic toxins at clinically relevant concentrations. Thus, the paradoxical findings in our previous clinical study might be at least in part explained by the direct inhibition of hepatic SN-38 uptake by uremic toxins, which are elevated in plasma of the end-stage patients. This is the first report to show uremic toxins to inhibit SN-38 uptake in human hepatocytes.

We examined the contribution of OATPs to overall uptake of SN-38 in human hepatocytes by using two OATP inhibitors, E3S and RIF, since E3S can inhibit

OATP1B1, but RIF can inhibit both OATP1B1 and OATP1B3 when appropriate concentrations of E3S and RIF are used (9, 14). It was confirmed that 10 μM E3S is sufficient to inhibit OATP1B1-mediated uptake of SN-38, while 100 μM RIF is sufficient to inhibit both OATP1B1- and OATP1B3-mediated uptake (Fig. 4A, 4B). The fact that the SN-38 uptake in human hepatocytes was significantly inhibited by 10 μM E3S or 100 μM RIF (Fig. 2A, 2B) indicates a larger contribution of OATP1B1 to hepatic SN-38 uptake, as compared with OATP1B3. Kinetic analyses revealed that the uptake of SN-38 by OATP1B1 was the highest, followed by OATP1B3, and the uptake by OATP2B1 was negligible (Fig. 3). Therefore, OATP1B1 may be a major contributor to the hepatic uptake of SN-38 in humans. However, there could be limitation of using transporter selective inhibitors to assess relative contribution of the hepatic transporter, and E3S could be also the inhibitor for other transporters than OATP1B1 and OATP1B3. Exact contribution of these transporters should be estimated by further analyses.

Then, another hypothesis for the elevated SN-38 concentration in patients with end-stage renal failure that gene expressions of *SLCO1B1* and *SLCO1B3* might be down-regulated in such patients came to us. As expected, we first demonstrated that the

mRNA levels of OATP1B1 and OATP1B3 in hepatocytes treated with uremic plasma were significantly lower than those in hepatocytes incubated with normal plasma (Fig. 8), although the detailed mechanism(s) of the reduced gene expression at present remains unclear. Further studies are warranted to elucidate the mechanism(s). We are now investigating the effects of uremic toxins on the *SLCO1B1* and *SLCO1B3* gene expressions. Taking these results into account, the direct inhibition by uremic toxins of hepatic SN-38 uptake mediated by OATP1B1, together with the down-regulation of *SLCO1B1* gene expression might partly contribute to the delayed SN-38 elimination observed in patients with severe renal dysfunction. Nevertheless, SN-38 has both lactone and carboxylate forms, which may display different transporter properties and show inter-conversion during the experiment. Although the present study was performed at the neutral pH condition which mimics human plasma, more quantitative analysis should be performed at the same inter-conversion condition between the uptake study and human plasma. Further studies are necessary to wholly understand the mechanisms of the phenomena.

Considering the reports by us (6) and de Jong et al. (16), the increase in

plasma SN-38 concentration was only observed in patients with severe renal failure having creatinine clearance (CL_{cr}) less than 20 mL/min. A similar phenomenon has been reported for rosuvastatin, an HMG-CoA reductase inhibitor, that is predominantly taken up into liver by OATP1B1 (17). The area under the concentration-time curve of rosuvastatin elevated only in patients with severe renal failure having CL_{cr} less than 30 mL/min (18). These results might suggest an analogy in mechanism(s) for elevated pharmacokinetic profiles of medicines that are predominantly taken up by OATP1B1 into the liver.

In our previous study, the plasma concentration profile of SN-38, but not irinotecan or SN-38 glucuronide, was significantly delayed in patients with severe renal failure who underwent hemodialysis, as compared with those with normal kidney (6). If activity or expression of carboxylesterase is altered by the patient's renal function, plasma concentration profiles of irinotecan and SN-38 should be simultaneously altered. Similarly, pharmacokinetics of SN-38 and SN-38 glucuronide should be simultaneously altered when UGT1A1 activity or expression was changed. Accordingly, we at present rule out the possibility of the alteration of drug-metabolizing enzyme activity(s) in

patients with severe renal failure comparing to that in patients with normal renal function. However, SN-38 is excreted into bile by transporters such as MRP2 and BCRP, which are expressed in canalicular membranes (1, 2). Since uremic toxins have been shown to inhibit BCRP (19), the inhibition of SN-38 efflux transport by uremic toxins might complicate the interpretation of the mechanism for the change of SN-38 elimination in patients with severe renal failure.

Inhibition of OATP1B1 by uremic toxins exhibited two characteristic features. First, the inhibition exhibits substrate-dependence, the inhibitory effect being greater for uptake of SN-38 than for uptake of E3S (Fig. 5). Indeed, the IC_{50} of SN-38 for inhibition of OATP1B1-mediated E3S uptake was 21.0 μM, which was different from the K_m for SN-38 uptake (~5 μM) (Fig. 3D). Similar substrate-dependent inhibition has been proposed in other cases, and can be accounted for by the presence of multiple binding sites at the catalytic site in OATP1B1 (20, 21). The present finding implies that uremic toxins may potently inhibit the uptake of other certain OATP1B1 substrates than SN-38. Since the plasma concentrations of these uremic toxins are elevated during severe renal failure, their interaction with other OATP1B1 substrate drugs may alter the

pharmacokinetics of such drugs during renal failure, even if the substrates are mainly eliminated in the liver. The other interesting feature of OATP1B1 inhibition by uremic toxins is its sigmoidal profile (Fig. 5); in particular, the inhibition by CMPF at 3 ~ 30 μM showed a relatively gentle slope (Fig. 5A). A similar inhibition profile by CMPF was also found for SN-38 uptake in human hepatocytes (Fig. 7A). This atypical inhibition profile may also be explained by the presence of multiple binding sites on the transporter molecule, and leads to difficulty in identification of the type of inhibition from Lineweaver–Burk plots (Fig. 6). The inhibition of rat renal uptake transporters OAT1 and OAT3 by CMPF, Indox, HA and IA showed simple Michaelis-Menten type kinetics (22). Therefore, this sigmoidal profile could be a specific feature of OATP1B1. Further study will be needed to examine the mechanism for the substrate-dependence and the sigmoidal character of the inhibition by CMPF.

