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Effect of a New Hepatoprotective Agent, YH-439, on the Hepatobiliary Transport of Organic Cations (OCs): Selective Inhibition of Sinusoidal OCs Uptake without Influencing Glucose Uptake and Canalicular OCs Excretion

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The effect of a new hepatoprotective agent, YH-439, on the hepatobiliary transport of a model organic cation (OC), TBuMA (tributylmethylammonium), was investigated. The area under the plasma concentration-time curve (AUC) from time zero to 4 h following iv administration of TBuMA (6.6 μmol/kg) was increased significantly when YH-439 in corn oil (300 mg/kg) was orally administered to rats 24 h prior to the experiment. Nevertheless, the cumulative biliary excretion of TBuMA remained unchanged. As a consequence, the apparent biliary clearance (CL_b) of TBuMA was decreased significantly as a result of YH-439 pretreatment, consistent with the fact that the *in vivo* excretion clearance of TBuMA across the canalicular membrane (CL_{exc}) was not changed by the pretreatment. The in vitro uptake of TBuMA into isolated hepatocytes was decreased by one half by the pretreatment, owing to a decrease in the apparent V_{max} and CL_{linear}, but the K_m for the process remained constant. Most interestingly, however, the sinusoidal uptake of glucose, a nutrient, into hepatocytes was not influenced by the pretreatment, suggesting the YH-439 pretreatment specifically impaired the sinusoidal uptake of OCs. Thus, the OC-specific inhibition of hepatic uptake, without influencing the uptake of glucose, a nutrient, appeared to be associated with the hepatoprotective activity of YH-439.

Key words: YH-439, Hepatoprotective agent, Sinusoidal uptake, Organic cation, TBuMA, Selective inhibition

INTRODUCTION

Isopropyl-2-(1,3-dithioethane-2-ylidene)-2-[*N*-(4-methylthiazol-2-yl) carbamoyl] acetate (YH-439) is under development as a hepatoprotective agent by the Research Center of the Yuhan Co. (Seoul, Korea), and is currently being evaluated in phase II clinical trials (Fig. 1). The hepatoprotective mechanism of YH-439 is known to involve the protection of the liver against chemical-induced hepatic injury probably by suppressing the expression of CYP2E1, which is responsible for the toxicity of a number of xenobiotics, *via* metabolic activation and/or the accumulation of reactive metabolites (Choi *et al.*, 1996; Jeong *et al.*, 1996). It was concluded based on the fact that an oral administration of YH-439 for 2-3 consecutive days at a dose of 200 mg/kg suppressed the expression of hepatic CYP 2E1, and simultaneously caused an elevation in CYP1A levels (Lee *et al.*, 1996) and the activities of microsomal epoxide hydrolase and cytosolic glutathione S transferase (GST) in rats (Choi *et al.*, 1996). As the result, the pharmacokinetics of drugs is frequently influencd by YH-439 pretreatment (Cho *et al.*, 1996; Kim *et al.*, 1996), as exemplified by the accelerated

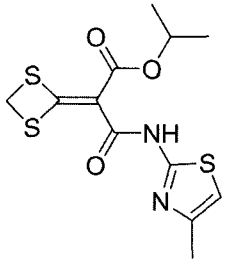

Fig. 1. Structure of YH-439

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systemic elimination of acetaminophen via an increase in glucronide conjugation (Cho *et al.*, 1996).

Hepatic enzymes are frequently linked with transporters in the liver in terms of both expression and functions (Benet *et al.*, 2003; Oude Elferink *et al.*, 1993). Thus, it is reasonable to hypothesize that the expression or function of hepatic transporters may be influenced by pretreatment with YH-439, as well as the case of enzymes. No reports concerning this issue, however, have been reported for YH-439. In the present study, therefore, the effect of YH-439 on the function of transporters that are expressed on the sinusoidal and canalicular membranes of hepatocytes was examined. For this purpose, the hepatobiliary transport of tributylmethylammonium (TBuMA, Mw: 200), a representative organic cation (OC), was determined, since the hepatic uptake and canalicular excretion of the compound are known to be mediated by a sinusoidal transporter (i.e., an organic cation transporter 1, OCT1) (Koepsell, 1998; Hong *et al.*, 2000), and a canalicular transporter (i.e., an ATP-dependent P-gylcoprotein, P-gp, system) (Song *et al.*, 1999). TBuMA has advantages as a model compound over the other organic compounds in that it does not bind to proteins in either the plasma or the liver cytosol, and is not metabolized in the body (Neef *et al.*, 1984). The sinusoidal uptake of glucose, a nutrient, into hepatocytes was also determined in order to compare the effect of YH-439 between the sinusoidal transporters.

