Different Activity of ATP Dependent Transport Across the Canalicular Membrane for Tributylmethylammonium and Triethylmethylammonium as a Potential Mechanism of the Preferential Biliary Excretion for Tributylmethylammonium in the Rat

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Purpose. The mechanism(s) responsible for the significantly higher biliary excretion of tributyl methyl ammonium (TBuMA) than of triethyl methyl ammonium (TEMA) was investigated in canalicular liver plasma membrane vesicles (cLPM).

Methods. The uptake of [^{3}H]TBuMA and [^{3}H]TEMA into cLPM in the presence of a pH gradient or ATP was measured by a rapid filtration technique.

Results. The uptake of substrates into the vesicle was significantly increased by an outwardly directed pH gradient. The pH dependent uptake was saturable and cross-inhibited by the other organic cation, indicating that TEMA and TBuMA share a common transport mechanism. Kinetic analysis revealed the two compounds show similar characteristics for the pH-gradient dependent uptake. Thus, the organic cation/H^+ exchange mechanism does not appear to explain the significant difference in biliary excretion of the organic cations. In the presence of ATP, however, uptake into cLPM was readily observed for TBuMA while TEMA uptake was negligible. Inhibition studies with typical P-glycoprotein substrates indicated the uptake may be mediated by the P-glycoprotein.

Conclusions. Differences between TBuMA and TEMA in reactivity for an ATP dependent transport process, rather than for an organic cation/H^+ exchanger, may be responsible for the markedly different biliary excretion of TBuMA and TEMA.

KEY WORDS: TBuMA; TEMA; cLPM; biliary excretion; P-glycoprotein; organic cation/H^+ exchanger.

INTRODUCTION

The extent of biliary excretion of organic cations (OCs) varies depending on the molecular weight (Mw) of the compounds (1,2). For example, tributyl methyl ammonium (TBuMA, Mw 200) is primarily excreted (in excess of 40% of dose) into the bile of rats whereas, only negligible excretion (about 0.3%) is observed for triethyl methyl ammonium (TEMA, Mw 116, 3). Currently, the underlying mechanism(s) for differences such as these is (are) unknown. In a previous study (4) we demonstrated the canalicular membrane transport process appears to be responsible for these differences. Since a number of carrier mediated mechanisms in the liver canalicular membrane have been suggested to participate in OC transport (5–11), it is logical to hypothesize these transporters may be involved in the marked variations in OC excretion into the bile. Among these transport systems, an OC/H^+ exchanger has been the most intensively studied. For example, the antiporter was reported to transport TBuMA (6) and tetraethyl ammonium (TEA, 5), a structural analog of TEMA. However, it was not clear whether this transport mechanism is responsible for the marked difference in biliary excretion of TBuMA and TEMA.

Recently, Smit *et al.* reported a P-glycoprotein (P-gp) mediated transport of some OCs including TBuMA in LLC-PK1 cell line in which P-gp was overexpressed by transfection of *mdr1a* and *mdr1b* genes (11). They also reported significantly decreased biliary excretion of TBuMA in *mdr1a* and *mdr1a/1b* gene knockout mice (12,13). Therefore, it is reasonable to suspect that the P-gp transport system in the liver canalicular membrane may also be involved in the biliary excretion of TBuMA, in addition to the OC/H^+ exchanger. However, P-gp mediated transport has never been examined for the presence of TEMA.

Therefore, the objective of this study is to identify the transport mechanism in canalicular liver plasma membrane vesicles (cLPM) responsible for the marked difference in biliary excretion of TBuMA and TEMA. Thus, we compared transport activity of OC/H^+ exchanger for TBuMA and TEMA. In addition, we were interested in examining whether the P-gp mediated transport mechanism in the liver could be demonstrated for the OCs and determining whether the transport system contributes to the preferential transport of TBuMA across the canalicular membrane.

MATERIALS AND METHODS

Chemicals

[^{3}H]TBuMA (0.2 Ci/mmol) and [^{3}H]TEMA (0.2 Ci/mmol) were synthesized according to the method of Neef *et al.* (14). [^{3}H]Methyl iodide (85 Ci/mmol) was purchased from Amersham (Arlington Heights, Illinois). Unlabelled methyl iodide, triethylamine, and tributylamine were purchased from Aldrich Chemical Co. (Milwakee, Wisconsin). [^{3}H]Taurocholate (3.7 Ci/mmol) and [^{3}H]daunomycin (4.4 Ci/mmol) were purchased from NEM Life Science products (Boston, Massachusetts). Unlabelled daunomycin was a gift from Dong-A Pharm Co. (Seoul, Korea). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Missouri).

