Pharmaceutical Research, Vol. 27, No. 5, May 2010 (© 2010)
DOI: 10.1007/s11095-010-0075-0

Research Paper

Increased Affinity to Canalicular P-gp via Formation of Lipophilic Ion-Pair Complexes with Endogenous Bile Salts is Associated with Mw Threshold in Hepatobiliary Excretion of Quaternary Ammonium Compounds

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Received July 31, 2009; accepted November 24, 2009; published online March 10, 2010

Objectives. We intended to elucidate the mechanism of the molecular weight (Mw) threshold (i.e., 200±50) for appreciable hepatobiliary excretion of quaternary ammonium compounds (QACs) in rats.
Methods. We measured the effect of ion-pair complexation of QACs with taurodeoxycholate (TDC), an endogenous anionic bile salt, on the apparent partition coefficients (APC) of QACs between n-octanol and phosphate buffer, and the inhibition of organic cation transporter1 (OCT1)- and P-glycoprotein (P-gp)-mediated transport of representative substrates.
Results. By measuring the APC, we demonstrated that there is a Mw threshold of 200±50 in the ion-pair complexation of QACs with an endogenous bile salt, TDC. We also demonstrated, by measuring the inhibition of relevant transports, that a Mw threshold of 200±50 exists for the binding of QACs to canalicular P-gp, but not for sinusoidal OCT1. The Mw threshold values for ion-pair formation and P-gp binding were identical and consistent with the reported Mw threshold value for appreciable biliary excretion of QACs in rats.
Conclusions. Mw-dependent binding of QACs to canalicular P-gp contributes in part to the mechanism of the Mw threshold of 200±50. The formation of lipophilic ion-pair complexes with bile salts, followed by stronger binding to canalicular P-gp, appears to accelerate biliary excretion of QACs with a high Mw.

KEY WORDS: hepato-biliary excretion; ion-pair complex; molecular weight threshold; P-gp.

INTRODUCTION

A molecular weight (Mw) threshold (i.e., Mw greater than 200±50) supposedly exists for the appreciable biliary excretion (more than 10% of dose) of quaternary ammonium compounds (QACs) in rats (1). For example, tributyl methyl ammonium (TBuMA, Mw 200) is primarily excreted (in excess of 40% of dose) into the bile, whereas only negligible excretion (about 0.3% of dose) is observed for triethyl methyl ammonium (TEMA, Mw 116) (2). Since organic cation transporter 1 (OCT1) and P-glycoprotein (P-gp) are reportedly involved in the sinusoidal uptake and canalicular excretion of QACs, respectively, it seems reasonable to assume the association of OCT1 and/or P-gp-mediated transport with the Mw-dependent hepatobiliary excretion of QACs (2–4).

We previously proposed that TBuMA forms lipophilic ion-pair complexes specifically with deoxyconjugated endogenous bile salts, such as taurodeoxycholate (TDC), and thereby undergoes substantial biliary excretion, probably via canalicular P-gp, while TEMA neither forms such ion-pair complexes nor undergoes appreciable biliary excretion (5,6). As a result of the formation of such ion-pair complexes, the apparent partition coefficient (APC) of TBuMA to n-octanol from phosphate buffer (pH 7.4) was dramatically increased in the presence of rat bile or TDC (5–7). The affinity of the ion-pair complex (i.e., TBuMA-TDC complex) to the excretory transporter (i.e., P-gp) was much greater than that of TBuMA itself, which substantially increased biliary excretion of TBuMA via the transporter (5,6). This hypothesis, i.e., the formation of ion-pair complexes with specific endogenous bile salts and the facilitated transport of the complexes via P-gp for large-Mw QACs was further validated in experimentally bile-salts-depleted rats (8). Bile salt concentration in the rat liver was lowered 38% by once-daily oral administration of cholestyramine at a dose of 0.5 g/kg for two consecutive

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ABBREVIATIONS: APC, apparent partition coefficients; CsA, cyclosporine A; MPP^+, methyl-4-phenylpyridinium acetate; Mw, molecular weight; OCT1, organic cation transporter 1; P-gp, P-glycoprotein; QACs, Quaternary Ammonium Compounds; TBA, tetrabutyl ammonium; TBuMA, tributylmethyl ammonium; TDC, taurodeoxy cholate; TEA, tetraethyl ammonium; TEER, transepithelial electrical resistance; TMA, tetramethyl ammonium; TPeA, tetrapentyl ammonium.

weeks (8). When TBuMA was administered intravenously to these rats, the biliary clearance of TBuMA was decreased significantly (8), probably due to reduced ion-pair formation with bile salts. On the other hand, canalicular excretion of TBuMA was not changed in rats with CCl_4-induced acute hepatic injury, despite significant induction of canalicular P-gp by the injury (9). Any potential increase in the excretion/clearance of TBuMA and its ion-pair complexes via induced P-gp might have been cancelled by a decrease in the hepatic concentration of bile salts, which would have reduced the formation of lipophilic ion-pair complexes, more favorable P-gp substrates than TBuMA, in the liver (9). These results (8,9) appear to support the validity of our hypothesis.

Nevertheless, the existence of a Mw threshold in the biliary excretion of QACs has not yet been fully examined. In order to verify the general applicability of our ion-pair hypothesis to other QACs, it is essential to perform experiments with various QACs. Therefore, in the present study, relevant experiments were conducted using six QACs with different Mws, chain length and lipophilicity (4,10) (Fig. 1). The issue of whether a Mw threshold exists for QACs in the formation of lipophilic ion-pair complexes was first examined by measuring the APC of QACs in the presence of a representative bile salt, TDC. Then, in order to examine whether a Mw threshold exists in the affinity of QACs and their ion-pair complexes to OCT1 and P-gp, the affinity of these compounds to relevant hepatic transporters was assessed by measuring the ability of each compound to inhibit the uptake of methyl-4-phenylpyridinium acetate (MPP^+), a representative substrate of OCT1, in MDCKII-OCT1 cells (4,11), and the basal-to-apical (B-to-A) transport of duanorubicin, a representative P-gp substrate (12–15), in MDCKII-MDR1 cells. The purpose of the present study is, therefore, to examine whether the existence of a Mw threshold in the hepatobiliary excretion of QACs can be explained in association with our hypothesis of ion-pair formation with endogenous bile salts and excretion via canalicular P-gp.

MATERIALS AND METHODS

Materials

[^{3}H]Daunorubicin (4.4 Ci/mmol) and [^{3}H]methyl-4-phenylpyridinium acetate (MPP^+, 3.21 Ci/mmol) were purchased from Perkin Elmer Inc. (Boston, MA). Tetramethyl ammonium bromide (TMA, Mw 74.1), tetraethyl ammonium chloride (TEA, Mw 130.2), tetrabutyl ammonium tribromide (TBA, Mw 242.5), tetrapentyl ammonium bromide (TPeA, Mw 298.6), taurodeoxycholate (TDC), and cyclosporine A (CsA) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO), and tetrapropyl ammonium bromide (TPrA, Mw 186.4) was obtained from Lancaster Synthesis (Morecambe, UK). Tributyl methyl ammonium (TBuMA, Mw 200.4) was purchased from Fluka (Buchs, Switzerland). All other reagents were analytical grade. Chemical structures and Mws of QACs examined in the present study are summarized in Fig. 1.

