# ATP-Dependent Transport of a Novel Thromboxane $A_2$ Receptor Antagonist, [2-(4-Chlorophenylsulfonylaminomethyl)-Indan-5-yl]Acetate (Z-335) and Its Xenobiotic Taurine Conjugate (Z-335-Tau) by Rat Bile Canalicular Membrane Vesicles

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*Received August 1, 2003; accepted November 13, 2003*

***Purpose.*** The characteristics of bile canalicular transport processes for xenobiotic taurine conjugates have not yet been clarified. To elucidate the biliary excretion characteristics of xenobiotic taurine conjugates, we investigated the transport of a novel thromboxane $A_2$ receptor antagonist, Z-335, and its taurine conjugate (Z-335-Tau) across the bile canalicular membrane.

***Methods.*** We examined the uptake of Z-335 and Z-335-Tau by isolated bile canalicular membrane vesicles (CMVs) from Sprague Dawley and Eisai-hyperbilirubinemic rats (EHBRs) which EHBRs have a hereditary defect of canalicular multidrug resistance-associated protein 2 (Mrp2) function. Also, the *in vitro* and *in vivo* kinetics of Z-335-Tau uptake and excretion were compared.

***Results.*** Z-335 uptake by CMVs from normal rats exhibited marked ATP-dependence, whereas ATP-dependent uptake of Z-335 into CMVs from EHBRs was not observed. In contrast, Z-335-Tau uptake into CMVs from both normal rats and EHBRs was ATP dependent. The initial uptake velocity was concentration-dependent, with an *in vitro* Michaelis constant for initial uptake of 189 μM, which was similar to the *in vivo* value.

***Conclusions.*** The biliary excretion of Z-335 involves Mrp2, whereas that of Z-335-Tau involves active transport systems that remain intact in EHBRs and show marked ATP dependence, which ATP-dependent transport is involved in the biliary excretion of Z-335-Tau *in vivo*.

**KEY WORDS:** biliary excretion; Bsep/Spgp; Mdr1a/1b; Mrp2; taurine conjugate.

## INTRODUCTION

[2-(4-Chlorophenylsulfonylaminomethyl)indan-5-yl]acetate (Z-335) is a new orally active thromboxane $A_2$ ($TXA_2$) receptor antagonist that is currently undergoing phase II clinical trials. Z-335 inhibits the specific binding of the $TXA_2$ antagonist [$^3$H]SQ-29548 to human platelets and inhibits human platelet aggregation induced by the $TXA_2$ receptor agonist U-46619. It also ameliorates experimental thrombosis without prolonging bleeding time (1,2). These observations suggest that Z-335 targets the intravascular compartment (i.e., the platelet), and therefore that its pharmacological action will be regulated by its plasma concentration. In rats, Z-335 is concentrated in the liver and eliminated by biliary excretion (3). A subsequent pharmacokinetic study in healthy male human volunteers showed that recovery of the unchanged drug in the urine within 24 h was minimal (4). These results indicate that Z-335 is taken up mainly into the liver and eliminated by metabolism or biliary excretion. We have recently demonstrated that Z-335 is taken up into hepatocytes by the sodium-independent active transport system, and that this uptake is inhibited by substrates of organic anion transporting polypeptides (oatps) such as estradiol 17β-glucuronide, estrone 3-sulfate, taurocholate, pravastatin, and bromosulfophthalein (BSP) (5). Biliary excretion is also important for the elimination of many xenobiotic compounds, most of which are converted into more hydrophilic metabolites by enzymes that catalyze oxidation (e.g., the cytochrome P-450 system) and/or conjugation (e.g., glutathione S-transferase or uridine 5′-phosphate-glucuronosyl-transferase) before being excreted into the bile.

A number of ATP-dependent transporters are expressed in the canalicular membrane of the hepatocyte, and these mediate the active transport of endogenous substrates and many exogenous compounds into the bile. These transporters include the bile salt export pump/sister gene of p-glycoprotein (Bsep/Spgp) and the multidrug resistance (Mdr) and multidrug resistance-associated protein (Mrp) families. Bsep/Spgp is thought to mediate the biliary excretion of bile salts such as taurocholate (6) whereas Mrp2 mediates the biliary excretion of a wide variety of amphipathic anion substrates, including BSP (7), methotrexate (8), and pravastatin (9). Mrp2 is defective in certain hereditary mutant rat strains, such as Eisai hyperbilirubinemic rats (EHBRs) (10). In rodents, the Mdr/p-glycoprotein (P-gp) family has three members: Mdr1a, Mdr1b, and Mdr2. Mdr1a/1b transports many hydrophobic drugs, such as rhodamine 123 (11) and digoxin (12), and is inhibited by verapamil (13,14), whereas Mdr2 is thought to play a role in the biliary excretion of phospholipids (15). Thus, these transporters may be responsible for a large proportion of the hepatobiliary transport of endogenous and exogenous organic compounds. The effects of drug–drug interactions on the biliary excretion process, in which these transporters regulate the hepatic elimination of xenobiotic compounds, must also be taken into consideration. Therefore, it is important to clarify the relative contributions of these transporters to the pathways by which xenobiotic compounds are excreted into bile.

After uptake into the rat liver, Z-335 is mostly metabolized to its taurine conjugate. Both Z-335 and its taurine conjugate (Z-335-Tau) are eliminated mainly by biliary excretion. In a previous study, we characterized the biliary excretion of Z-335 and Z-335-Tau *in vivo* after constant-rate infusion into normal rats and EHBRs and found that the biliary excretion of Z-335 may involve Mrp2, whereas Z-335-Tau is excreted into the bile by active transport systems that remain intact in EHBRs (16). However, the characteristics of bile canalicular transport processes including the driving force for transport have not yet been clarified for xenobiotic taurine conjugates, such as the taurine conjugate of Z-335. In

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order to elucidate the hepatobiliary transport system responsible for the excretion of Z-335 and its taurine conjugate, we investigated the biliary excretion characteristics of these compounds using bile canalicular membrane vesicles (CMVs) prepared from normal rats and EHBRs. We also examined the effects of inhibitors of various hepatobiliary transporters to clarify the mechanisms of biliary excretion of the taurine conjugate of Z-335.