MATERIALS AND METHODS

Materials

[^{3}H]TBuMA (0.2 Ci/mmol) was synthesized according to the method of Neef *et al.* (1984), and diluted for each experiment. YH-439 was generously donated by the Yuhan Research Center (Seoul, Korea). All other reagents employed were of the highest grade commercially available.

Pretreatment of animals

Male Sprague-Dawley rats, 7 to 8 weeks of age, were used in the study. YH-439 pretreatment involved the oral administration of a suspension of YH-439 in corn oil (60 mg/mL) at a dose of 300 mg/kg for 3 consecutive days. For the control rats, corn oil without YH-439 was administered in the same volume. All studies were performed 24 h after the last administration.

Systemic *in vivo* pharmacokinetic study

Under light ether anesthesia, the femoral artery and vein of control and YH-439 pretreated rats were cannulated with polyethylene tubing (PE-50) for blood sampling and TBuMA administration, respectively. In order to collect bile specimens, the common bile duct was also cannulated with PE-10 tubing. After recovery from the surgery, the animals received [^{3}H]TBuMA at a bolus dose of 6.6 μmol (13.2 mCi)/kg *via* the femoral vein.

Blood samples and bile were collected at appropriate intervals over a 4 h period. The concentrations of [^{3}H]TBuMA in the plasma and bile were quantified by means of a liquid scintillation counting (LSC System 1409, Wallac). The area under the plasma concentration-time curve from time zero to 4 h (AUC) was calculated by the trapezoidal rule. The apparent biliary clearance (CL_b) was calculated by dividing the amount of TBuMA excreted into the bile during 4 h by the AUC up to 4 h.

In vivo biliary excretion across canalicular membrane

For the estimation of biliary excretion clearance (CL_{exc}), the rats were given an i.v. bolus injection, followed by infusion, in order to obtain a value for the steady state concentration in the liver. [^{3}H]TBuMA was infused to the control or the YH-439 pretreated rats at a rate of 1.5 mmole (13.2 μCi)/h/kg after a bolus administration of 1.5 mmole (13.2 μCi)/kg. Plasma and bile were collected at 30 min intervals up to 3 h, and blood and liver samples were immediately collected after the last sampling for the determination of TBuMA concentration. Radioactivity in the liver, plasma, and bile was determined, and the CL_{exc} was calculated by dividing the biliary excretion rate by the concentration of TBuMA in the liver.

In vitro uptake into hepatocytes

Hepatocytes were obtained according to the procedure described by Han et al. (1999) from control and YH-439 pretreated rats. The cell suspension (2.5×10^6 cells/mL) was pre-incubated in the medium for 5 min at 37°C. [^{3}H]TBuMA (20 μL) was added to the suspension to give a final medium concentration of 1~100 μM. An aliquot (200 μL) of the suspension was sampled at 30, 60, 90, and 120 sec, and the level of radioactivity in the hepatocytes was determined as described previously. The initial rate of uptake of TBuMA into hepatocytes, which was calculated from the linear portion (i.e., generally up to 1 min) of the plot, was then plotted against the initial concentration of the substrate in the medium. A nonlinear regression analysis was performed, to fit the plot to the following equation using WINNONLIN (version 1.0; SCI Software, Lexington, KY).

$$V_o = V_{max} \cdot S/(K_m + S) + CL_{linear} \cdot S \quad \text{(Eq. 1)}$$

Where V_o is the initial uptake rate of the substrate (pmole/min/10^6 cells), S is the concentration of TBuMA in the medium (μM). V_{max} and K_m are the maximum rate of uptake and the medium concentration at the half maximal rate, respectively, and CL_{linear} represents the linear uptake clearance.

In order to briefly elucidate whether the effect of YH-439 pretreatment is specific for the uptake of TBuMA, the uptake of glucose, a nutrient, was also investigated. [^{3}H] glucose (11.5 Ci/mmol, 20 μL) was added to a suspension of hepatocytes to give a glucose concentration of 1 mM, and aliquots (200 μL) of the suspension were sampled at 30, 60, 90, and 120 sec, and the level of radioactivity in the hepatocytes was determined.