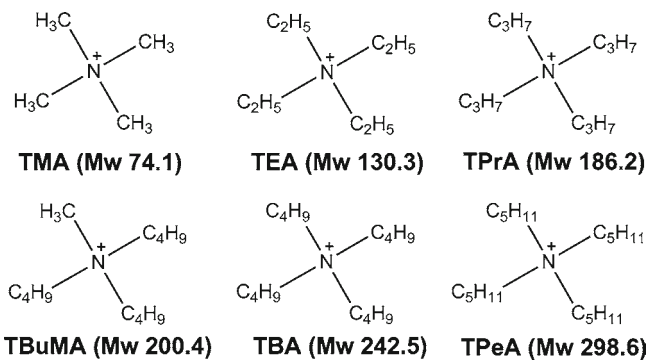

Fig. 1. Structures and Mws of QACs examined in the present study.

Determination of Apparent Partition Coefficient (APC) of QACs in the Presence of TDC

The effect of TDC on the APC of QACs between the aqueous and organic phases was investigated, as previously described (5). An equal volume of pre-saturated n-octanol was then added to each of the aqueous phases containing 10 μM TMA, TEA, TPrA, TBuMA, TBA, and TPeA, respectively, in the presence or absence of 100 μM TDC, and the mixture was vortexed vigorously for 5 min followed by shaking for 2 hr at 25°C in a temperature-controlled water bath. After standing for 30 min at 25°C in the water bath, the mixture was separated into two phases by centrifugation at 3,000 rpm for 10 min. The concentrations of TMA, TEA, TPrA, TBuMA, TBA, and TPeA in the aqueous and organic phases were quantified using an LC-MS system. A 20 μL aliquot of each aqueous and organic phase was diluted with 4 mL of acetonitrile. A 5 μL aliquot was injected directly into the LC-MS system. The APC was estimated from the concentration ratio of TMA, TEA, TPrA, TBuMA, TBA, and TPeA between the aqueous and organic phases.

The HPLC system consisted of a Waters 2695 separation module with quaternary pump and autoinjector. A Gemini C18 analytical column (2.0×150 mm, 3 μm; Phenomenex, Torrance, CA) was used. The flow rate of the mobile phase (i.e., MeOH:DDW=7:3, v/v) was set at 0.2 mL/min. A Finnigan LCQ ion-trap mass spectrometer equipped with an ESI source was used for mass analysis and detection. Mass spectrometric analysis was performed in the positive ion mode and set up in the selected ion monitoring mode. On the basis of the full-scan mass spectra of each analyte, the most abundant ion was selected, and the mass spectrometer was set to monitor the ions as follows: m/z 74.1 for TMA, m/z 130.3 for TEA, m/z 186.2 for TPrA, m/z 200.4 for TBuMA, m/z 242.5 for TBA, and m/z 298.6 for TPeA. For all compounds, calibration studies indicated that the detector response was linear over 5–2,000 nM for TMA, 1-1,000 nM for TEA, 0.3–1,000 nM for TPrA, 0.1–200 nM for TBuMA and TBA, and 1–200 nM for TPeA below 15.0% of interday coefficients of variation.

OCT1-Mediated MPP^+ Uptake Study

Preparation and characterization of OCT1 stably expressing MDCKII cells (MDCKII-OCT1) were described in our previous report (10). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin. For experiments, 10^4 cells were seeded in 96-well plates. After the cells reached 95% confluence, the growth media were discarded, and the

attached cells were washed with Dulbecco's modified phosphate-buffered saline (DPBS) and preincubated for 30 min in DPBS at 37°C. The uptake of 1 μM [^{3}H]MPP$^+$ was initiated by the addition of medium (200 μL) containing [^{3}H]MPP$^+$. The inhibition of the uptake of 1 μM [^{3}H]MPP$^+$ by various concentrations of QACs (0.1–1,000 μM) was measured at 37°C in the presence and absence of a 10-fold molar excess of TDC (i.e., 1 μM–10 mM). The effect of TDC (1 μM–10 mM) on the uptake of 1 μM [^{3}H]MPP$^+$ was also measured. The cells were washed three times with 200 μL of ice-cold PBS immediately after placing the plates on ice. The radioactivity of the [^{3}H]MPP$^+$ in the cells was measured by liquid scintillation counting after lysing the cells with 50 μL of cell lysis buffer [100 mM KH_2PO_4 (pH 7.8), 1 mM EDTA, 7 mM 2-mercaptoethanol, 1% (v/v) Triton X-100, 10% (v/v) glycerol] (Promega, Madison, WI). The affinities of QACs to OCT1 were assessed by measuring the concentration of each QAC that inhibited uptake of MPP$^+$ (IC_{50} values) by 50% in the presence or absence of a 10-fold molar excess of TDC (4,11,16).

Western Blot Analysis

MDCKII-mock cells and MDCKII cells stably expressing MDR1 (MDCKII-MDR1) were obtained from Dr. A.H. Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air, in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin–streptomycin. MDCKII-mock and MDCKII-MDR1 cells were harvested by centrifugation at 6,000 rpm for 3 min at 4°C, and cell pellets were swollen in one volume of RIPA buffer [50 mM Tris-Cl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and protease inhibitor] for 10 min. Aliquots that contained 30 μg of protein were separated by SDS-PAGE in a 4–12% gradient gel (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% nonfat milk and probed with anti-P-gp (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-actin antibodies (Cell Signaling Technology, Beverly, MA). The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) and visualized using the ECL system (Santa Cruz Biotechnology).

P-gp-Mediated Transport Study

Vectorial transport study of daunorubicin was performed in MDCKII-mock and MDCKII-MDR1 cells after the trans-epithelial electrical resistance (TEER) value of the seeded cells reached 200–250 Ω·cm^2. For measurement of the apical-to-basal (A-to-B) transport of daunorubicin, 0.5 mL of DPBS containing 100 nM [^{3}H]daunorubicin was added on the apical side, and 1.5 mL of DPBS without daunorubicin was added on the basal side of the insert. The insert was transferred to a well containing fresh DPBS medium every 15 min for 1 hr. For measurement of the B-to-A transport, 1.5 mL of DPBS containing 100 nM [^{3}H]daunorubicin was added on the basal side, and 0.5 mL of DPBS without daunorubicin was added on the apical side. The transport medium in the apical side was replaced with 0.35 mL of fresh incubation medium every 15 min for 1 hr. The radioactivity of an aliquot (50 μL) of the apical side after each replacement was measured. To confirm the P-gp functionality of the cells, the inhibitory effect of cyclosporine A (CsA, 0.1–20 μM), a representative inhibitor of P-gp, on the B-to-A transport of danorubicin (100 nM) was examined. The inhibitory effect of QACs (0.1–100 μM) on the B-to-A transport of daunorubicin (100 nM) was then measured in the presence and absence of a 10-fold molar excess of TDC (i.e., 1–1000 μM). The effect of TDC (1–1000 μM) on the B-to-A transport of daunorubicin (100 nM) was also measured. The affinities of QACs to P-gp were assessed by measuring the concentration of each QAC that inhibited transport of daunorubicin (IC_{50} values) by 50% in the presence or absence of TDC (4,11,16).