Preparation of Canalicular Liver Plasma Membrane Vesicles

Canalicular liver plasma membrane vesicles (cLPM) were prepared from male SPF SD rats (Dae-Han Experimental Animal, Seoul, Korea; 250–270 g in body weight) by the method of Inoue *et al.* (15). The activity of alkaline phosphatase (16), a marker enzyme for canalicular membrane, was enriched approximately 52-fold in the vesicle preparation, compared with that in crude liver homogenates. Protein concentration of the vesicle preparation was 0.14 ± 0.03

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mg/g of liver when measured by the Lowry method (17) using bovine serum albumin as a standard. The inside-out proportion of vesicles was more than 30% (i.e., 69.8 ± 8.6% for rightside-out vesicles, mean ± SD for four measurements) when determined by measurement of exposed sialic acid concentration (18), which is consistent with the previous reports (19,20). Functional activity of the vesicle preparation was confirmed as evidenced by at least a 5-fold higher uptake of [^{3}H]taurocholate in the presence of ATP regenerating system compared with the uptake in the absence of an ATP (21,22). Immediately after the preparation, the cLPM was suspended in a membrane suspension buffer (MSB) containing (in mM) 250 sucrose, 10 Hepes, 10 Tris, 10 $MgCl_2$, and 0.2 $CaCl_2$ (pH 7.4) to yield a protein concentration of 4–6 mg/ml. The suspension was stored at 70°C for up to 2 weeks until transport studies were carried out.

Vesicle Uptake Studies

The uptake of [^{3}H]TBuMA and [^{3}H]TEMA into cLPM vesicles in the presence of a pH gradient or ATP was measured by a rapid filtration technique (23). A frozen suspension was quickly thawed by immersing it into a 37°C water bath, revesiclized by passing it 20 times through a 25-gauge needle, and diluted with MSB to give 1–1.5 mg/ml of protein. For 4 min 20 μl of the diluted suspension was preincubated in a test tube at 37°C. Then, 80 μl of an incubation buffer, containing 5 μM [^{3}H]TBuMA or [^{3}H]TEMA (0.080 μCi each), was added to the diluted vesicle suspension. In studies involving pH gradient dependent uptake, buffers containing (in mM) 82 sucrose, 100 K^+-gluconate, 91 MES, 14 Hepes, 29 Tris, 0.2 Ca-gluconate (for pH 5.9 conditions), and 70 sucrose, 100 K^+-gluconate, 76 Hepes, 70 Tris, 0.2 Ca-gluconate (for pH 7.9 conditions) were used. MSB (pH 7.4) was used as an incubation buffer for ATP dependency studies. After predetermined time intervals, uptake was quenched by the addition of 4 ml of ice-cold stop solution, which consisted of (in mM) 250 sucrose, 10 Hepes, 10 Tris, 10 $MgCl_2$, 0.2 $CaCl_2$, 1 TBuMA, or TEMA (pH 7.4, for ATP dependency studies), 204 sucrose, 150 K^+-gluconate, 10 Hepes, 10 Tris, 5 Mg-gluconate, 0.2 Ca-gluconate, 1 TBuMA, or TEMA (pH 7.5, for uptake studies involving pH gradient). The entire contents were then rapidly filtered through a 0.45 μm MF-MEMB 25 mm filter (Seoul Science, Seoul), which was presoaked 2 h in ice cold stop solution. The tube was rinsed again with 4 ml of respective ice cold stop solution and filtered. After washing twice with 4 ml of the ice cold stop solution, the filter was dissolved in 4 ml of a scintillation cocktail (Ultima Gold, Packard, Meriden, Connecticut, and the radioactivity of the mixture was measured using a Wallac 1409 liquid scintillation counter (Wallac, Turku). Presoaking and rinsing the filter with the ice cold stop solution, which contains 1 mM TBuMA or TEMA, resulted in almost negligible nonspecific binding of OCs to the filter (i.e., negligible radioactivity in the filter; data not shown). The binding of TEMA or TBuMA to the surface of vesicles at equilibrium (i.e., 60 min) was approximately 15% of the peak uptake value, when it was estimated from the relationship between the uptake and the osmolarity of the incubation medium. Thus, the estimated transport is not likely to be affected by the presence of bound OCs to the surface. When it was necessary to examine the concentration dependency for the uptake, OC uptake for 30 sec was examined for 0.01–5 mM TBuMA and TEMA (while fixing the [^{3}H] specific activity at 0.16 μmCi for each substrate) in the presence of a pH gradient or ATP. The effect of representative P-gp substrates (50 μM), OCs (100 μM), and an organic anion (benzyl penicillin, 100 μM) on a 30 sec period of uptake of 10 μM [^{3}H]TBuMA (0.16 μ Ci) was also measured in the presence