RESULTS AND DISCUSSION

Systemic *in vivo* pharmacokinetics of TBuMA

Temporal profiles for the plasma concentration of TBuMA after intravenous administration to the control and the YH-439 pretreated rats at a dose of 6.6 μmol/kg are shown in Fig. 2A, and the relevant pharmacokinetic parameters are listed in Table I. The plasma concentration of TBuMA followed a triexponential decline in both the control and pretreated rats. The mean plasma concentrations were higher in the pretreated rats compared to control rats, leading to a significant increase in AUC values for the pretreatment (Table I). On the other hand, no differences were observed between the control and pretreated groups for the cumulative biliary excretion of TBuMA (Fig. 2B and Table I). As a consequence, the biliary excretion clearance (CL_b) of TBuMA in the pretreated rats became significantly ($p < 0.01$) smaller compared to the control rats, suggesting that not only the enzyme activity (Choi *et al.*, 1996; Jeong *et al.*, 1996; Lee *et al.*, 1996) but also the hepatobiliary transport of OCs were significantly influenced by the YH-439 pretreatment.

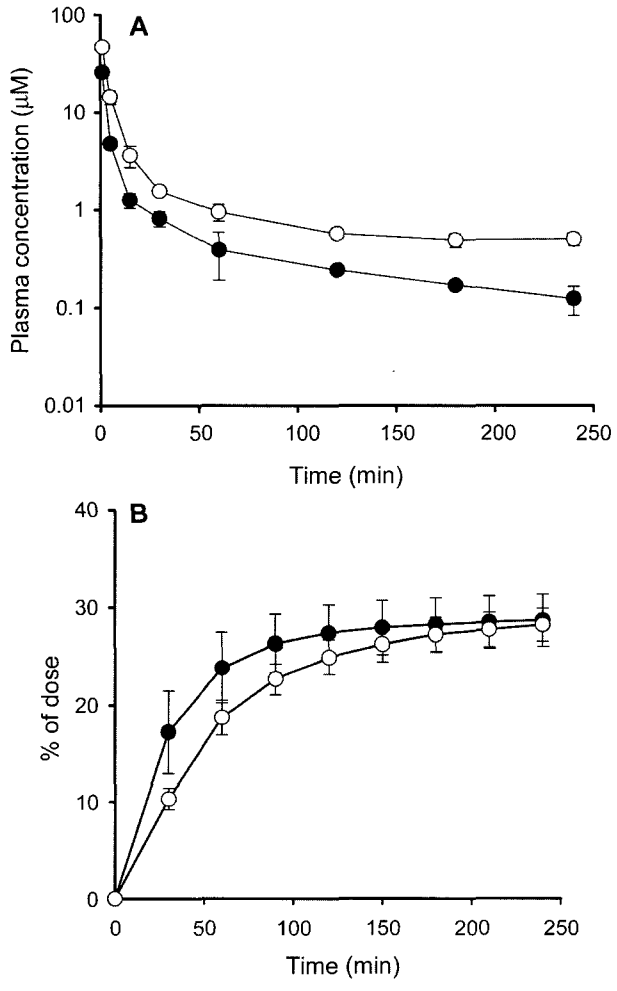

Fig. 2. A: Temporal profiles for the plasma concentration (A) and cumulative biliary excretion (B) of TBuMA in the control (●) and the YH-439 pretreated rats (○). Each data point represents the mean ± S.D. of four animals. B: cumulative biliary excretion of TBuMA

Table I. The effect of YH-439 pretreatment on the pharmacokinetics of TBuMA after i.v. administration (6.6 $_m$mol/kg)[a]

Pharmacokinetic parameters	Control	YH-439 pretreated
AUC (μM×min)[b]	198 ± 9	452 ± 30 *
Cumulative biliary excretion (% of dose)[b]	28.7 ± 2.6	28.2 ± 1.7
CL_b (mL/min/kg)	9.6 ± 1.2	4.1 ± 0.4 *

[a] Each data is expressed as the mean ± S.E. of four separate experiments.
[b] Calculated up to 4 h.
*$p<0.01$ for the control group by the unpaired student's *t*-test.