We also investigated the transepithelial transport of QACs in the presence or absence of TDC. For measurement of A-to-B transport, 10 μM QACs in the presence and absence of TDC was added to the top of the insert and moved to the next well every 15 min for 1 h. The B-to-A transport of 10 μM QACs in the presence and absence of TDC was also measured by replacing transported medium (0.35 mL) in the apical side of the insert with 0.35 mL of fresh medium every 15 min for 1 h. Two volume of acetonitrile were added to the aliquots of transport medium, mixed vigorously for 5 min, and centrifuged at 15,000 g for 10 min. A 10 μL aliquot of the supernatant was directly injected into a LC-MS system to measure the concentration of the QACs.

To investigate the functional consequences of the QACs-TDC complexes, cellular accumulation of QACs in the presence and absence of TDC was measured. For the experiments, 10^6 cells were seeded in 12-well plates and incubated until cells reached 90% confluence. After preincubating the cells with DPBS for 30 min, QACs uptake was initiated by the addition of medium containing 10 μM TEA, TPrA, TBuMA, TBA, and TPeA with and without 100 μM TDC to the cells, followed by incubation at 37°C for 30 min. Cells were washed twice with ice-cold PBS and scraped in 150 μL PBS. Two volumes of acetonitrile were added to the cells, which were then sonicated, followed by centrifugation at 15,000 g for 10 min. A 10 μL aliquot of the supernatant was directly injected into a LC-MS system to measure the concentrations of the QACs.

Data Analysis

To determine the IC_{50} values of QACs for the uptake by MDCKII-OCT1 cells and B-to-A transport in MDCKII-MDR1 cells, relevant data were fitted to an inhibitory effect model (Eq. 1) using a Winnonlin 5.2 (Pharsight, Mountain View, CA).

$$v = V_{max} \times \left(1 - \frac{[S]}{[S] + IC_{50}}\right) \quad (1)$$

In Eq. 1, as shown above, v, V_{max}, [S], and IC_{50} represent the transport rate, maximum transport rate, concentration of inhibitor, and the inhibitor concentration that inhibited 50% of the maximum transport rate, respectively.

Statistical significance was analyzed using unpaired *t*-tests, and values for $p<0.05$ were considered to be statistically significant. The reproducibility of the results was confirmed in at least three separate experiments. Data are expressed as means ± SD.

RESULTS

Apparent Partition Coefficients (APCs) of QACs in the Presence of TDC

The APCs of 10 μM QACs (i.e., TMA, TEA, TPrA, TBuMA, TBA, and TPeA) in the presence or absence of 100 μM TDC were measured and are shown in Fig. 2. TDC was selected as an anion component that represents endogenous conjugated deoxy bile salts, because it formed lipophilic ion-pair complexes with TBuMA (5). The APCs of TBuMA, TBA and TPeA were greatly increased by the presence of a 10-fold molar excess of TDC as the Mw increased, whereas the APCs of TMA, TEA and TPrA were not influenced by the presence of TDC. These results demonstrate the existence of a Mw threshold (i.e., Mw>200) for QACs to form lipophilic ion-pair complexes with bile salts such as TDC.

Characteristics of MDCKII-OCT1 and MDCKII-MDR1 Cells

Since OCT1 and P-gp are two major transporters that govern sinusoidal uptake and canalicular excretion, respectively, of organic cations including QACs, we further investigated whether ion-pair complexation of QACs with TDC can affect the transport of QACs via OCT1 and P-gp. For this purpose, we characterized the expression and functionality of these transporters in MDCKII-OCT1 and MDCKII-MDR1 cells. The overexpression of OCT1 and transport activity of MDCKII-OCT1 cells was confirmed, as published previously (10). The overexpression of P-gp in MDCKII-MDR1 cells was confirmed by a Western blot analysis as described in the Materials and Methods section. The expression level of P-gp in the cells was much greater than that in MDCKII mock cells (Fig. 3A). The transport activity of the MDCKII-MDR1 cell monolayer was confirmed by measuring the B-to-A transport of daunorubicin, a representative substrate for P-gp (12). As shown in Fig. 3B, the B-to-A transport of daunorubicin in MDCKII-MDR1 cells was 4-fold higher than the A-to-B transport, while the B-to-A transport of daunorubicin in MDCKII-mock cells was similar to the A-to-B transport in the cells. To evaluate the functionality of P-gp in MDCKII-MDR1 cells, the inhibitory effect of CsA on the B-to-A transport of daunorubicin (100 nM) was measured as a function of CsA concentration (0.1–20 μM). The presence of CsA significantly inhibited the B-to-A transport of daunorubicin, and the IC_{50} value was estimated to be 0.641±0.301 μM (Fig. 3C), consistent with previous reports (13–15). Taken together, our MDCKII-MDR1 cell system was confirmed as appropriate to examine the B-to-A transport of P-gp substrates.

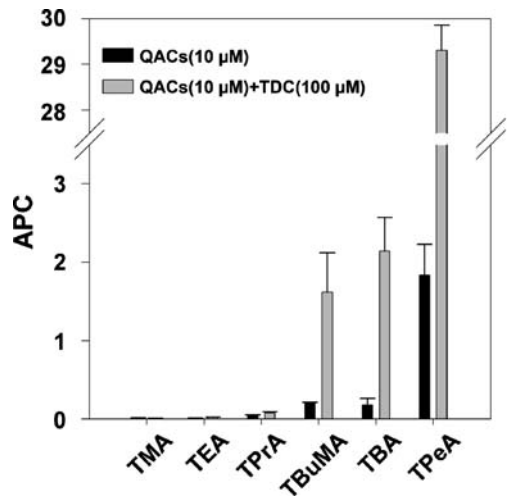

Fig. 2. Apparent partition coefficient (APC) of QACs (10 μM) in the presence or absence of TDC (100 μM).