## MATERIALS AND METHODS

### Chemicals

Sodium[2-(4-chlorophenylsulfonylaminomethyl)indan-5-yl]acetate (Z-335; Fig. 1A), its taurine conjugate (Z-335-Tau; Fig. 1B), and ID910096 (sodium[2-(4-chlorophenylsulfonylaminomethyl)indan-5-yl]ethylcarboxylate, used as the internal standard for the assay) were synthesized in the central research laboratories of ZERIA Pharmaceutical Co., Ltd. (Saitama, Japan). [$^{14}$C]Z-335 (827 MBq/mmol) and [$^{14}$C]Z-335-Tau (3.8 GBq/mmol) were synthesized by Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). [$^{3}$H]Taurocholate (1850 GBq/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO, USA). [$^{3}$H]Digoxin (1369 GBq/mmol) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). Estradiol 17β-glucuronide, estrone 3-sulfate, probenecid, taurocholate, cholate, glycocholate, digoxin, verapamil, and cyclosporin A were purchased from Sigma Chemical Co., Ltd. (St Louis, MO, USA). ATP, creatine phosphate, creatine phosphokinase, ADP, AMP, and GTP were also purchased from Sigma. Bromosulfophthalein (BSP) and sodium orthovanadate were purchased from Aldrich (Milwaukee, WI, USA). Glutathione (GSH) was purchased from Wako Pure Chemical (Osaka, Japan). All other chemicals were of reagent grade.

### Animals

Male Sprague Dawley rats (weighing 230–300 g) were obtained from Charles River Japan, Inc. (Kanagawa, Japan), and male EHBRs (also weighing 230–300 g) were obtained from SLC Co. Ltd. (Shizuoka, Japan). All animals were treated in accordance with the guidelines for animal experimentation stipulated by the ethics committee of ZERIA Pharmaceutical Co., Ltd.

### Preparation of CMVs

CMVs were prepared from the livers of normal rats and EHBRs as described previously (17). They were then suspended in 50 mM Tris-HCl (pH 7.4) containing 250 mM sucrose and frozen at –80°C until required for use. To check the purity of the prepared CMVs, alkaline phosphatase was determined by the method of Yachi *et al.* (18). As Yachi *et al.* had used CMVs with enriched alkaline phosphatase activity (mean ± SE) 49.8 ± 9.6 times as that in the homogenate, only CMVs exhibiting more than 30 times as the alkaline phosphatase activity of the homogenate were used.

Their transport activity was also checked by measuring the ATP-dependent uptake of a standard substrate, [$^{3}$H]taurocholate (1 μM), during a 3-min incubation at 37°C. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard.

### Uptake Study

Uptake studies were performed using [$^{14}$C]Z-335 (100 μM) and [$^{14}$C]Z-335-Tau (10 μM), as reported previously (19,20). The transport medium (10 mM Tris-HCl 250 mM sucrose and 10 mM $MgCl_2 \cdot 6H_2O$, pH 7.4) contained the ligands, 5 mM ATP, and an ATP-regenerating system (10 mM creatine phosphate and 100 μg/ml creatine phosphokinase). An aliquot of transport medium (18 μl) was mixed rapidly with the vesicle suspension (10 μg protein in 2 μl). The transport reaction was stopped by addition of 1 ml of ice-cold buffer containing 250 mM sucrose, 10 mM Tris-HCl, and 100 mM NaCl (pH 7.4). The stopped reaction mixture was passed through a 0.7-μm GF/F filter (Whatman International Ltd., Kent, UK) and then washed twice with 5 ml of stop-solution. Radioactivity retained on the filter and the reaction mixture were mixed with scintillation cocktail (Clear-sol II; Nacalai Tesque, Kyoto, Japan), and the radioactivity in the medium and CMVs was measured using a liquid scintillation counter (2900; Packard Instrument Co., Ltd., Downer Grove, IL, USA). Initial uptake velocities of [$^{14}$C]Z-335-Tau and [$^{3}$H]digoxin were calculated at 30 s and 5 min, respectively. Uptake of ligands was normalized with respect to the amount of membrane protein.

### *In vivo* Experiments

Normal rats were anesthetized with pentobarbital (50 mg/kg), and the common bile duct was cannulated with polyethylene tubing (PE10) to collect the bile. Z-335 was dissolved in physiological saline (0.9% NaCl) and infused through the femoral vein cannula at a flow rate of 20 μl/min, and the infusion rates were set at 10, 20, 50, 100, and 200 nmol $min^{-1}$ $kg^{-1}$. The rats were killed at 65 min after starting the infusion. Their livers were immediately excised, rinsed with 250 mM sucrose containing 50 mM Tris-HCl buffer (pH 7.4), weighed, and homogenized in 250 mM sucrose containing 50 mM Tris-HCl buffer (pH 7.4) to form a 25% W/V homogenate.