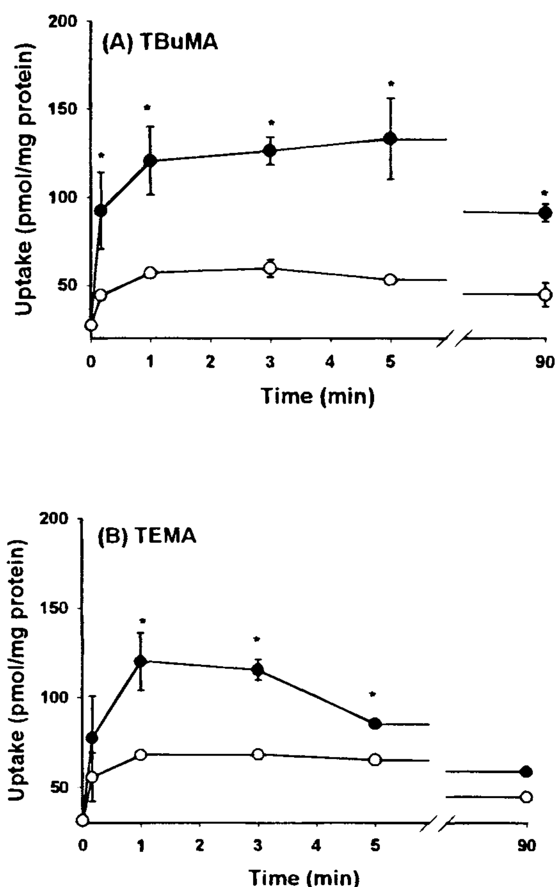

Fig. 1. Uptake of OCs (5 μM) into cLPM in the presence (●) and absence (○) of an outwardly directed pH gradient for TBuMA (A) and TEMA (B). Membrane vesicles were treated with valinomycin (5 μg/mg protein) for 10 min at 37°C. Each data point is expressed as means ± S.D. of triplicate measurements for three different batches of cLPM. * $p < 0.01$ from two-way ANOVA.

Table I. Inhibitory Effect of OCs on TBuMA Uptake into cLPM in the Presence of a pH Gradient[a]

Inhibitors	Concentration	TBuMA uptake	
		Pmole/mgprotein/30s	% of control
Control	—	239.7 ± 11.2	—
TBuMA	100 μM	118.7 ± 9.0*	49.4
TPMA	100 μM	122.3 ± 10.7*	51.0
TEMA	100 μM	133.3 ± 6.7*	55.6

[a] Each data point is expressed as means ± S.D. of triplicate measurements of three batches of membrane vesicle preparations.
* $p < 0.01$ from Student's t-test.

of ATP. To examine whether P-gp transport activity was present in our cLPM, a control experiment was carried out. In this control study, the uptake of a standard P-gp substrate, i.e., [^{3}H]daunomycin, into cLPM was measured. The experimental conditions (e.g., the composition of the ATP regenerating system, buffer composition etc.) were identical to those for TBuMA uptake study except, that TBuMA was replaced by daunomycin in the uptake medium and an ice-cold stop solution containing 20 μM daunomycin was used.