The SN-38 uptake observed in HEK293/OATP1B1 or HEK293/OATP1B3 cells (Fig. 3A, 3B) seemed to be relatively lower than that observed in human hepatocytes (Fig. 1A), given that the uptake studies were conducted up to 5 and 120 min for hepatocytes and HEK293 cell, respectively. Although the exact reason for such

discrepancy is unknown, it should be noted that the uptake of [^{3}H]E3S is also higher in hepatocytes (Fig. 1B) than HEK293/OATP1B1 cells (see Results). In addition, we observed non-saturable portion of SN-38 uptake in human hepatocytes (Fig. 1C, 1D). Therefore, unknown transport or intracellular sequestration mechanisms that were not mediated by OATPs might contribute to the SN-38 uptake observed in human hepatocytes.

Given that OATP1B1 predominantly mediates hepatic uptake of SN-38, genetic polymorphisms in *SLCO1B1* that causes decreased function might greatly affect the pharmacokinetics of SN-38. In fact, *SLCO1B1*15*, which causes reduced transport activity (23), is reported to influence the area under the plasma concentration-time curve of SN-38 and irinotecan-induced toxicity (24, 25). Further studies are warranted to examine the effects of genetic polymorphisms in *SLCO1B1* on the pharmacokinetics of as well as toxicity of irinotecan.

In conclusion, we have shown for the first time that uremic toxins, CMPF, Indox, HA and IA inhibit SN-38 uptake in human hepatocytes at clinically relevant concentrations. Our results indicate that OATP1B1 is a major contributor to the

saturable uptake of SN-38 in human hepatocytes. The gene expression of *SLCO1B1* was down-regulated by uremic plasma. The inhibition by uremic toxins of OATP1B1-mediated SN-38 uptake and the down-regulated *SLCO1B1* gene expressions may be part of the mechanisms causing the delay of SN-38 elimination observed in patients with severe renal dysfunction.

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Table 1. Inhibition potency of uremic toxins for OATP1B1-mediated uptake of SN-38 and [^{3}H]E3S[a)]

Substrate	SN-38[b)]	[^{3}H]E3S[c)]
CMPF (μM)	158	>300
Indox (μM)	2,290	4,750
HA (μM)	6,710	>3,000
IA (μM)	>3,000	>3,000

Inhibition potencies of the respective uremic toxins were expressed as IC_{50} (μM).

a) OATP1B1-mediated uptake was measured by subtracting the uptake in HEK293/mock cells from that in HEK293/OATP1B1 cells.

b) OATP1B1-mediated uptake of SN-38 for 80 min was measured.

c) OATP1B1-mediated uptake of [^{3}H]E3S for 3 min was measured.

LEGENDS TO FIGURES

Figure 1

Uptake of SN-38 or [^{3}H]E3S in Human Hepatocytes

Uptake of SN-38 (0.03 μM) and [^{3}H]E3S (1.6 nM) by Lot#706 (●, ○) and Lot#694 (▲, △) of cryopreserved human hepatocytes is shown in panels (A) and (B), respectively. Panels (C) and (D) show Eadie-Hofstee plots for the uptake of SN-38 by Lot#706 (●) and Lot#694 (▲) of human hepatocytes, respectively. Incubations were performed for 5 min. Each uptake value was obtained by subtracting that of [^{14}C]inulin, as an extracellular marker, and represents the mean ± SEM (n = 3).

Figure 2

Inhibition by Mixture of Uremic Toxins and OATP Inhibitors of SN-38 Uptake in Human Hepatocytes

Panels (A) and (B) show the uptake of SN-38 (0.03 μM) by Lot#706 (A) and Lot#694 (B) of cryopreserved human hepatocytes, respectively, in the presence of mixture of uremic toxins and OATP inhibitors, E3S and RIF. The concentrations of CMPF, Indox, HA and IA were 100, 100, 100 and 10 μM for Mix1 and 300, 1,000, 3,000 and 100 μM

for Mix2. The incubation period was 5 min. Panel (C) shows the uptake of [^{3}H]E3S (1.6 nM) and the inhibition by OATP inhibitors in human hepatocytes (#694) (positive control study). The incubation was performed for 2 min. Each uptake value was obtained by subtracting that of [^{14}C]inulin, as an extracellular marker, and represents the mean ± SEM (n = 3-9). *, Significantly different from the control ($p < 0.05$).

Figure 3

Uptake of SN-38 by OATP1B1, OATP1B3 and OATP2B1

Symbol (■) shows the uptake of SN-38 (0.03 μM) by HEK293/OATP1B1 (A), HEK293/OATP1B3 (B), and HEK293/OATP2B1 cells (C). The uptake of SN-38 by HEK293/mock cells (□) was also measured. OATP1B1-, OATP1B3-, and OATP2B1-mediated uptake (●) were calculated by subtracting the uptake by HEK293/mock cells from that by HEK293/OATP1B1, HEK293/OATP1B3, and HEK293/OATP2B1 cells, respectively. OATP1B1-mediated (D) and OATP1B3-mediated (E) uptakes of SN-38 for 80 min were measured at various SN-38 concentrations (0.03-30 μM). Each point represents the mean ± standard error of the

mean (SEM) (n = 3-6). When error bars are not shown, they are smaller than symbols.

Figure 4

Inhibition by Mixture of Uremic Toxins and OATP Inhibitors of OATP1B1- and OATP1B3-mediated SN-38 Uptake

Panels (A) and (B) show respective OATP1B1- and OATP1B3-mediated SN-38 (0.03 μM) uptakes, measured in the presence of mixture of uremic toxins or each OATP inhibitors at the indicated concentrations. OATP1B1- and OATP1B3-mediated uptake was calculated by subtracting the uptake by HEK293/mock cells from that by HEK293/OATP1B1 and HEK293/OATP1B3 cells, respectively. The concentrations of CMPF, Indox, HA and IA were 100, 100, 100 and 10 μM for Mix1 and 300, 1,000, 3,000 and 100 μM for Mix2. Incubation period was 80 min. Each value represents the mean ± SEM (n = 3-15).