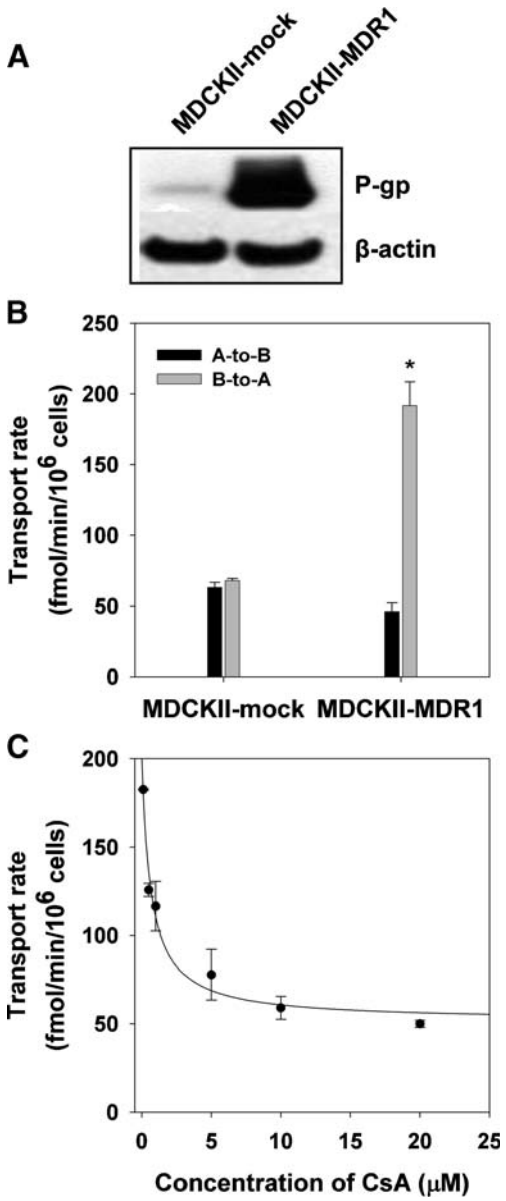

Fig. 3. (**A**) Western blot analysis of MDCK cells that stably express MDR1. Lane 1 and 2 represent vector-transfected control cells (MDCKII-mock) and cells overexpressing P-gp (MDCKII-MDR1), respectively, and β-actin was used as a loading control. (**B**) Apical-to-basal (A-to-B; ■) and basal-to-apical (B-to-A; □) transport of 100 nM [^{3}H] danorubicin across MDCKII-mock and MDCKII-MDR1 cell monolayers. Each data point represents the mean ± S.D. of three independent experiments. *: $p<0.05$ (**C**) Concentration-dependent inhibitory effect of cyclosporine A (0.1–20 μM) on the B-to-A transport rate of 100 nM [^{3}H]daunorubicin in the MDCKII-MDR1 cell monolayer.

Inhibitory Effect of QACs on MPP$^+$ Uptake in MDCKII-OCT1 Cells

First, we measured the inhibitory effect of TBA (0.1–1000 μM), in the presence or absence of a 10-fold molar excess of TDC, on the OCT1-mediated MPP$^+$ (1 μM) uptake. TBA itself significantly reduced the MPP$^+$ uptake in a concentration-dependent manner (Fig. 4A), and the IC_{50} value was calculated to be 20.6±7.2 μM, consistent with a previous report (10). TDC alone, up to 10 mM, did not inhibit the OCT1-mediated MPP$^+$ uptake. The presence of TDC did not affect the inhibitory effect of TBA either, resulting in an IC_{50} value of 19.4±8.0 μM. These results suggest that the formation of ion-pair complexes with TDC does not influence the inhibitory potential of TBA in terms of OCT1-mediated uptake.

We then measured the inhibitory effect of the other QACs on the MPP$^+$ uptake in a similar manner. Again, the presence of TDC did not affect the inhibitory potential of the respective QACs (data not shown). Therefore, it was concluded that the formation of ion-pair complexes with TDC does not affect the inhibitory potential of QACs in terms of OCT1-mediated MPP$^+$ uptake.

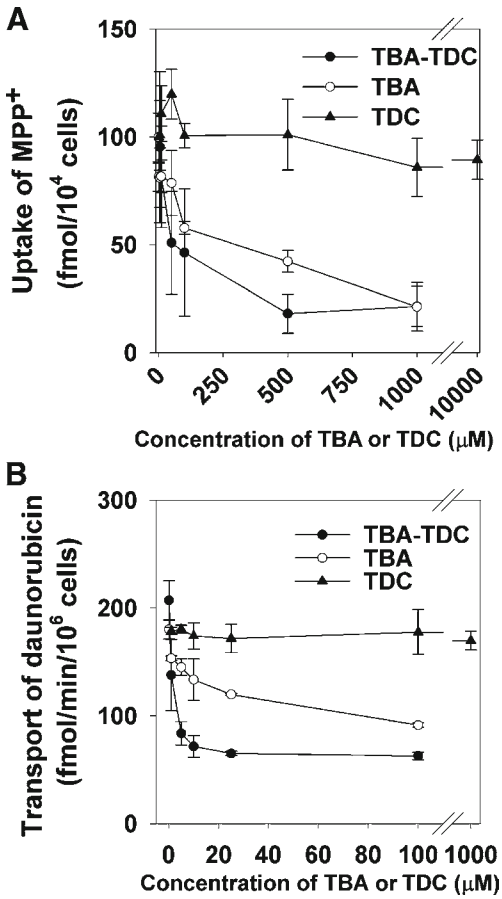

Fig. 4. (**A**) Concentration-dependent inhibitory effect of TBA (0.1–1,000 μM) in the presence or absence of a 10-fold molar excess of TDC (i.e., 1–10,000 μM) as well as TDC itself (1–10,000 μM) on the uptake of 1 μM [^{3}H]MPP$^+$ in MDCKII-OCT1 cells. (**B**) Concentration-dependent inhibitory effect of TBA (0.1–100 μM) in the presence or absence of a 10-fold molar excess of TDC (i.e., 1–1,000 μM) as well as TDC itself (1–1,000 μM) on the B-to-A transport rate of 100 nM [^{3}H]daunorubicin in MDCKII-MDR1 cell monolayer. Each data point represents the mean ± S.D. of three independent experiments.

Inhibitory Effect of QACs on Daunorubicin Transport in MDCKII-MDR1 Cells

First, we measured the inhibitory effect of TBA (0.1–100 μM) in the presence or absence of a 10-fold molar excess of TDC on the B-to-A transport of daunorubicin. As shown in Fig. 4B, a significant inhibitory effect was observed for TBA alone, with an IC_{50} value of 169±24.1 μM. However, TDC (1–1000 μM) alone did not inhibit the P-gp-mediated daunorubicin transport. On the other hand, a much stronger inhibitory effect was observed for TBA in the presence of TDC, resulting in a much lower IC_{50} value of 10.3±5.3 μM. This suggests that, when compared with TBA alone, the formation of ion-pair complexes with TDC results in a greater inhibition potential.

We then measured the inhibitory effect of the other QACs on daunorubicin transport, in a similar manner. Again, significant inhibition was observed for TBuMA and TPeA in the absence of TDC, and the inhibition was potentiated by the presence of TDC (data not shown). We concluded, therefore, that the formation of ion-pair complexes with TDC would enhance the inhibitory potential of certain QACs in terms of P-gp-mediated daunorubicin transport.