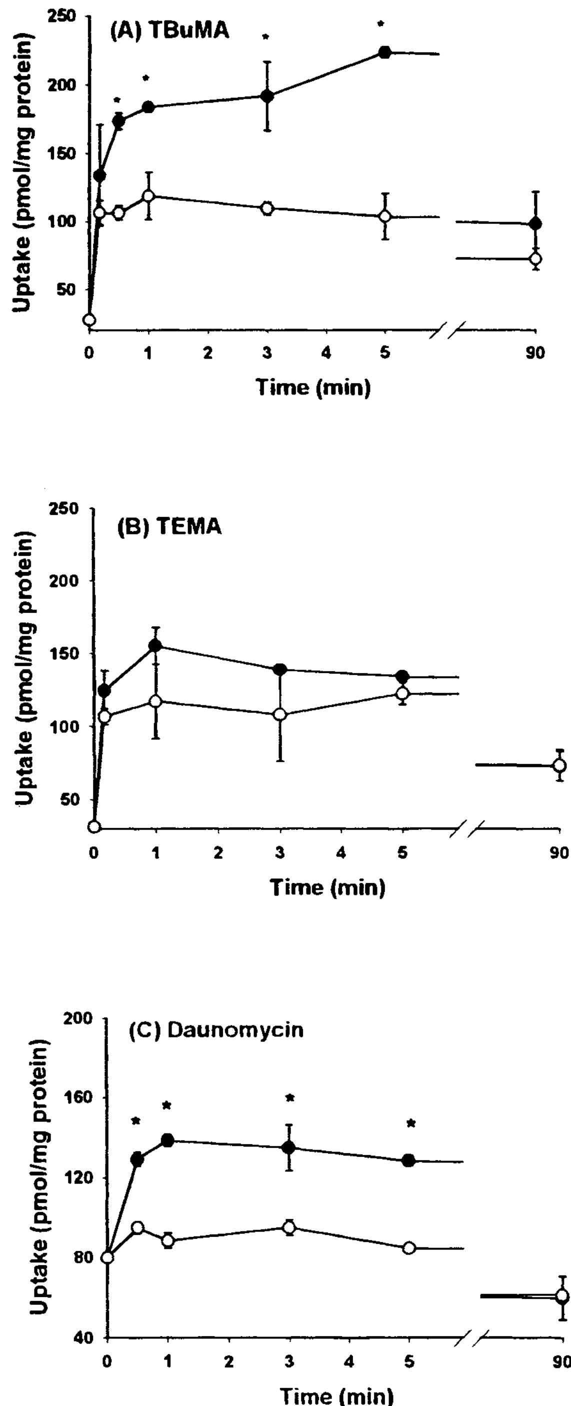

Fig. 2. Uptake of OCs into cLPM in the presence (●) and absence (○) of 1.2 mM ATP and an ATP regenerating system (3 mM phosphocreatine and 3.6 μg/100 μl creatine phosphokinase) for 5 μM TBuMA (A), 5 μM TEMA (B) and 0.1 μM daunomycin (C). Membrane vesicles were preincubated for 4 min at 37°C. Each data point is expressed as means ± S.D. of triplicate measurements for three different batches of cLPM. * $p < 0.01$ from two-way ANOVA.