### Measurement of Z-335-Tau

The homogenate specimens (50 μl) were added to methanol (1 ml). After centrifuging the mixture, the supernatant was evaporated to dryness under nitrogen gas in a

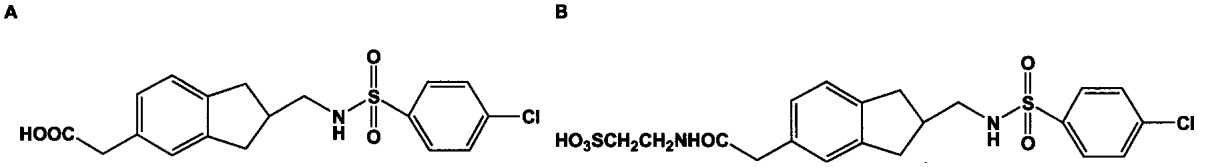


**Fig. 1.** Structures of (A) Z-335 and (B) Z-335-Tau.

water bath at 40°C, and the residue was dissolved in 100 μl of mobile phase (see below for composition). The bile specimens were diluted with saline to 5 ml. The concentration of Z-335-Tau in each sample was determined by high-performance liquid chromatography (HPLC) using a reverse phase column (Capcell Pak MF C8; Shiseido, Tokyo, Japan). A solvent delivery system (LC-9A), auto-injector (SIL-6B), and UV detector (SPD-10A; all from Shimazu, Kyoto, Japan) were also included in the HPLC system. The UV absorbance was monitored at 230 nm. The mobile phase consisted of 0.1 M $KH_2PO_4$ (pH 7.0), $CH_3CN$, and isopropanol (85:11:4) pumped at a flow rate of 0.9 ml/min.

### Determination of Kinetic Parameters

All fittings described below were calculated by a nonlinear least squares method using the MULTI program (21).

*In vitro Experiments*

The kinetic parameters for Z-335-Tau and digoxin uptake were estimated according to the following equation:

$$v_0 = V_{max} \cdot S/(Km + S) + P_{dif.} \cdot S \quad (1)$$

where $v_0$ is the initial uptake velocity of Z-335-Tau (nmol $min^{-1}$ $mg^{-1}$ protein) and digoxin (pmol $min^{-1}$ $mg^{-1}$ protein), S is the concentration of Z-335-Tau and digoxin in the medium (μM), Km is the Michaelis constant (μM), $V_{max}$ is the maximum uptake velocity (nmol $min^{-1}$ $mg^{-1}$ protein or pmol $min^{-1}$ $mg^{-1}$ protein), and $P_{dif.}$ is the nonspecific uptake clearance (μl $min^{-1}$ $mg^{-1}$ protein).

*In vivo Experiments*

The kinetic parameters for Z-335-Tau biliary excretion were estimated according to the following equation:

$$v_{bile} = V_{max,\,in\ vivo} \cdot C_{liver}/(Km_{,in\ vivo} + C_{liver}) + P_{dif.,\,in\ vivo} \cdot C_{liver} \quad (2)$$

where $v_{bile}$ is the biliary excretion rate of Z-335-Tau (nmol $min^{-1}$ $kg^{-1}$) between 55 and 65 min, $C_{liver}$ is the concentration of Z-335-Tau in the liver (μM), assuming that 1 g liver = 0.71 ml (22), and $Km_{,in\ vivo}$ (μM), $V_{max,in\ vivo}$ (nmol $min^{-1}$ $kg^{-1}$), and $P_{dif.,in\ vivo}$ (ml $min^{-1}$ $kg^{-1}$) represent the Michaelis constant (defined as $C_{liver}$ for the purposes of the current study), the maximum excretory velocity, and the nonspecific diffusion clearance, respectively.

### Inhibition Study

The inhibition constants (Ki), assuming competitive inhibition due to previously identified transport systems of inhibitors (6,7,12–14,24–26,28), were calculated according to the following equation from the data obtained by varying the inhibitor concentration (I; digoxin added at 0.1, 0.5, 1, 5, 10, 50, and 100 μM, and all other inhibitors added at 1, 5, 10, 50, 100, 500, and 1000 μM to the incubation medium) with the Z-335-Tau concentration kept constant (10 μM).

$$v_0 = V_{max} \cdot S/[Km \cdot (1 + I/Ki) + S] + P_{dif.} \cdot S \quad (3)$$

where the Km (189 μM), $V_{max}$ (9.45 nmol $min^{-1}$ $mg^{-1}$ protein) and $P_{dif.}$ (14.8 μl/min/mg protein) values obtained as described (Fig. 6A) were used.

## RESULTS

### Time Course of Uptake of Z-335 and Z-335-Tau into CMVs Prepared from Normal Rats and EHBRs

The time courses of ATP-dependent uptake of [$^{14}$C]Z-335 (100 μM) and [$^{14}$C]Z-335-Tau (10 μM) by CMVs prepared from normal rats and EHBRs are shown in Figs. 2 and 3. ATP-stimulated uptake of [$^{14}$C]Z-335 was observed in CMVs prepared from normal rats, but not in those prepared