Mw-Dependent Inhibitory Effect of QACs on MPP$^+$ Uptake in MDCKII-OCT1 Cells

We measured the inhibitory effect of various QACs (0.1–1000 μM), in the presence and absence of a 10-fold molar excess of TDC, on the uptake of MPP$^+$ in MDCK-OCT1 cells, and the data were fitted to Eq. 1 to determine the IC_{50} values of QACs and their ion-pair complexes for OCT1. The log IC_{50} values were then plotted as a function of the Mw of QACs (Fig. 5A). Regardless of the presence of TDC, the log IC_{50} values decreased linearly as the Mw of QACs increased with good correlations of $\log(IC_{50})=-0.011x+4.052$ ($r^2=0.995$, $p<0.001$) for QACs alone, and $\log(IC_{50})=-0.012x+4.267$ ($r^2=0.991$, $p<0.001$) for QAC in the presence of TDC. No significant difference in IC_{50} values was observed between the QACs and their ion-pair complexes.

Mw-Dependent Inhibitory Effect of QACs and Their TDC Ion-Pair Complexes on Daunorubicin Transport in MDCKII-MDR1 Cells

We measured the inhibitory effect of various QACs (0.1–100 μM), in the presence and absence of a 10-fold molar excess of TDC, on the B-to-A transport of daunorubicin (100 nM) in MDCK-MDR1 cells, and the data were fitted to Eq. 1 to determine the IC_{50} values of QACs and their ion-pair complexes for P-gp. The log IC_{50} values thus obtained were then plotted as a function of the Mw of QACs (Fig. 5B). An apparent Mw threshold of around 200 was observed for the inhibitory potential of QACs to P-gp in the absence of TDC. Interestingly, an identical but much more distinct Mw threshold (i.e., around 200 as the Mw of QACs) in the IC_{50} values was observed for QACs in the presence of TDC. This appears to indicate that the affinity of QACs (e.g., TBuMA, TBA, and

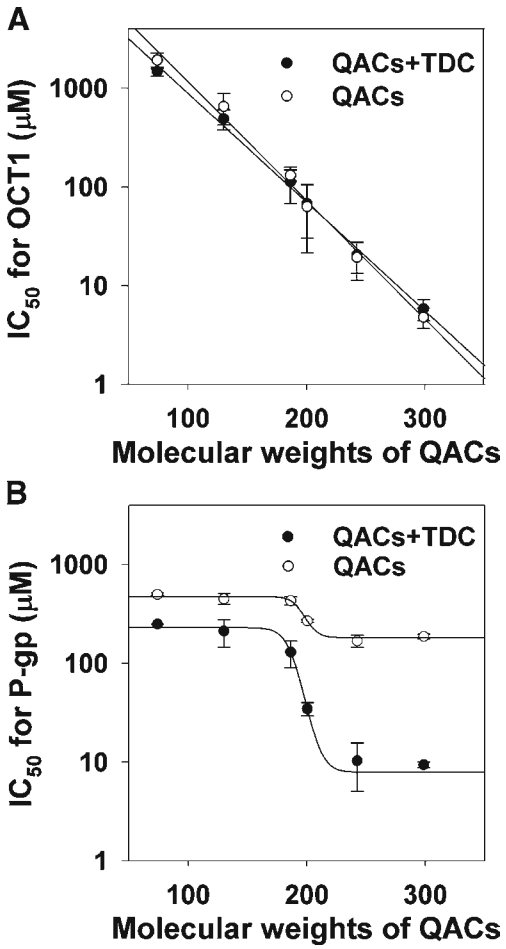

Fig. 5. (**A**) Relationship between molecular weights of QACs and IC_{50} values in inhibiting 1 μM [^{3}H]MPP^+ uptake in MDCKII-OCT1 cells. (**B**) Relationship between molecular weights of QACs and IC_{50} values in inhibiting the B-to-A transport rate of 100 nM [^{3}H] daunorubicin in MDCKII-MDR1 cell monolayer. Each data point represents the mean ± S.D. of three independent experiments.

TPeA) to P-gp increases when the Mw is higher than 200. Moreover, the affinity of these compounds to P-gp is increased significantly by the presence of TDC, probably via the formation of respective lipophilic ion-pair complexes. In any case, a significant reduction in TEER values was not observed after the experiments (data not shown).

Effect of Ion-Pair Complexation of QACs with TDC on the Transepithelial Transport of QACs across the MDCKII-MDR1 Cell Monolayer

To investigate the functional consequences of QACs-TDC complexes with increased lipophilicty and increased affinity for P-gp, we measured the effect of QACs-TDC complexes on the transepithelial transport of QACs. Fig. 6A shows that B-to-A transport rates of TBuMA, TBA, and TPeA are greater than the A-to-B transport rates of the respective compounds, indicating that these high Mw QACs themselves are substrates for P-gp, consistent with a significant decrease in IC_{50} values, as shown in Fig. 5B. The B-to-A transport rates of these compounds were significantly increased by the addition of TDC with magnitudes of 1.4-fold for TBuMA, 1.5-fold for TBA, and 1.8-fold for TPeA. The presence of 100 μM TDC appeared to have no influence on the integrity of the cell membranes, because no changes in TEER values were observed (data not shown). These results suggest that the increased affinity for P-gp due to the formation of QACs-TDC complexes enhanced the B-to-A transport of QACs. However, A-to-B transport rates of QACs were not changed by the presence of TDC. To investigate the effect of increased efflux via P-gp on the cellular accumulation of QACs, we measured the cellular accumulation of QACs for 30 min in the presence and absence of TDC. Cellular accumulations of TBuMA, TBA,