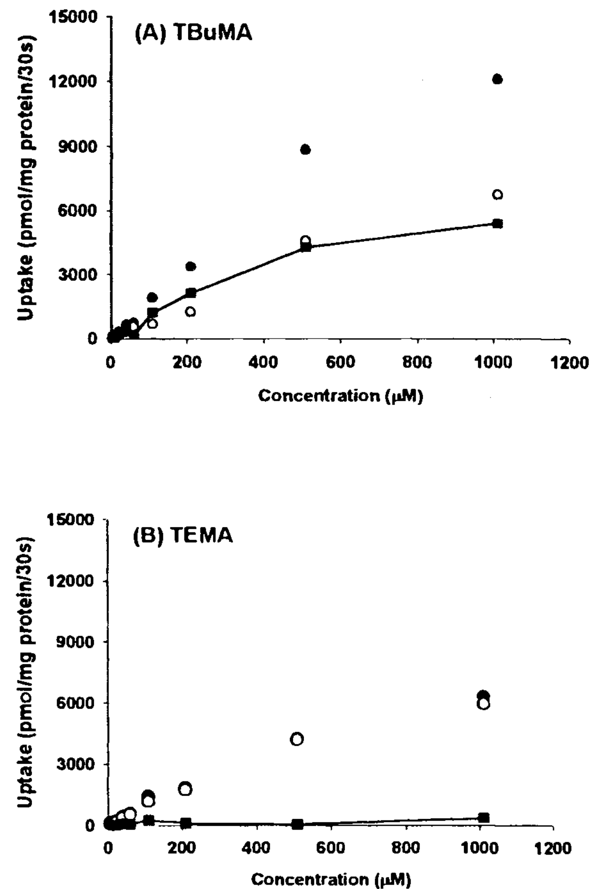

Fig. 3. Concentration (0.005–1 mM) dependency of OC uptake into cLPM at 37°C in the presence (●) and absence (○) of 1.2 mM ATP and an ATP regenerating system (3 mM phosphocreatine and 3.6 μg/100 μl creatine phosphokinase) for TBuMA (A) and TEMA (B). ATP dependent uptake (■) was estimated by subtracting OC uptake in the absence of ATP from that in the presence of ATP. Each data point is expressed as the mean ± S.D. of triplicate measurements for three different batches of cLPM.

Data Analysis

All data are expressed as mean ± S.D. Two-way ANOVA was performed to test differences between transport conditions. The student's t-test was used to test the difference for mean uptake value in inhibition studies. In all cases, $p < 0.01$ was accepted as representing a statistical difference.

RESULTS AND DISCUSSION

Outwardly Directed pH Gradient Dependent OC Uptake

The uptake of 5 μM TBuMA (Fig. 1A) or TEMA (Fig. 1B) into cLPM vesicles was significantly enhanced by an outwardly directed pH gradient (pH 5.9 in/pH 7.9 out) under voltage-clamped conditions ($K^+_{in} = K^+_{out}$, pretreated with valinomycin, a K^+ ionophore, at 5 μg/mg protein for 10 min at

Table II. Inhibitory Effect of P-gp Substrates and Other OCs on TBuMA Uptake into cLPM in the Presence of ATP[a]

	Inhibitors	Concentration	TBuMA uptake	
			pmol/mg prot/30s	% of control
	Control	—	244.4 ± 6.8	—
P-gp substrates	Doxorubicin	50 μM	125.3 ± 11.2*	51.3
	Daunomycin	50 μM	134.1 ± 4.7*	54.9
	Verapamil	50 μM	135.0 ± 7.4*	55.2
Organic cations	Ranitidine	100 μM	202.9 ± 0.5	83.0
	TBuMA	100 μM	104.7 ± 2.5*	42.8
	TEMA	100 μM	272.6 ± 6.5	111.5
Organic anion	Benzylpenicillin	100 μM	249.4 ± 3.1	102.0

[a] Each data point is expressed as means ± S.D. of triplicate measurements of three batches of membrane vesicle preparations.
* $p < 0.01$ from Student's t-test.

37°C). A concentration dependency was found for the pH gradient-driven uptake of TBuMA and TEMA in the substrate concentration range of 0.01–5 mM (data not shown). The concentration dependency data were fitted to a kinetic model involving both, a simple Michaelis-Menten saturable process and a linear uptake mechanism by a non-linear regression analysis to obtain kinetic parameters. The statistical analysis indicated the kinetic characteristics for the two substrates are nearly identical (V_{max}; 0.97 nmol/mg protein/30 s, K_m; 1.4 mM, and Cl_{linear}; 6.3 μl/mg protein/30 s for TBuMA, V_{max}; 0.80 nmol/mg protein/30 s, K_m; 1.3 mM and Cl_{linear}; 9.9 μl/mg protein/30 s for TEMA). In a preliminary study, we have maintained the hepatic concentration of the OCs at the range of 1–550 μM by intravenous infusion (4). Under this condition, the biliary excretion of TBuMA was approximately 188-fold greater than that of TEMA (4). Since these OCs are not bound in liver cytosol (3), the free concentration in the liver is expected to be very close to the liver concentration. Despite the high OC concentrations in the liver, the canalicular transport processes are not expected to be saturated because of the relatively high Km values (i.e., 1.3—1.4 mM for the pH dependent process) found in this study. The uptake of 10 μM TBuMA into cLPM was inhibited to a similar extent by TBuMA, TPMA (tripropyl methyl ammonium), and TEMA (100 μM each) in the presence of an outwardly directed pH gradient (Table I), indicating TEMA, as well as TBuMA and TEA (5–7), is transported via the OC/H^+ exchanger with a similar affinity. Taken together with the comparison of kinetic parameters, differences in the canalicular transport found for these compounds (4) are unlikely to be explained by the OC/H^+ exchanger mediated mechanism.