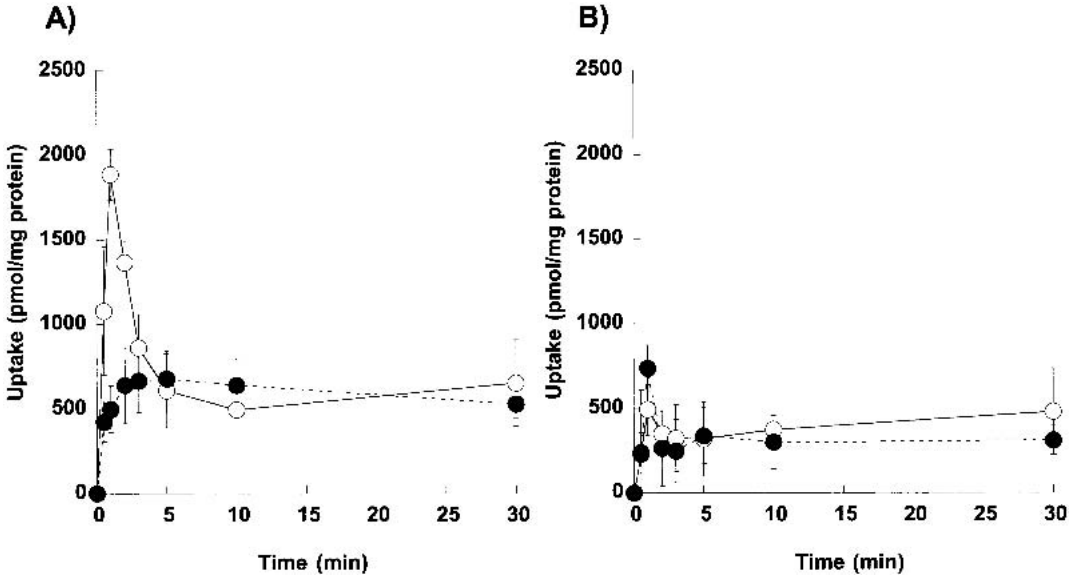


**Fig. 2.** Time course of [$^{14}$C]Z-335 (100 μM) uptake by canalicular membrane vesicles (CMVs) prepared from (A) normal rats and (B) Eisai-hyperbilirubinemic rats. After a 3-min preincubation, the uptake reaction was started by adding the CMVs (10 μg protein). The reaction mixtures were incubated at 37°C with (open circles) or without (closed circles) ATP (5 mM) and an ATP-regenerating system (10 mM creatine phosphate and 100 μg/ml creatine phosphokinase). Each point and vertical bar represents the mean ± SE for three determinations.

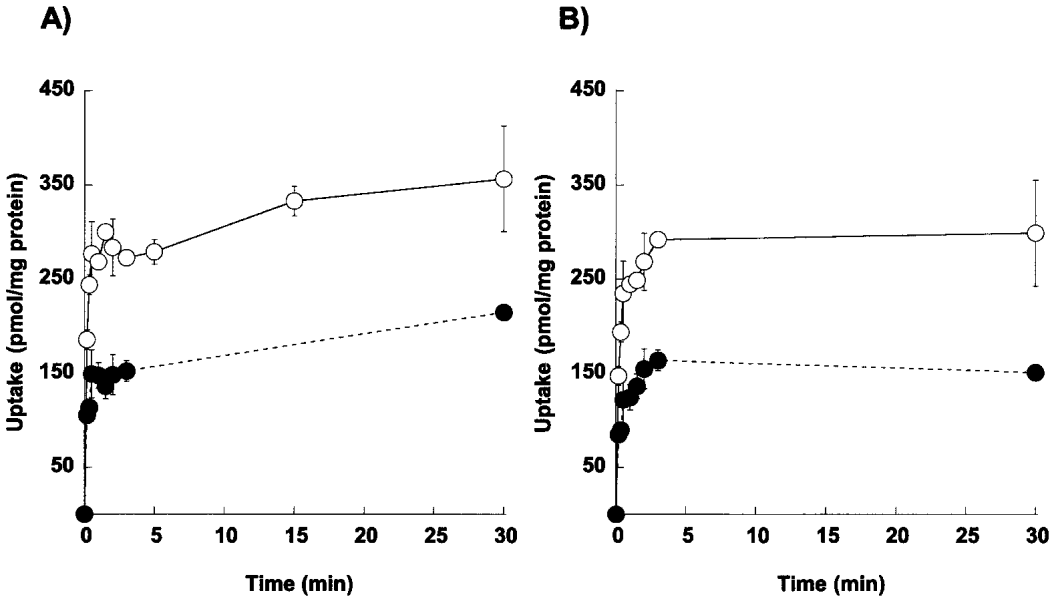


**Fig. 3.** Time course of [$^{14}$C]Z-335-Tau (10 μM) uptake by canalicular membrane vesicles (CMVs) prepared from (A) normal rats and (B) Eisai-hyperbilirubinemic rats. After a 3-min preincubation, the uptake reaction was started by adding the CMVs (10 μg protein). The reaction mixtures were incubated at 37°C with (open circles) or without (closed circles) ATP (5 mM) and an ATP-regenerating system (10 mM creatine phosphate and 100 μg/ml creatine phosphokinase). Each point and vertical bar represents the mean ± SE for three determinations.

from EHBRs. In contrast, ATP-stimulated uptake of [$^{14}$C]Z-335-Tau was observed in CMVs prepared from both normal rats and EHBRs.

### Osmolarity Sensitivity of Z-335-Tau Uptake into CMVs

To confirm that the vesicle-associated uptake of [$^{14}$C]Z-335-Tau reflected transport into the vesicular space, rather than binding to the surface, [$^{14}$C]Z-335-Tau uptake was measured in transport media containing several different concentrations of sucrose and was found to decrease as the sucrose concentration increased (Fig. 4). Moreover, the *y* intercept for the relationship between the vesicle-associated uptake of Z-335-Tau and the reciprocal of the sucrose concentration in the medium, which indicates the binding of [$^{14}$C]Z-335-Tau to the vesicular surface, was similar in the presence (57.3 pmol/mg protein) and absence (60.8 pmol/mg protein) of ATP.

### Nucleotide Specificity of Z-335-Tau Uptake into CMVs and the Effect of Vanadate

To establish the nucleotide dependence of [$^{14}$C]Z-335-Tau transport and to determine whether ATP hydrolysis was required, [$^{14}$C]Z-335-Tau uptake was measured in the presence of various nucleotides and vanadate. Of the four nucleotides tested, [$^{14}$C]Z-335-Tau uptake was most efficient in the presence of ATP (Fig. 5). This ATP-dependent uptake of [$^{14}$C]Z-335-Tau was inhibited by vanadate, suggesting that ATP hydrolysis is required for uptake to proceed (Fig. 5).