Figure 5

Inhibitory Effects of Uremic Toxins on OATP1B1-mediated Uptake of SN-38 and

[^{3}H]E3S

Uptake of SN-38 (0.03 μM, ●) or [^{3}H]E3S (1.6 nM, ○) by HEK293/OATP1B1 or HEK293/mock cells was measured in the presence of various concentrations of CMPF (A), Indox (B), and HA (C). OATP1B1-mediated uptake was then calculated by subtracting the uptake by HEK293/mock cells from that by HEK293/OATP1B1 cells. Each value represents the mean ± SEM (n = 3-4). Uptake period was 80 min.

Figure 6

Characterization of the Inhibition by CMPF of OATP1B1-mediated SN-38 Uptake

Uptake of SN-38 (0.03-0.3 μM) by HEK293/OATP1B1 or HEK293/mock cells was measured in the presence of various concentrations of CMPF (0–300 μM). Incubation period was 80 min. OATP1B1-mediated uptake was then calculated by subtracting the uptake by HEK293/mock cells from that by HEK293/OATP1B1 cells. Data were expressed as Lineweaver–Burk plots. Each value represents the mean ± SEM (n = 3-13).

Figure 7

Inhibitory Effect of CMPF on SN-38 and [^{3}H]E3S Uptake by Human Hepatocytes

Panel (A) and (B) show the uptake of SN-38 or [^{3}H]E3S by Lot#706 (●) and Lot#694 (▲) of human hepatocytes, respectively, in the presence of various concentration of CMPF. Incubations periods for SN-38 and [^{3}H]E3S were 5 and 2 min, respectively. Each uptake value was obtained by subtracting that of [^{14}C]inulin, as an extracellular marker, and represents the mean ± SEM (n = 3-6).

Figure 8

The mRNA Levels of OATP1B1, OATP1B3 and ABCB1 in Plateable Human Hepatocytes Incubated with Normal or Uremic Human Plasma

Plateable human hepatocytes were incubated for 48 hr with each of 10% human normal plasma obtained from 3 patients (N1, N2 and N3) or uremic plasma from 3 patients (R1, R2 and R3). eGFR values in N1, N2 and N3 were 89.9, 97.5 and 66.9 mL/min, respectively, and those in R1, R2 and R3 were 7.0, 10.8 and 11.1 mL/min, respectively. The mRNA levels of OATP1B1 (A), OATP1B3 (B) and ABCB1 (C) were then quantified by RT-PCR. These mRNA levels were normalized by that of 36B4. The copy numbers in human hepatocytes incubated with 10% normal plasma were arbitrarily

defined as 100%. Data represents the mean ± SEM (n = 3-4). The up- and down-regulations of OATP1B1 mRNA by respective addition of phenobarbital and colhichine were confirmed as positive and negative controls (http://tools.invitrogen.com/content/sfs/brochures/ISSX2009InductionofTransporterExpressionandModulationofHepatobiliaryDisposition.pdf). Panel (D) represents the average of mRNA levels for respective transporters seen in panels (A-C). Data represents the mean ± SEM (n=11-12). *, Significantly different from patients with normal kidney function.