At present, it is not clear whether the OC transport driven by outwardly directed proton gradient occurs *in vivo*. However, our preliminary study (4) suggested approximately 188-fold higher clearance in *in vivo* canalicular excretion for TBuMA. If the excretion process is entirely mediated by the proton driven transport, such a marked difference would not be expected. Therefore, it is possible to hypothesize that the process is a minor component in TBuMA transport across the bile canalicular membrane.

ATP Dependent OC Uptake

Although the presence of P-gp in cLPM has been indicated (24,25), the functional activity of the transporter has been somewhat controversial. For example, Moseley *et al.* (6) failed to demonstrate an ATP dependent OC uptake in a cLPM while daunomycin uptake has been reported in other studies (24,25). Therefore, a control study, involving the measurement of daunomycin uptake in our cLPM was carried out in order to confirm the presence of P-gp mediated transport. The uptake of daunomycin, a model substrate for P-gp, was significantly increased in the presence of ATP (Fig. 2C), indicating P-gp activity can be readily measured in our experimental conditions. The transport activity in this study appeared comparable to the transport activity described previously (24). Under similar experimental conditions, the uptake of 5 μM TBuMA by cLPM was significantly enhanced in the presence of an ATP regenerating system (Fig. 2A). In contrast, the uptake of 5 μM TEMA does not depend on the presence of ATP (Fig. 2B). Uptake velocity vs. substrate concentration profile indicates uptake is mediated by saturable and nonsaturable processes for TBuMA (Fig. 3A) while uptake is apparently linear for TEMA (Fig. 3B). Nonlinear regression analysis of the uptake, fitted to an equation involving both a saturable and a nonsaturable process, indicated the kinetic parameters for V_{max}; 0.32 nmol/mg protein/30 s, K_m; 0.76 mM, and Cl_{linear}; 0.0001 μl/mg protein/30 s best described the TBuMA uptake into the cLPM. Therefore, it can be concluded ATP-dependent transport across the canalicular membrane is involved in the case for TBuMA, but not for TEMA. Our previous study (4) suggested a significantly higher biliary excretion is mediated by a preferential transport of TBuMA across the canalicular membrane. Therefore, it is possible the ATP dependent TBuMA transport in cLPM may be responsible for the preferential transport of the OC, which was observed in our *in vivo* study (4). Considering the relatively high hepatic concentration of TBuMA in our previous experiment involving intravenous infusion (4), the ATP dependent transport process is likely to play a role in the canalicular excretion process despite its relatively high Km value.

The ATP-dependent uptake of 10 μM TBuMA into cLPM was inhibited significantly by typical P-gp substrates (e.g., doxorubicin, daunomycin, verapamil) and TBuMA, but not by TEMA, ranitidine, and benzylpenicillin (Table II). A significant inhibition of TBuMA uptake by typical P-gp substrates suggests TBuMA and P-gp substrates may share a common transport mechanism. This result is consistent with the observations that the uptake of TBuMA is enhanced in P-gp overexpressed LLC-PK1 cell line (11), and the biliary excretion of TBuMA decreases in *mdr1a* and *mdr1a/1b* gene knockout mice (12,13). Ranitidine, a substrate for type 2 OC hepatic uptake carrier,

and benzyl penicillin, an organic anion, did not affect the ATP dependent uptake of TBuMA. No appreciable inhibition of ATP dependent TBuMA uptake was found for TEMA. Therefore, these observations are consistent with a hypothesis that a P-gp related transport mechanism is involved in the ATP dependent TBuMA uptake.

CONCLUSIONS

TBuMA and TEMA were studied for their carrier-mediated uptake characteristics in cLPM. These compounds were actively transported into the vesicles by an OC/H^+ exchanger which showed comparable kinetic characteristics (i.e., K_m, V_{max}, and Cl_{linear}). Therefore, the OC/H^+ exchange mechanism fails to explain the differences in the canalicular transport of these compounds observed in a previous *in vivo* study (4). In addition to the antiport mechanism, TBuMA was transported via an ATP dependent transport process. The ATP dependent mechanism possesses characteristics which are consistent with a P-gp transporter. In contrast, an ATP dependent transport was not observed for TEMA. Therefore, the ATP-dependent transport of TBuMA, but not an OC/H^+ exchange mechanism, may be responsible for differences in the biliary excretion of TBuMA and TEMA.

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