### Concentration Dependence of Z-335-Tau Uptake into CMVs Prepared from Normal Rats

The initial uptake velocity for [$^{14}$C]Z-335-Tau was concentration-dependent (Fig. 6A), indicating a two-component process. The estimated kinetic parameters (mean ± SE) were

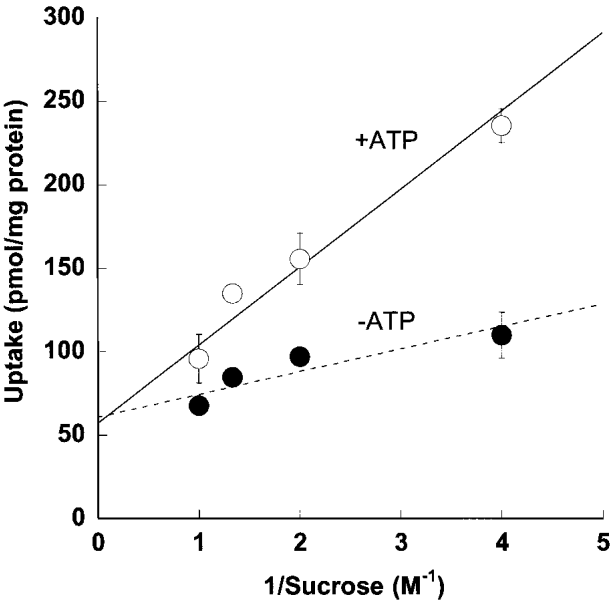


**Fig. 4.** Effects of medium osmolarity on the uptake of [$^{14}$C]Z-335-Tau into canalicular membrane vesicles (CMVs) prepared from normal rats. The uptake was measured in CMVs (10 μg protein) preincubated for 15 min at 37°C with 0.25–1 M sucrose. After preincubation, the reaction was started by adding the ligand. The reaction mixtures were incubated for 2 min at 37°C with (open circles) or without (closed circles) ATP (5 mM) and an ATP-regenerating system (10 mM creatine phosphate and 100 μg/ml creatine phosphokinase). Each point and vertical bar represents the mean ± SE for three determinations.

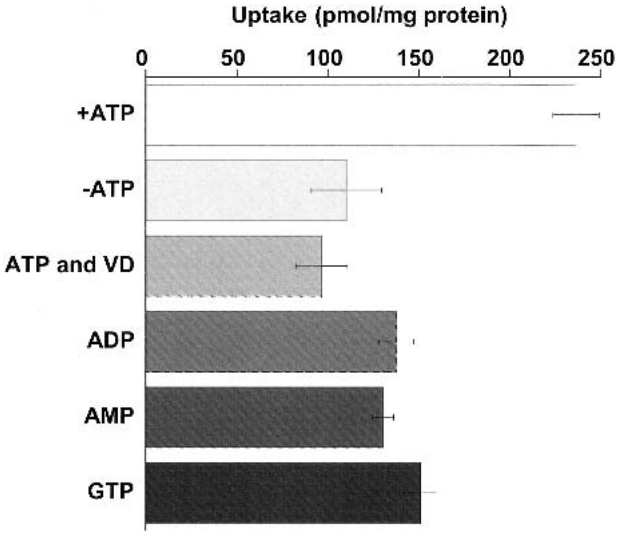


**Fig. 5.** Effects of various nucleotides and vanadate (VD) on the uptake of [$^{14}$C]Z-335-Tau by canalicular membrane vesicles (CMVs) prepared from normal rats. After a 3-min preincubation, the uptake reaction was started by adding the CMVs (10 μg protein). The reaction mixtures were incubated at 37°C with or without the nucleotides (5 mM) and an ATP-regenerating system (10 mM creatine phosphate and 100 μg/ml creatine phosphokinase). The concentration of VD in the incubation medium was 1000 μM. Each column and vertical bar represents the mean ± SE for five determinations.

Km = 189 ± 3 μM, $V_{max}$ = 9.45 ± 0.31 nmol min$^{-1}$ mg$^{-1}$ protein, and $P_{dif.}$ = 14.8 ± 0.8 μl min$^{-1}$ mg$^{-1}$ protein (Table I).

### Concentration Dependence of the Biliary Excretion of Z-335-Tau

Figure 6B shows the concentration dependency of the biliary excretion of Z-335-Tau. At any infusion rate, the biliary excretion rate of Z-335-Tau ($v_{bile}$) saturated with the increase in the liver concentration of Z-335-Tau and indicated a two-component process. The estimated kinetic parameters were: $Km_{,in\ vivo}$ = 167 μM, $V_{max,in\ vivo}$ = 87.9 nmol min$^{-1}$ kg$^{-1}$, and $P_{dif.,in\ vivo}$ = 0.04 ml min$^{-1}$ kg$^{-1}$ (Table I).

### Inhibition of the Initial Uptake Velocity of Z-335-Tau by Organic Anions, Bile Acids, and Other Substrates

To investigate the specificity of [$^{14}$C]Z-335-Tau uptake, the effects of other substrates on its uptake were studied. [$^{14}$C]Z-335-Tau uptake was decreased by addition of taurocholate, glycocholate, cholate, estrone 3-sulfate, estradiol 17β-glucuronide, or BSP, with Ki values of 337, 531, 395, 405, 530, and 140 μM, respectively (Fig. 7A–C; Table II). To compare the Ki value of digoxin on Z-335-Tau uptake with its own Km value, we also estimated the kinetic parameters of digoxin on the uptake into CMVs. The estimated kinetic parameters (mean ± SE) were Km = 0.57 ± 0.13 μM, $V_{max}$ = 14.5 ± 1.4 pmol min$^{-1}$ mg$^{-1}$ protein, and $P_{dif.}$ = 3.34 ± 0.33 μl min$^{-1}$ mg$^{-1}$ protein. [$^{14}$C]Z-335-Tau uptake was also decreased by addition of digoxin, although the Ki value was 119.1 μM, considerably higher than the Km value for digoxin (Fig. 7C; Table II). In contrast, probenecid (Fig. 7C; Table II),