Fig. 1

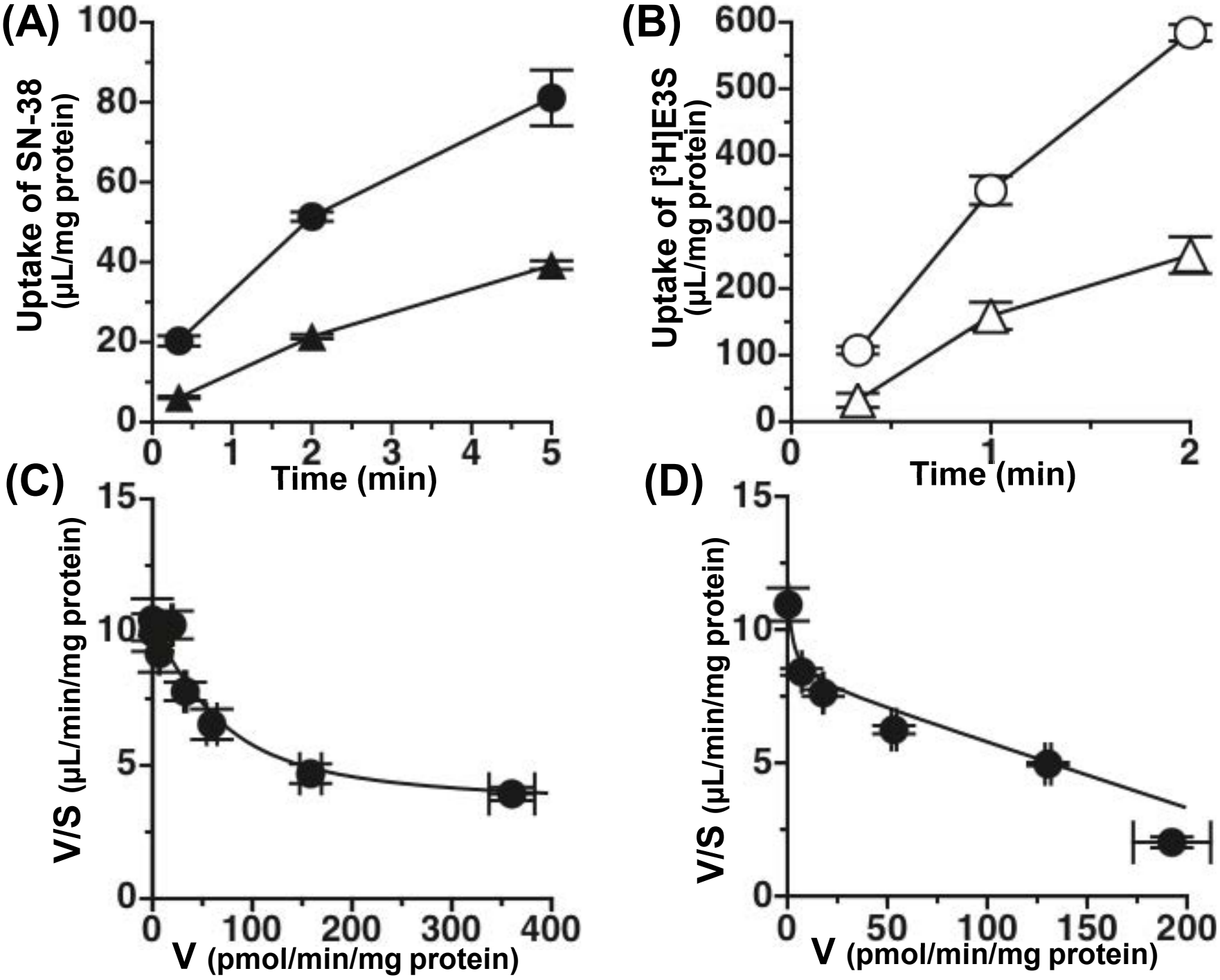

(A)
Uptake of SN-38 (μL/mg protein)
Time (min)
(B)
Uptake of [3H]E3S (μL/mg protein)
Time (min)
(C)
V/S (μL/min/mg protein)
V (pmol/min/mg protein)
(D)
V/S (μL/min/mg protein)
V (pmol/min/mg protein)

Fig. 2

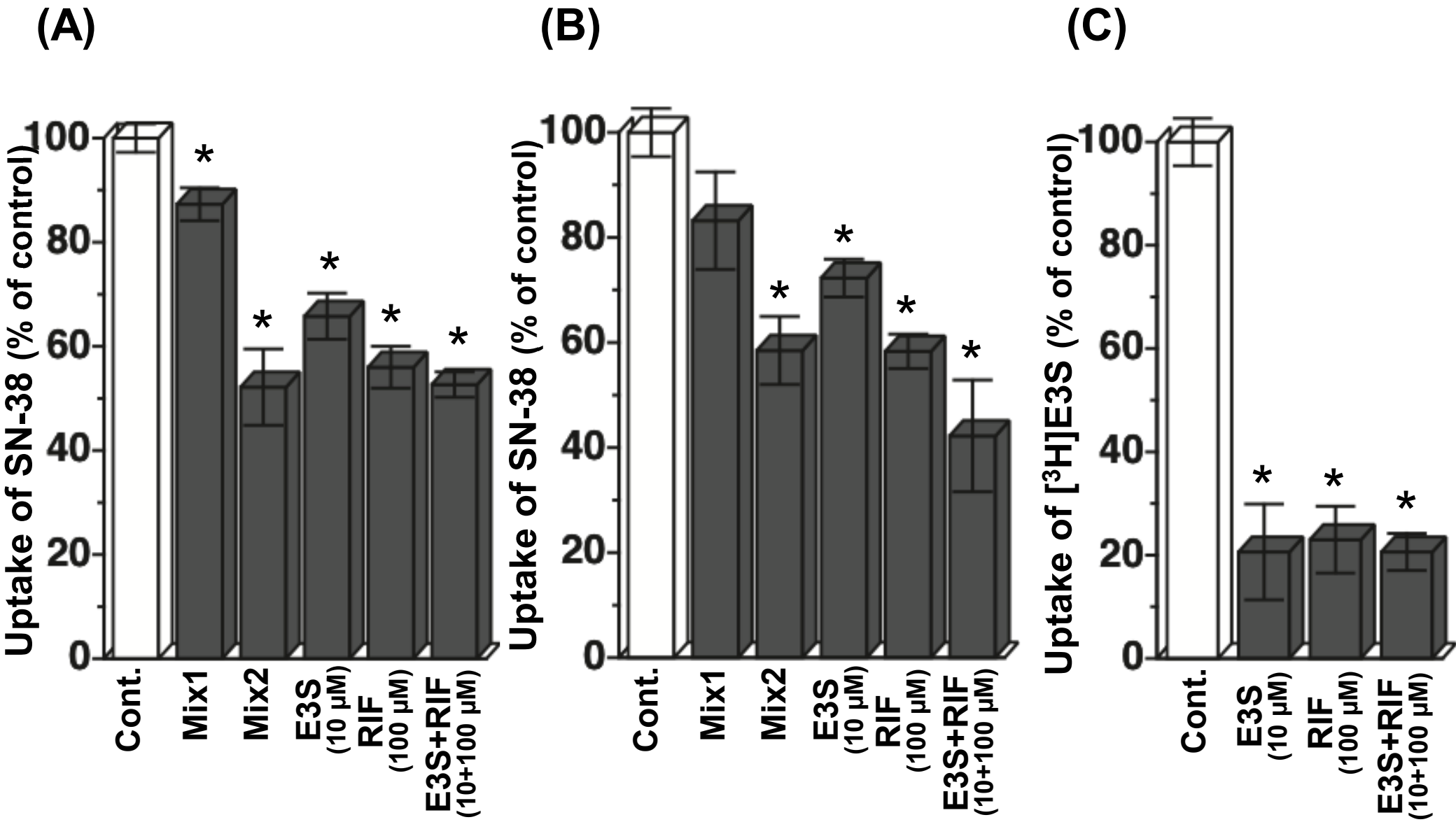

(A)
(B)
(C)
Uptake of SN-38 (% of control)
Uptake of [3H]E3S (% of control)
100
80
60
40
20
0
*
Cont.
Mix1
Mix2
E3S (10 μM)
RIF (100 μM)
E3S+RIF (10+100 μM)