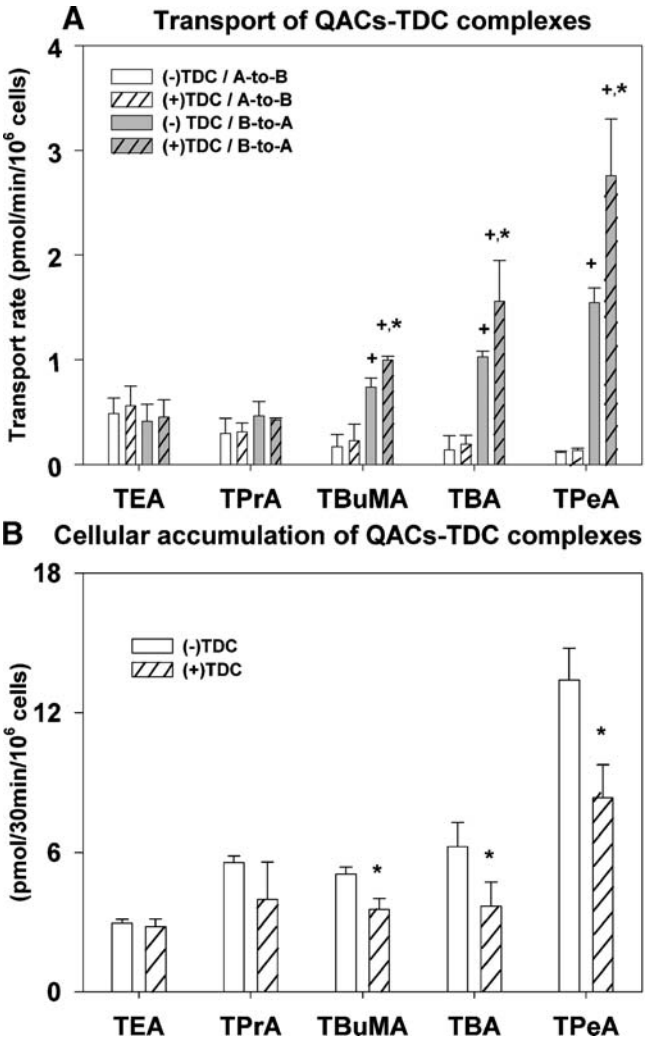

Fig. 6. (**A**) Effect of TDC (100 μM) on the transepithelial transport of TBuMA (10 μM) and TEMA (10 μM) across the MDCKII-MDR cell monolayers. White symbols indicate A-to-B transport, and gray symbols the B-to-A transport. The open bar indicates the transport rate in the absence of TDC, and the hatched bar indicates the transport rate in the presence of TDC. (**B**) Cellular accumulation of 10 μM TEA, TPrA, TBuMA. TBA, and TPeA in the presence and absence of 100 μM TDC in MDCKII-MDR cells. The open bar indicates the transport rate in the absence of TDC, and the hatched bar indicates the transport rate in the presence of TDC. Data are means ± S.D. of three determinations. *: $p<0.05$, statistically different compared with TDC-untreated groups by unpaired *t*-test, +: $p<0.05$, statistically different compared with A-to-B transport groups by unpaired *t*-test.

and TPeA were significantly decreased by the presence of TDC, which was consistent with our previous results (6), whereas the cellular accumulation of neither TEA nor TPrA was changed by the addition of TDC (Fig. 6B). These results reconfirm that formation of a lipophilic ion-pair complex increases the P-gp-mediated efflux of QACs and, as a result, decreases cellular accumulation of high-Mw QACs.

DISCUSSION

Biliary excretion is one of the major elimination pathways for both endogenous compounds and xenobiotics. Biliary excretion is highly dependent on the Mw of these compounds, and a threshold for the Mw is observed for the biliary excretion of organic cations as well as organic anions: organic cations and anions with a Mw higher than 200±50 and 325±50, respectively, showed appreciable excretion into the bile of rats (1,17,18). For organic anions, the involvement of canalicular Mrp2 and Bcrp was suggested as the mechanism of the Mw threshold (19). However, no mechanism other than our hypothesis has been proposed for the Mw threshold of organic cations. We previously reported that the ability of QACs to form lipophilic ion-pair complexes with bile salts in the liver appears to determine the extent of biliary excretion of such compounds: the higher the Mw of QACs, the more ion-pair complexes are formed with bile salts, followed by greater biliary excretion (5).

OCT1 and P-gp are transporters responsible for hepatobiliary excretion of many QACs in humans as well as in rats (4,11,16,20). Therefore, in this study, we examined the effect of ion-pair formation with TDC on the affinity of QACs to these transporters. First, we examined the relationship between the Mw of QACs and ion-pair formation. The increase in the APC between n-octanol and phosphate buffer (pH 7.4) was utilized as evidence of ion-pair formation. TDC was selected as a representative endogenous bile salt that forms lipophilic ion-pair complexes with TBuMA, based on an interesting finding that only conjugated salts of deoxy bile acids (e.g., TDC) can form lipophilic ion-pair complexes with high-Mw QACs (5). Using the APC values in Fig. 2, the APC ratio (i.e., the ratio of APC between the presence of TDC and absence of TDC) was calculated and plotted as a function of the Mw of QACs (Fig. 7A). The ratios for lower Mw QACs (i.e., TMA, TEA, and TPrA) were generally low, while those for higher Mw QACs (i.e., TBuMA, TBA, and TPeA) were generally high, and a sharp increase in the ratio was observed at a Mw of 200±50 (Fig. 7A). This indicates that a Mw threshold exists for QACs to form lipophilic ion-pair complexes with relevant bile salts. It should be noted that this threshold is highly consistent with the well-known Mw threshold for appreciable hepatobiliary excretion of QACs in rats (1).

We then investigated whether ion-pair complexation can modulate the affinity of QACs to hepatic transporters (OCT1 and P-gp) in a Mw-dependent manner. The modulation was assessed by measuring the inhibitory effect of QACs (i.e., IC_{50} values) in the presence of TDC on the OCT1 and P-gp-mediated transport of MPP^+ and daunorubicin, respectively (4,11,16). For this purpose, we used MDCKII cells that overexpress OCT1 and MDR1 based on insignificant species differences of the transporters (11,16). In other words, the

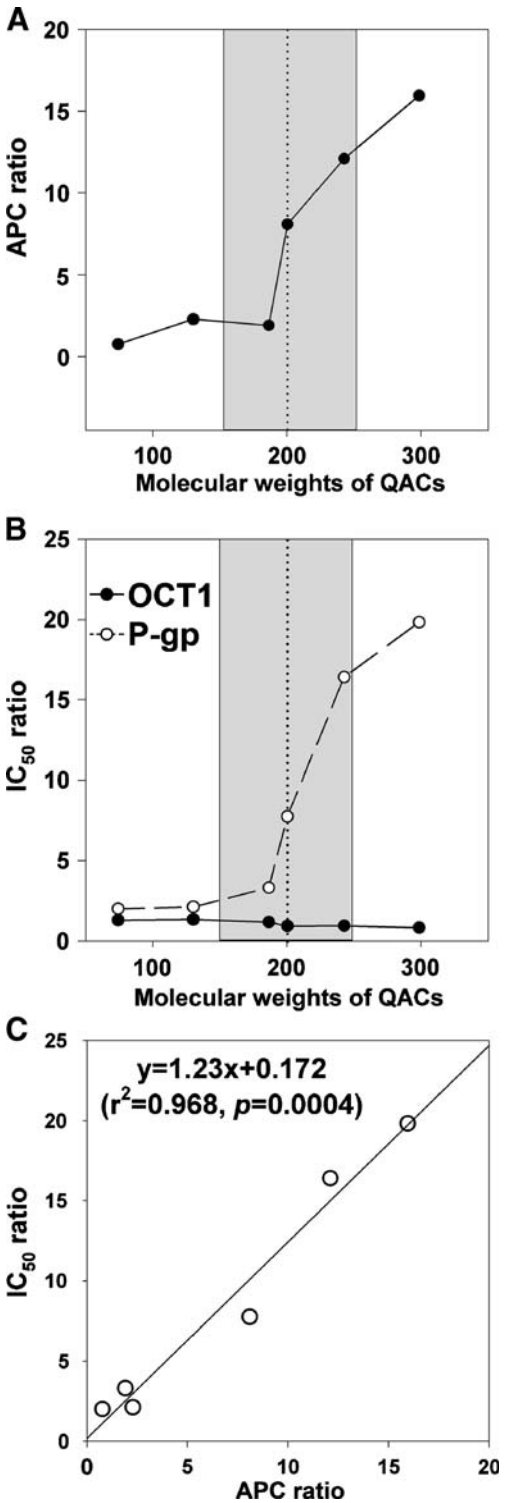

Fig. 7. (**A**) The relationship between molecular weights of QACs and their APC ratio. APC ratios were calculated by dividing APC of 10 μM QACs in the presence of 100 μM TDC by APC of 10 μM QACs in the absence of TDC. (**B**) The relationship between the molecular weights of QACs and their IC_{50} ratios for OCT1 (●) or P-gp (○). IC_{50} ratios were calculated using the data of Fig. 5 from the concentration dependence in the inhibitory effect of QACs on the OCT1 or P-gp-mediated transport of MPP^+ or daunorubicin, respectively. (**C**) Correlation between APC ratios and IC_{50} ratios for P-gp.

tendency for a correlation between the inhibitory potency and Mw of QACs was quite similar in OCT1 from different species, such as mice, rats, rabbits, and humans, and the apparent K_i values of QACs were comparable among species (4,11). Moreover, both human MDR1 and rat mdr1 were able to transport the monoquaternary model drugs, and both the affinity for P-gp and the inhibitory potential of the monoquaternary model drugs were similar between human MDR1 and rat mdr1, with noted exception of N-methylquinine (16).