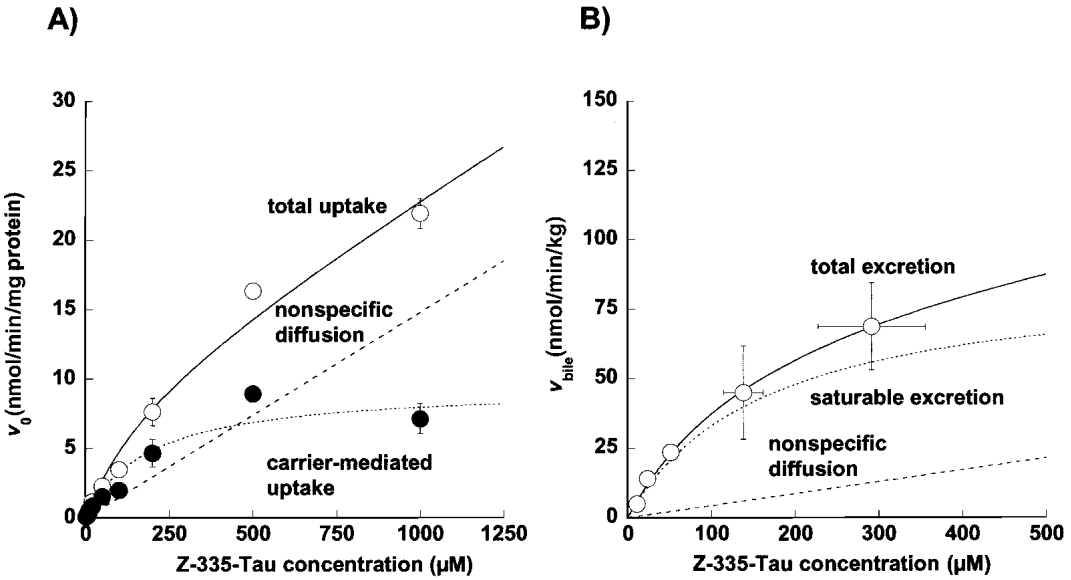


**Fig. 6.** Concentration dependence of ATP-dependent [$^{14}$C]Z-335-Tau uptake by canalicular membrane vesicles prepared from (A) normal rats and (B) steady-state biliary excretion of Z-335-Tau during the *in vivo* experiments. The relationship between the initial uptake velocity ($v_0$) and the concentration of [$^{14}$C]Z-335-Tau in the incubation medium is shown. Each point and vertical bar represents the mean ± SE for three (*in vitro*) or five (*in vivo*) experiments. The solid line shows the least-square fit of the data used in Eqs. (1) (*in vitro*) and (2) (*in vivo*) (see "Materials and Methods" section for details). The dashed line represents nonspecific diffusion calculated using the value for nonspecific uptake clearance ($P_{dif.}$). Carrier-mediated uptake (closed circles) was calculated by subtracting nonspecific diffusion from total uptake (open circles) and indicates saturable uptake. The dotted line shows the theoretical curve for saturable uptake.

**Table I.** Kinetic Parameters for CMVs Uptake and *in vivo* Biliary Excretion of Z-335-Tau

| | |
|---|---|
| *In vitro** | |
| $V_{max}$ (nmol/min/mg protein) | 9.45 ± 0.31 |
| Km (μM) | 189 ± 3 |
| $P_{dif.}$ (μl/min/mg protein) | 14.8 ± 0.8 |
| *In vivo*† | |
| $Vmax_{,in\ vivo}$(nmol/min/kg) | 87.9 |
| $Km_{,in\ vivo}$ (μM) | 167 |
| $P_{dif.,\ in\ vivo}$ (ml/min/kg) | 0.04 |

CMVs, canalicular membrane vesicles.
* Mean ± SE from three determinations, calculated from data shown in Fig. 6A by use of Eq. (1).
† Calculated from data shown in Fig. 6B by use of Eq. (2).

GSH, verapamil, and cyclosporin A had no inhibitory effects on [$^{14}$C]Z-335-Tau uptake (Table III).

## DISCUSSION

The biliary excretion of several organic anions is mediated by the canalicular organic anion transporter (cMOAT/Mrp2), which shows a hereditary defect in some mutant rat strains, including EHBRs. In a previous study, we characterized the biliary excretion of Z-335 and Z-335-Tau *in vivo* after constant-rate infusion into normal rats and EHBRs and found that the biliary excretion of Z-335 may involve Mrp2, whereas Z-335-Tau is excreted into the bile by active transport systems that remain intact in EHBRs (16). However, the characteristics of bile canalicular transport processes including the driv-