Fig. 3

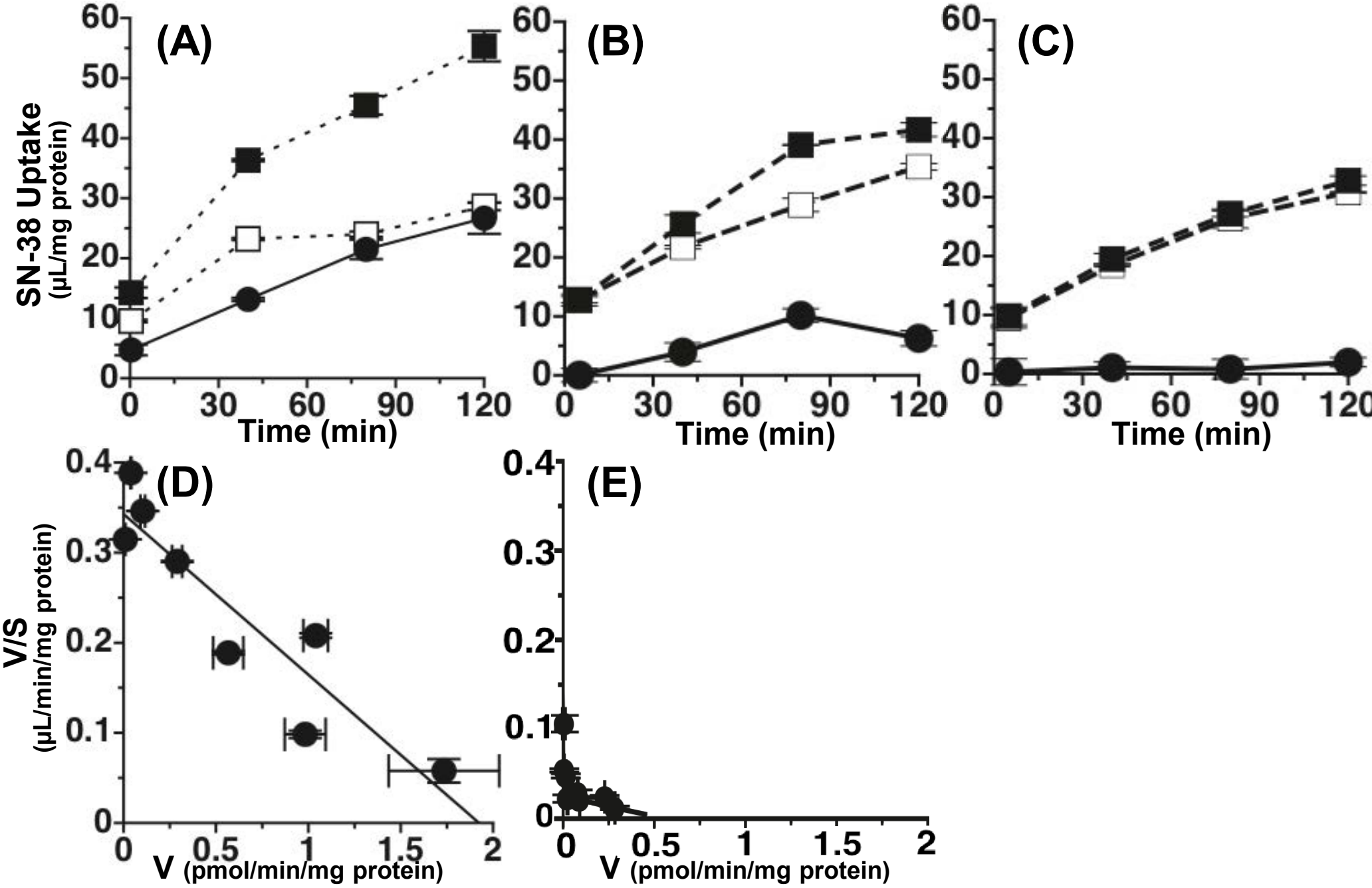

(A)
(B)
(C)
SN-38 Uptake
(μL/mg protein)
Time (min)
Time (min)
Time (min)
(D)
(E)
V/S
(μL/min/mg protein)
V (pmol/min/mg protein)
V (pmol/min/mg protein)