In vivo biliary excretion across canalicular membrane

Under the given experimental conditions, the concentration of TBuMA in the plasma and liver reached steady states, respectively, at 120 min after the start of the infusion in both the control and YH-439 pretreated rats (1.0 ± 0.1 *vs* 2.1 ± 0.3 μM for the plasma, n = 4 and 6.6 ± 1.2 *vs* 14.3 ± 3.8 nmole/g for the liver, n = 4). No difference was found for the biliary excretion clearance (CL_{exc}) of TBuMA at the steady state, i.e., the biliary excretion rate divided by the liver concentration, between the control (181 ± 28 μL/min/kg) and YH-439 pretreated (170 ± 57 μL/min/kg) groups, suggesting that the pretreatment had no influence on the transport of TBuMA across the canalicular membrane, despite the significant change in the systemic pharmacokinetics (Fig. 2 and Table I). Considering the fact that TBuMA excretion across the canalicular membrane is mediated by the canalicular P-gp system (Song *et al.*, 1999), YH-439 does not appear to influence the transport of OCs *via* this system. The CL_{exc} in the control ratrs was consistent with findings reported in our previous study (Hong *et al.*, 2000).

In vitro uptake of TBuMA and glucose into hepatocytes

When the initial uptake rate of glucose (1 μM) into

hepatocytes was calculated from the slope in initial times (i.e., generally up to 2 min), it was not influenced significantly by YH-439 pretreatment (Fig. 3A), while a significant decrease was observed for the uptake of TBuMA (Fig. 3B). The uptake profiles for TBuMA were apparently linear over at least 90 sec for both the control and YH-439 pretreated rats (Fig. 3B), where the slopes of the lines represent the initial rate of uptake (velocity, V_0). Plots of the rate of uptake, V_0, vs. the initial substrate concentration (S) exhibited curvi-linear relationships for the control and YH-439 pretreated rats (Fig. 4), suggesting that uptake is mediated by both saturable and non-saturable processes. Eadie-Hofstee plots for the uptake were consistent with the mixed kinetic processes (data not shown). Table II summarizes the results of the fitting of the data (Fig. 4) to Eq. 1. A significant (more than one-half) decrease in V_{max} with a constant K_m was observed in the pretreated rats, suggesting a decrease in the quantity of carriers (V_{max}) that are responsible for hepatic uptake,

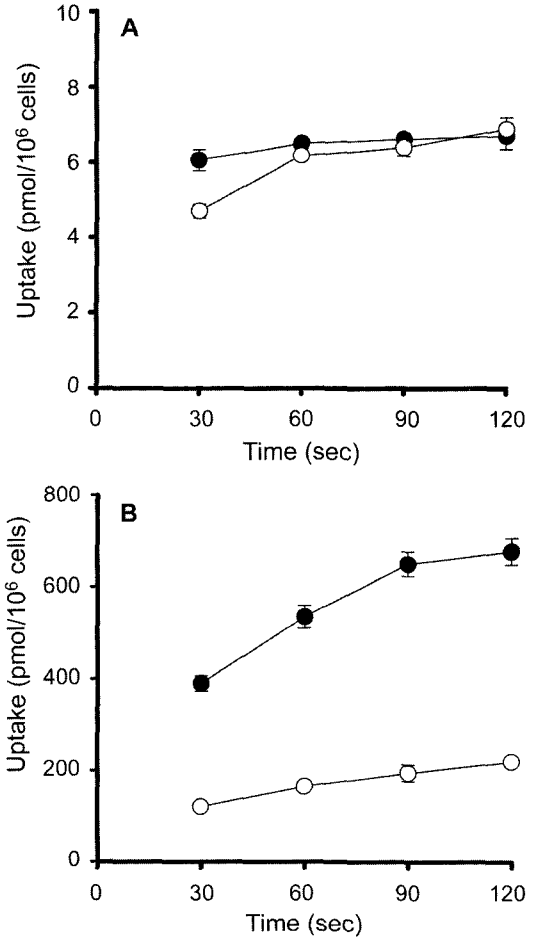

Fig. 3. Time course for the uptake of glucose (A) and TBuMA (B) into isolated hepatocytes for control (●) and YH-439 pretreated rats (○). Each data point represents the mean ± S.D. of three different preparations.

Table II. Effect of the YH-439 pretreatment on the kinetic constants for TBuMA uptake into isolated hepatocytes[a].