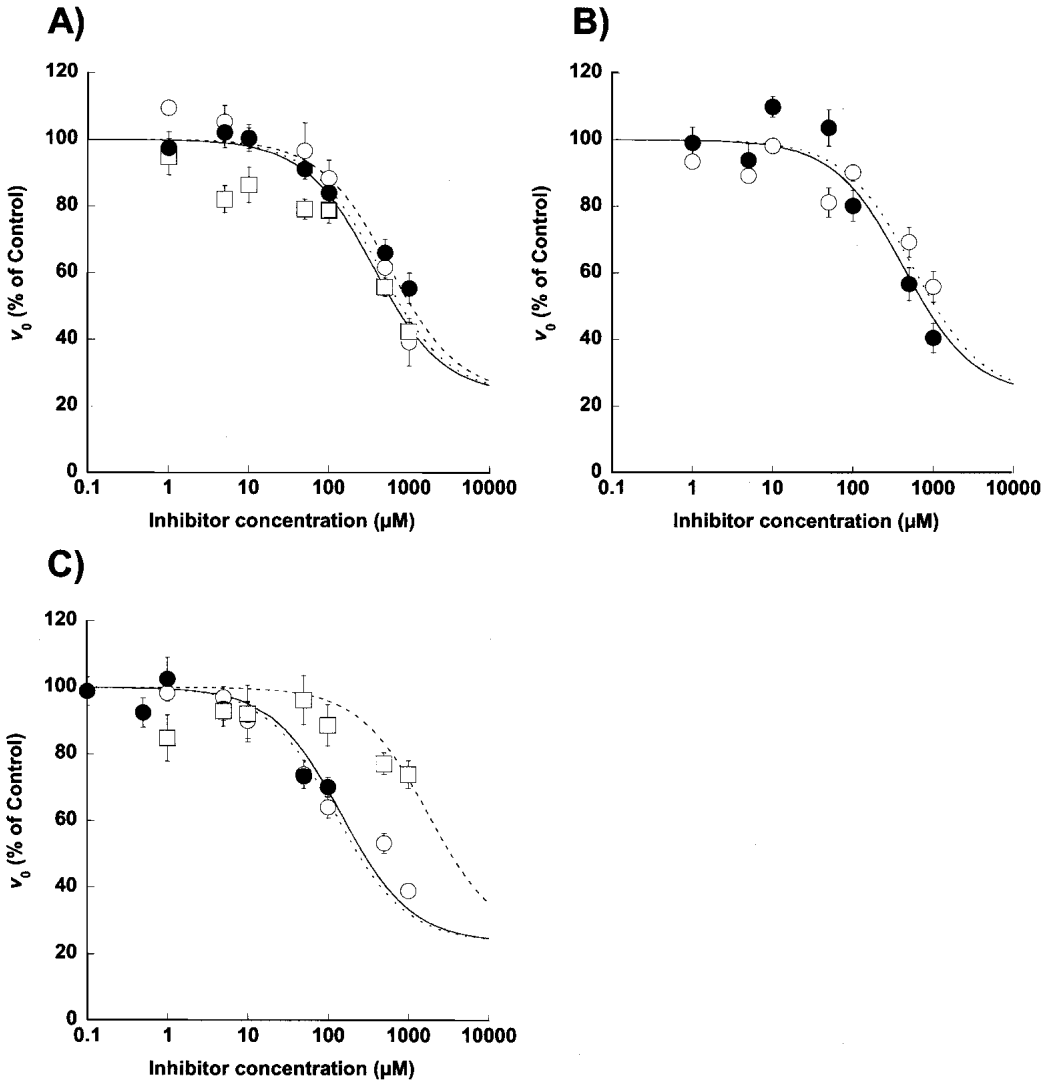


**Fig. 7.** Inhibition of [$^{14}$C]Z-335-Tau (10 μM) uptake by (A) bile acids, (B) conjugated steroids, and (C) other substrates. Each point and vertical bar represents the mean ± SE for six determinations, calculated by use of Eq. (3), assuming that all compounds competitively inhibit the Z-335-Tau. Where vertical bars are not shown, the SE is within the limits of the symbols. (A) initial uptake velocity ($v_0$) of [$^{14}$C]Z-335-Tau as a function of the concentration of taurocholate (1–1000 μM; open circles), glycocholate (1–1000 μM; closed circles), and cholate (1–1000 μM; open squares) in the medium. (B) $v_0$ of [$^{14}$C]Z-335-Tau as a function of the concentration of estrone 3-sulfate (1–1000 μM; open circles) and estradiol 17β-glucuronide (1–1000 μM; closed circles) in the medium. (C) $v_0$ of [$^{14}$C]Z-335-Tau as a function of the concentration of bromosulfophthalein (1–1000 μM; open circles) and digoxin (0.1–100 μM; closed circles), probenecid (1–1000 μM; open squares) in the medium.

**Table II.** Relationships Among Ki for [$^{14}$C]Z-335-Tau Uptake and Km Values for Various Drugs and Endogenous Substrates

| | Ki (μM)* | Km (μM) |
|---|---|---|
| Taurocholate | 337 ± 37 | 47 (29) |
| Glycocholate | 531 ± 90 | — |
| Cholate | 395 ± 89 | — |
| Estrone 3-sulfate | 405 ± 24 | — |
| Estradiol 17β-glucuronide | 530 ± 99 | 6.32 (24) |
| BSP | 140 ± 26 | 31 (7) |
| Digoxin | 119 ± 17 | 0.57 ± 0.13† |
| Probenecid | >1000 | — |

Km values for taurocholate, estradiol 17β-glucuronide, and BSP with reference to previous reports (29,24,7). BSP, bromosulfophthalein.
* Mean ± SE from six determinations, calculated by Eq. (3), assuming that all compounds competitively inhibit the uptake of Z-335-Tau.
† Mean ± SE from three determinations, calculated using Eq. (1).

ing force for transport have not been clarified for xenobiotic taurine conjugates such as the taurine conjugate of Z-335. In the current study, we investigated the biliary excretion characteristics of Z-335 and Z-335-Tau using CMVs from normal rats and EHBRs. The uptake of Z-335 by CMVs prepared from normal rats was clearly ATP-dependent, and over-shoot phenomenon was observed, whereas uptake by CMVs from EHBRs did not occur (Fig. 2). This finding is consistent with our previous results (16). In contrast, Z-335-Tau uptake by CMVs from both normal rats and EHBRs was ATP-dependent (Fig. 3), indicating that the Z-335-Tau uptake mechanism remains intact in EHBRs. However, Z-335-Tau uptake did not exhibit an over-shoot phenomenon. This result indicating that Z-335-Tau was more hydrophilic than Z-335, and that efflux of Z-335-Tau into medium from CMVs did not occur. Taken together, these results suggest that Z-335-Tau is taken up into CMVs by ATP-dependent active transport systems other than Mrp2.

To support this hypothesis, the uptake of Z-335-Tau was measured in the presence of several different concentrations of sucrose as well as various nucleotides and vanadate. In the presence of ATP, Z-335-Tau uptake fell as the osmolarity of the medium increased (Fig. 4). ATP-dependent uptake was also inhibited by vanadate, which has previously been shown to inhibit many ATPases (23), and nucleotides other than ATP did not stimulate uptake (Fig. 5). Furthermore, the uptake of Z-335-Tau was shown to be saturable (Fig. 6). These results suggested that the ATP stimulation observed in the uptake of Z-335-Tau was not due to binding to the surface of the CMVs but to carrier-mediated primary active transport into the CMVs.