Fig. 4

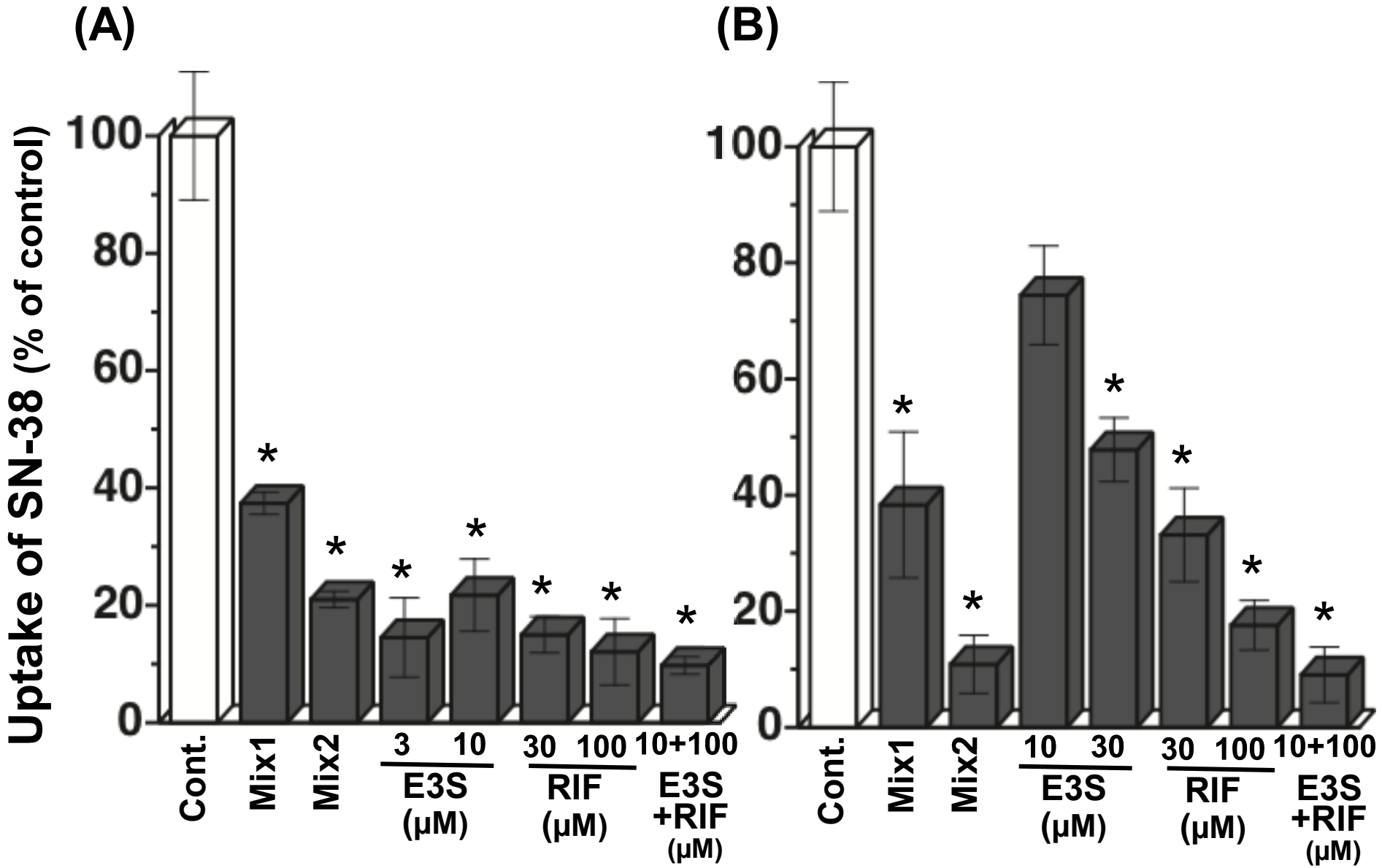

(A)
(B)
Uptake of SN-38 (% of control)
100
80
60
40
20
0
*
Cont.
Mix1
Mix2
3
10
E3S
(μM)
30
100
RIF
(μM)
10+100
E3S
+RIF
(μM)

Fig. 5

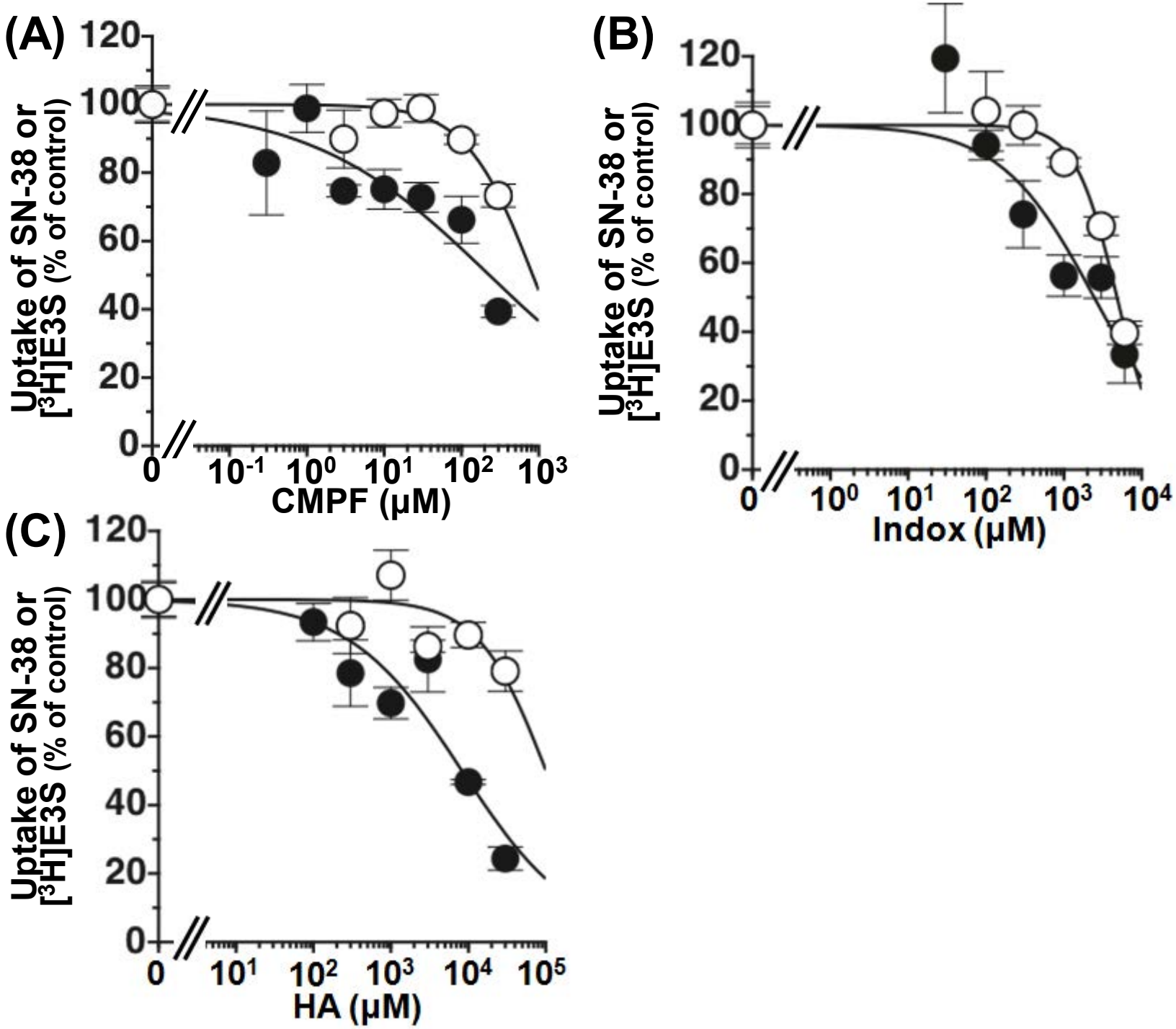

(A)
Uptake of SN-38 or [3H]E3S (% of control)
120
100
80
60
40
20
0
0
10-1
100
101
102
103
CMPF (μM)
(B)
Uptake of SN-38 or [3H]E3S (% of control)
120
100
80
60
40
20
0
0
100
101
102
103
104
Indox (μM)
(C)
Uptake of SN-38 or [3H]E3S (% of control)
120
100
80
60
40
20
0
0
101
102
103
104
105
HA (μM)