As shown in Fig. 5A, log IC_{50} values for OCT1-mediated MPP^+ uptake in MDCKII-OCT1 cells decreased linearly with an increasing Mw. No differences in the affinity of QACs to OCT1 were observed in the presence of TDC. These results suggest that OCT1-mediated uptake is not associated with the Mw threshold in the hepatobiliary excretion of QACs and that the presence of endogenous bile salts or ion-pair formation in the hepatocytes would not affect the sinusoidal uptake of QACs via OCT1. On the other hand, in the case of P-gp, log IC_{50} values did not decrease linearly with the Mw of QACs; instead, the value could be divided into two phases, one for QACs with a Mw smaller than 200 and the other for QACs with a Mw greater than 200 (Fig. 5B), suggesting P-gp mediated B-to-A transport for higher Mw QACs (Fig. 6A). However, the IC_{50} values of P-gp for QACs with a higher Mw were much lower in the presence of TDC when compared with those of QACs alone (Fig. 5B), indicating a higher affinity of ion-pair complexes to P-gp for higher Mw QACs. These results imply that the underlying mechanism of the Mw threshold for appreciable biliary excretion (1) is associated with Mw-dependent binding of QAC itself and lipophilic QAC-bile salt complexes to canalicular P-gp.

Next, we further examined the transport rate of QACs in the absence and the presence of TDC. The B-to-A transport rates were significantly greater than the A-to-B transport rates for high Mw QACs (i.e., TBuMA, TBA, and TPeA) (Fig. 6A) in the absence of TDC, suggesting a Mw-dependent and P-gp-mediated excretion mechanism in the excretion of QACs themselves. By the presence of TDC, the B-to-A transport rates of high Mw QACs were increased significantly, but not for low Mw QACs (Fig. 6A), suggesting that P-gp substrate specificity of high Mw QACs can be enhanced by the formation of ion-pair complexes with relevant bile salts. As a consequence of the enhanced P-gp functionality, the cellular accumulation of high Mw QACs was decreased by the addition of TDC (Fig. 6B). We previously reported similar results using TBuMA as a model compound in LLC-PK1 cells (6). Data from the study of Biedler and Riehm (21) showed that a great decrease in the accumulation in P-gp overexpressed cells was observed only for high Mw anticancer drugs (21), which is consistent with results from the present study.

To compare the affinities of QACs and QAC-bile salts complexes to P-gp, the IC_{50} ratios of ion-pair complexes over corresponding QACs were calculated and plotted as a function of the Mw of QACs (Fig. 7B). A rapid increase in the IC_{50} ratio was observed for ion-pair complexes, compared to corresponding QACs, for P-gp but not for OCT1, with a distinct Mw threshold at a Mw of 200±50. These results suggest that increased affinity of QACs to P-gp via ion-pair formation with bile salts is a governing mechanism of biliary excretion of high Mw QACs.

Finally, in order to elucidate the relationship between the lipophilicity and affinity of ion-pair complexes, the IC_{50} ratios were plotted against the APC ratios of QACs-TDC complexes (Fig. 7C). The only significant ($p=0.0004$) linear correlation existed between the ratios for P-gp with r^2 values of 0.968. This indicates that an increase in lipophilicity caused by the formation of ion-pair complexes may govern the affinity of the ion-pair complexes to P-gp. According to the 'hydrophobic vacuum cleaner' model of P-gp action, substrates first partition into the lipid bilayer and interact with P-gp within the membrane, then efflux to the extracellular side (22). Loo and Clarke suggested that the drug-binding pocket is located within the membrane-bound regions of P-gp and is made up of several transmembrane segments (23). Therefore, the functionality of P-gp is inextricably linked to the lipid bilayer, and the binding affinity of P-gp for a certain drug is related to its lipid-water partition coefficient (22,24). In this study, the increased lipophilicity that is due to formation of the QACs-TDC complexes resulted in accelerated partitioning into the lipid bilayer, thereby increasing the effective concentration around the P-gp substrate binding site. In fact, this might be the mechanism by which the P-gp affinity of some QACs was increased in the presence of TDC. Similar results were reported by Smit *et al*. That is, hepatic excretion of doxorubicin was inhibited by the presence of organic cations with various degrees of lipophilicity (i.e., quinidine, quinine, varapamil, digoxin, rocuronium, vinblastine, tributylmethylammonium, procainamidethobromide), and a positive correlation was observed between the log P value of these inhibitors and the inhibitory effect on biliary excretion of P-gp substrates (25).

In summary, a Mw threshold of 200±50 was necessary for the P-gp-mediated transport of QACs and for the formation of lipophilic ion-pair complexes with bile salts in the liver, and, thus, for appreciable hepatobiliary excretion of QACs (1). Ion-pair formation, on the other hand, did not

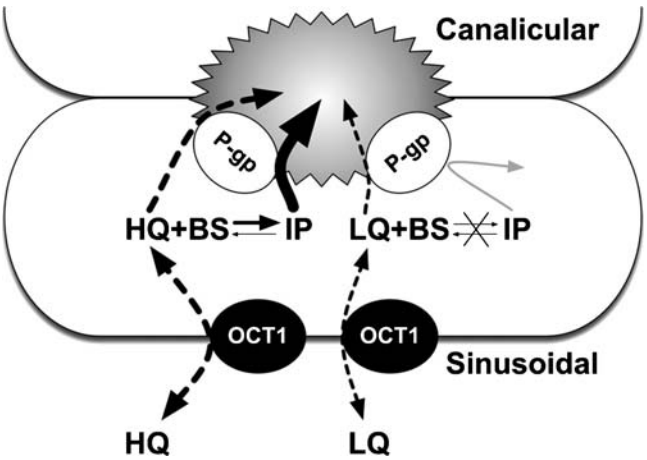

Fig. 8. Schematic illustration of the mechanism of molecular weight (Mw)-dependent hepatobiliary excretion of quarternary ammonium compounds (QACs) in rats. HQ: high Mw QACs (Mw>200), LQ: low Mw QACs (Mw<200), BS: bile salts (deoxy conjugated form), IP: ion-pair complexes of QACs with BS. Solid arrows indicate the transport in the forms of ion-pair complexes, dotted arrows the transport in the forms of QACs. Thickness of arrows indicates the magnitude of the transport.

affect the transport of high Mw QACs across sinusoidal OCT1 (Figs. 5A and 7B). Along with the P-gp-mediated transport of high Mw QACs, the formation of lipophilic ion-pair complexes with specific bile salts, followed by stronger binding to canalicular P-gp and facilitated transport rate probably due to increased lipophilicity of the complex, was proposed as a principal mechanism of appreciable biliary excretion of QACs (1) (Fig. 8). Overall, ion-pair formation with endogenous bile salts in the liver appears to be a mechanism that accelerates biliary excretion of QACs with a high Mw.