**Table III.** Effect of Various Substrates and Inhibitors on the ATP-Dependent Uptake of [$^{14}$C]Z-335-Tau Uptake into CMVs

| | % of control |
|---|---|
| Control | 100 |
| Cyclosporin A | |
| 100 μM | 88.1 ± 5.3 |
| 1000 μM | 83.4 ± 3.9 |
| Verapamil | |
| 100 μM | 100 ± 5 |
| 1000 μM | 116 ± 5 |
| GSH | |
| 100 μM | 100 ± 2 |
| 1000 μM | 109 ± 4 |

Mean ± SE from six determinations. CMVs, canalicular membrane vesicles.

To demonstrate directly that ATP-dependent transport is involved in the biliary excretion of Z-335-Tau *in vivo*, we compared *in vitro* and *in vivo* kinetic parameters for the uptake and excretion processes. The $Km_{,in\ vivo}$, (defined as the total concentration in the liver) for normal rats was 167 μM (Table I). We had previously evaluated the fraction of Z-335-Tau in the liver cytosol during a constant rate infusion study and found it to be 0.71 (16). Based on this previous result, the Km value was calculated to be approximately 119 μM. In the current study, $Km_{,in\ vitro}$ was 189 μM (Table I) and was thus similar to $Km_{,in\ vivo}$. The contributions of carrier-mediated uptake to total uptake in a linear range *in vitro* $\{[(V_{max,in\ vitro} / Km_{,in\ vitro})/(V_{max,in\ vitro} / Km_{,in\ vitro} + P_{dif.,in\ vitro.})]\}$ and *in vivo* $\{[(V_{max,in\ vivo} / Km_{,in\ vivo})/(V_{max,in\ vivo} / Km_{,in\ vivo} + P_{dif.,in\ vivo.})]\}$ can therefore be calculated as 77.2% and 92.4%, respectively, indicating that the excretion of Z-335-Tau into the bile would occur mainly via the carrier-mediated transport system. Thus, ATP-dependent transport appears to be involved in the biliary excretion of Z-335-Tau in *in vivo*.

Subsequently, to investigate the specificity of the biliary excretion pathway of Z-335-Tau, the effects of taurocholate, glycocholate, cholate, estrone 3-sulfate, estradiol 17β-glucuronide, BSP, digoxin and probenecid on its uptake by CMVs from normal rats were studied. Z-335-Tau uptake was inhibited by all of these substrates; however, the Ki values for taurocholate, BSP, and estradiol 17β-glucuronide determined in the Z-335-Tau uptake study were higher than their own Km values (Fig. 7; Table II). Probenecid had hardly any inhibitory effect on Z-335-Tau uptake (Ki > 1000 μM) (Fig. 7C; Table II). In this study, we also estimated the Km value of digoxin, whose Km value for uptake into CMVs was 0.57 μM. The Ki value of digoxin for Z-335-Tau uptake was 119 μM, considerably higher than the Km value of digoxin (Fig. 7C; Table II). Furthermore, GSH, verapamil, and cyclosporin A had hardly any inhibitory effect on Z-335-Tau uptake (Table III). Mrp2 is known to mediate the biliary excretion of BSP (7), methotrexate (8), estradiol 17β-glucuronide (24) and pravastatin (9), and the Mrp2-mediated uptake of S-(2,4-dinitrophenyl)-glutathione (DNP-SG) is inhibited by probenecid (25). Bsep/Spgp is thought to mediate the biliary excretion of taurocholate (6), whereas Mdr1a/1b transports many hydrophobic drugs, such as rhodamine 123 (11), cyclosporin A (26), and digoxin (12) and is inhibited by verapamil (13,14). Therefore, these results indicate that Z-335-Tau and taurocholate, glycocholate, cholate, estradiol 17β-glucuronide, BSP, digoxin, and probenecid are taken up into CMVs by different carrier proteins. On the other hand, the uptake of Z-335-Tau into CMVs was inhibited by estrone 3-sulfate with an estimated Ki value of 405.4 μM (Fig. 7B; Table II). Recently, it has been reported that the breast cancer resistance protein (BCRP/ABCG2) is expressed in bile canalicular membranes (27), although its role in the liver has not been clarified as yet. Nevertheless, its expression in the canalicular membrane indicates that, like other ABC transporters such as Mdr1a/1b and Mrp2, BCRP may be involved in the hepatic excretion processes of endogenous substrates and many exogenous

compounds. Takenaka *et al.* reported that the uptake of E3040-glucuronide into CMVs was decreased in EHBRs compared with normal rats, and that uptake of E3040-glucuronide into CMVs was involved in Mrp2 (20). However, slight ATP-dependent uptake of E3040-glucuronide into CMVs prepared from EHBRs still remained the same as Z-335-Tau. More recently, Suzuki *et al.* demonstrated that the uptake of estrone 3-sulfate into human ABCG2 expressed membrane vesicles was stimulated by ATP (28), that E3040-glucuronide and estradiol 17β-glucuronide were transported by human ABCG2, and that E3040-glucuronide inhibited ABCG2 mediated transport of estrone 3-sulfate slightly, although E3040-glucuronide and estradiol 17β-glucuronide were transported to a much lesser extent than estrone 3-sulfate and E3040-sulfate. Therefore, E3040-glucuronide uptake into CMVs may also be involved in BCRP. In this study, the uptake of Z-335-Tau into CMVs was inhibited by estrone 3-sulfate and estradiol 17β-glucuronide. Taken together, these results indicate that Z-335-Tau is taken up into CMVs by carrier proteins other than Mrp2, bsep/spgp, and Mdr1a/1b, which may be involved in BCRP.

In conclusion, the biliary excretion of Z-335 involves Mrp2, whereas Z-335-Tau is excreted into the bile by active transport systems that remain intact in EHBRs and show marked ATP-dependence. Furthermore, we clarified that Z-335-Tau is taken up into CMVs by carrier protein other than Mrp2, Bsrp/Spgp, and Mdr1a/1b, and that ATP-dependent transport appears to be involved in the biliary excretion of Z-335-Tau *in vivo*.

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