Fig. 6

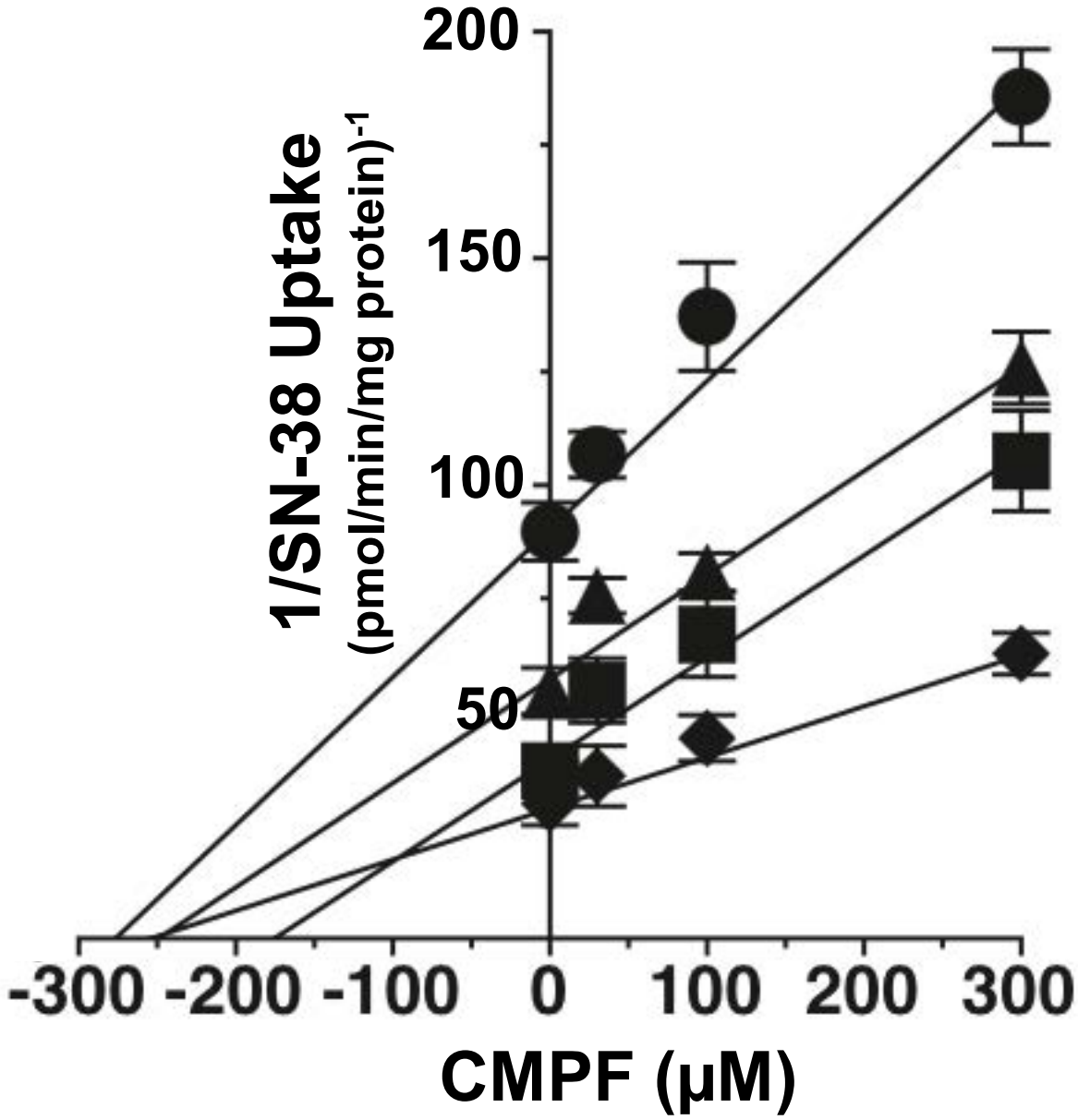

200
150
100
50
1/SN-38 Uptake
(pmol/min/mg protein)-1
-300 -200 -100 0 100 200 300
CMPF (μM)