ACKNOWLEDGMENTS

This work was supported by a grant (No. CBM33-B4000-01-01-00) from the Center for Biological Modulators of the 21st Century Frontier R&D Program, Ministry of Science and Technology, Korea, and the Korea Health 21 R&D Project, Ministry of Health and Welfare (A030001), and by a grant from the Korean Ministry of Science and Technology through the National Research Laboratory Program (ROA-2006-000-10290-0).

REFERENCES

1. Hughes RD, Millburn P, Williams RT. Molecular weight as a factor in the excretion of monoquaternary ammonium cations in the bile of the rat, rabbit and guinea pig. Biochem J. 1973;136:967–78.
2. Han YH, Chung SJ, Shim CK. Canalicular membrane transport is primarily responsible for the difference in hepatobiliary excretion of triethylmethylammonium and tributylmethylammonium in rats. Drug Metab Dispos. 1999;27:872–9.
3. Song IS, Chung SJ, Shim CK. 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. Pharm Res. 1999;16:540–4.
4. Zhang L, Gorset W, Dresser MJ, Giacomini KM. The interaction of n-tetraalkylammonium compounds with a human organic cation transporter, hOCT1. J Pharmacol Exp Ther. 1999;288:1192–8.
5. Song IS, Chung SJ, Shim CK. Contribution of ion pair complexation with bile salts to biliary excretion of organic cations in rats. Am J Physiol Gastrointest Liver Physiol. 2001;281:G515–25.
6. Song IS, Han YH, Chung SJ, Shim CK. Contribution of ion-pair complexation with bile salts to the transport of organic cations across LLC-PK1 cell monolayers. Pharm Res. 2003;20:597–604.
7. Shim WS, Choi MK, Kim IW, Kwon TS, Song IS, Han L, *et al.* Decreased secretory transport of a quarternary ammonium, TBuMA, across LLC-PK1 cells by the anionic kidney extract. Arch Pharm Res. 2008;31:671–7.
8. Choi MK, Song IS, Kim DD, Chung SJ, Shim CK. Decreased biliary excretion of tributylmethyl ammonium in cholestyramine pretreated rats due to reduced formation of ion-pair complexes with hepatic bile salts. Biopharm Drug Dispos. 2007;28:485–90.
9. Choi MK, Song IS, Park SR, Hong SS, Kim DD, Chung SJ, *et al.* Mechanism of the stationary canalicular excretion of tributylmethyl ammonium in rats with a CCl4-induced acute hepatic injury. J Pharm Sci. 2005;94:317–26.
10. Choi MK, Jin QR, Jin HE, Shim CK, Cho DY, Shin JG, *et al.* Effects of tetraalkylammonium compounds with different affinities for organic cation transporters on the pharmacokinetics of metformin. Biopharm Drug Dispos. 2007;28:501–10.
11. Dresser MJ, Gray AT, Giacomini KM. Kinetic and selectivity differences between rodent, rabbit, and human organic cation transporters (OCT1). J Pharmacol Exp Ther. 2000;292:1146–52.
12. Choi MK, Song IS, Kim DD, Chung SJ, Shim CK. Altered pharmacokinetics of daunorubicin in rats with CCl4-induced hepatic injury. J Pharm Pharm Sci. 2007;10:443–54.
13. Tang F, Horie K, Borchardt RT. Are MDCK cells transfected with the human MDR1 gene a good model of the human intestinal mucosa? Pharm Res. 2002;19:765–72.
14. Wang EJ, Casciano CN, Clement RP, Johnson WW. Active transport of fluorescent P-glycoprotein substrates: evaluation as markers and interaction with inhibitors. Biochem Biophys Res Commun. 2001;289:580–5.
15. Kusunoki N, Takara K, Tanigawara Y, Yamauchi A, Ueda K, Komada F, *et al.* Inhibitory effects of a cyclosporin derivative, SDZ PSC 833, on transport of doxorubicin and vinblastine via human P-glycoprotein. Jpn J Cancer Res. 1998;89:1220–8.
16. Hooiveld GJ, Heegsma J, van Montfoort JE, Jansen PL, Meijer DK, Muller M. Stereoselective transport of hydrophilic quaternary drugs by human MDR1 and rat Mdr1b P-glycoproteins. Br J Pharmacol. 2002;135:1685–94.
17. Hirom PC, Millburn P, Smith RL, Williams RT. Species variations in the threshold molecular-weight factor for the biliary excretion of organic anions. Biochem J. 1972;129:1071–7.
18. Hirom PC, Millburn P, Smith RL, Williams RT. Molecular weight and chemical structure as factors in the biliary excretion of sulphonamides in the rat. Xenobiotica. 1972;2:205–14.
19. Kato Y, Takahara S, Kato S, Kubo Y, Sai Y, Tamai I, *et al.* Involvement of multidrug resistance-associated protein 2 (Abcc2) in molecular weight-dependent biliary excretion of beta-lactam antibiotics. Drug Metab Dispos. 2008;36:1088–96.
20. Smit JW, Schinkel AH, Muller M, Weert B, Meijer DK. Contribution of the murine mdr1a P-glycoprotein to hepatobiliary and intestinal elimination of cationic drugs as measured in mice with an mdr1a gene disruption. Hepatology. 1998;27:1056–63.
21. Biedler JL, Riehm H. Cellular resistance to actinomycin D in Chinese hamster cells *in vitro*: cross-resistance, radioautographic, and cytogenetic studies. Cancer Res. 1970;30:1174–84.
22. Sharom FJ. Shedding light on drug transport: structure and function of the P-glycoprotein multidrug transporter (ABCB1). Biochem Cell Biol. 2006;84:979–92.
23. Loo TW, Clarke DM. Recent progress in understanding the mechanism of P-glycoprotein-mediated drug efflux. J Membr Biol. 2005;206:173–85.
24. Romsicki Y, Sharom FJ. The membrane lipid environment modulates drug interactions with the P-glycoprotein multidrug transporter. Biochemistry. 1999;38:6887–96.
25. Smit JW, Duin E, Steen H, Oosting R, Roggeveld J, Meijer DK. Interactions between P-glycoprotein substrates and other cationic drugs at the hepatic excretory level. Br J Pharmacol. 1998;123:361–70.