Fig. 7

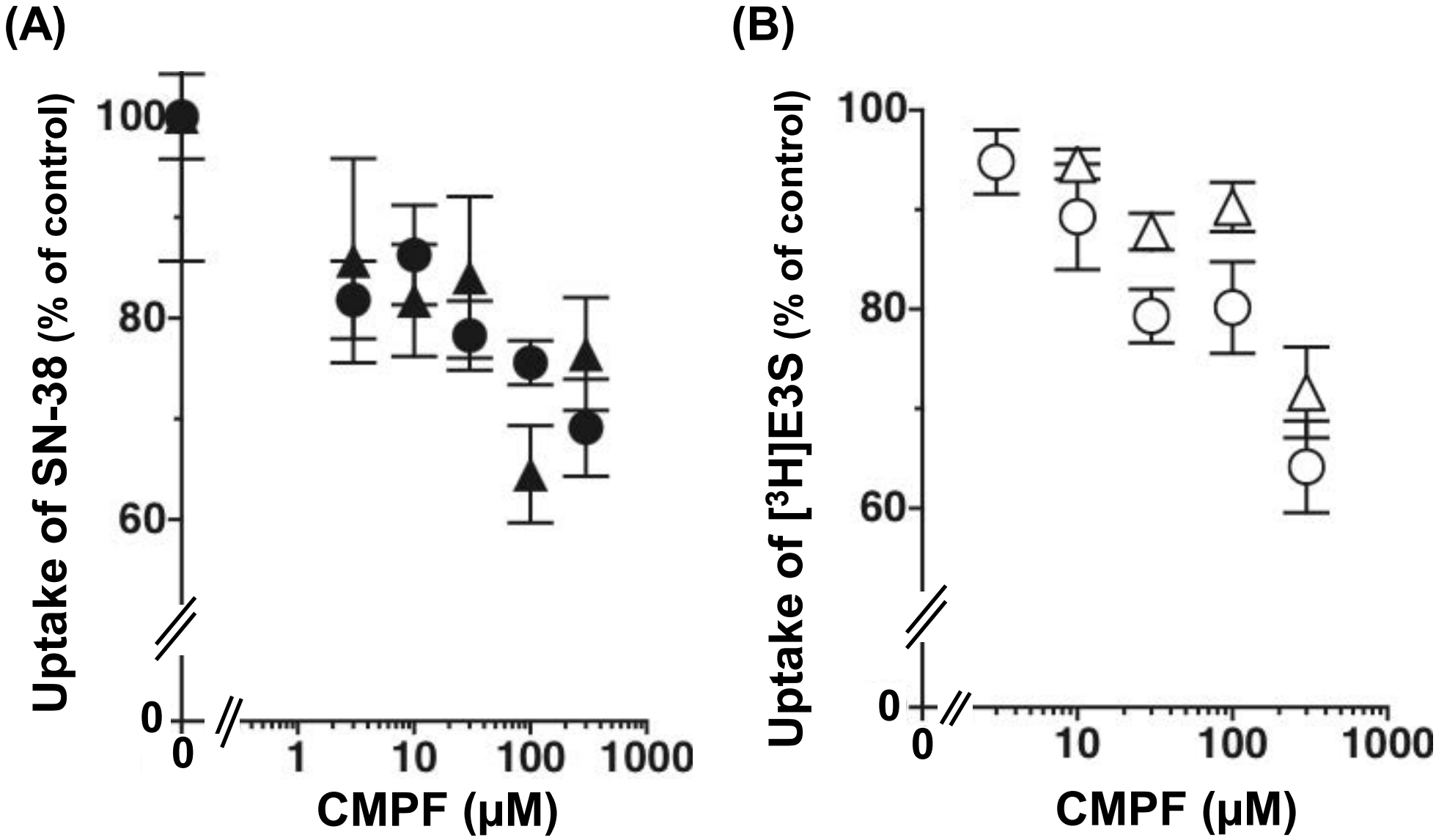
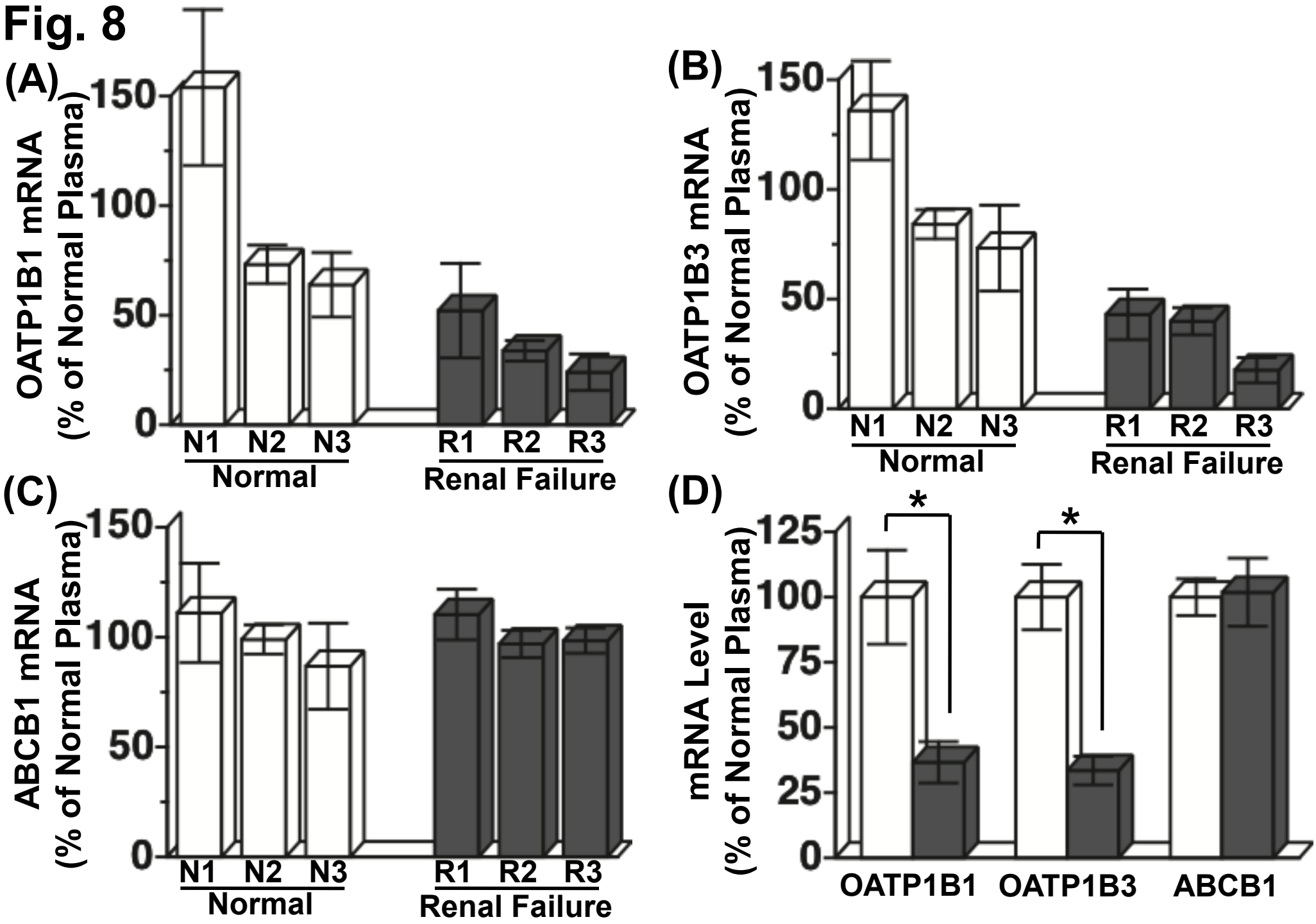

(A)
Uptake of SN-38 (% of control)
100
80
60
0
0
1
10
100
1000
CMPF (μM)
(B)
Uptake of [³H]E3S (% of control)
100
80
60
0
0
10
100
1000
CMPF (μM)

Fig. 8


(A)
OATP1B1 mRNA
(% of Normal Plasma)
150
100
50
0
N1 N2 N3
Normal
R1 R2 R3
Renal Failure
(B)
OATP1B3 mRNA
(% of Normal Plasma)
150
100
50
0
N1 N2 N3
Normal
R1 R2 R3
Renal Failure
(C)
ABCB1 mRNA
(% of Normal Plasma)
150
100
50
0
N1 N2 N3
Normal
R1 R2 R3
Renal Failure
(D)
mRNA Level
(% of Normal Plasma)
125
100
75
50
25
0
*
*
OATP1B1
OATP1B3
ABCB1