Kinetic parameters	Control	YH-439 pretreated
V_{max} (pmol/min/10^6 cells)	52.3 ± 4.5	20.0 ± 8.5*
K_m (μM)	1.85 ± 0.85	1.77 ± 0.66
CL_{linear} (μL/min/10^6 cells)	1.25 ± 0.04	0.47 ± 0.03*

[a] Each data is expressed as the mean ± S.E. of three separated experiments.
* $p<0.05$ from the control group by the unpaired student's *t*-test.

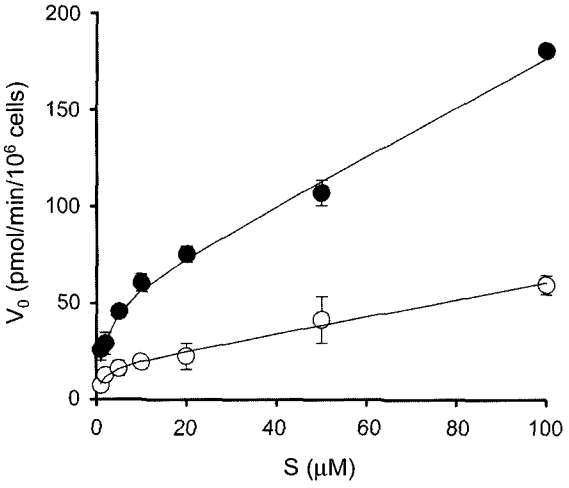

Fig. 4. Concentration dependency for the uptake of TBuMA into isolated hepatocytes for control (●) and YH-439 pretreated rats (○). Each data point represents the mean ± S.D. of three different preparations.

without influencing the affinity ($1/K_m$) of the compound to the carriers. OCT1 on the sinusoidal membrane could represent the transporter. A significant decrease in CL_{linear} by the pretreatment was also observed, suggesting that the non-saturable uptake, probably passive diffusion, was also impaired by the pretreatment. However, the contribution of nonsaturable uptake to overall uptake was minimal, less than 10 %.

It is noteworthy that TBuMA uptake, but not glucose uptake, was decreased as a result of the YH-439 pretreatment. This suggests that the sinusoidal uptake of specific xenobiotics (TBuMA in the present study) would be selectively impaired, without influencing the uptake of nutrients (e.g., glucose) by the YH-439 pretreatment. Considering that the hepatic uptake of TBuMA and glucose is mediated predominantly by an OCT1 (Hong *et al.*, 2000; Han *et al.*, 1999) and glucose transporter (GLUT2) (Lachaal *et al.*, 2000), respectively, the above results suggest that YH-439 selectively impairs OCT1-mediated transport (Hong *et al.*, 2000; Song *et al.*, 1999; Neef *et al.*, 1984; Han *et al.*, 1999) without influencing

GLUT-mediated transport. This is consistent with transporter-specific changes in the expression of hepatobiliary transporters by experimental hepatic failure (Song *et al.*, 2003). In the present study, YH-439 was confirmed to be absent in the blood, liver and bile at the time when the *in vivo* pharmacokinetic and *in vitro* hepatic uptake experiments were performed. Moreover, in our previous experiments, it was confirmed that YH-439 and TBuMA do not share identical transport systems (Park *et al.*, 2001). Therefore, a scenario in which YH-439 interferes with the pharmacokinetics and uptake of TBuMA can be excluded from the mechanism of the effect of YH-439. Pathophysiological changes induced by pretreatment with YH-439 appear to be responsible for the mechanism.

CONCLUSION

In vivo biliary excretion clearance (CL_b) of TBuMA was decreased significantly by pretreatment with YH-439, as a result of a decreased sinusoidal uptake. The canalicular excretion of TBuMA, on the other hand, was not impaired by the pretreatment. This suggests first that the effect of YH-439 is sinusoidal membrane-specific. The results of the *in vitro* hepatic uptake experiment revealed a decrease in V_{max} rather than a change in K_m, suggesting the decrease in the expression of relevant transporter, possibly OCT1. Contrary to the case for TBuMA, the uptake of glucose was not influenced by the pretreatment, suggesting that the effect of YH-439 on the sinusoidal membrane is selective for specific transporters. This might be related to the hepatoprotective nature of YH-439, i.e., the protection of the liver from xenobiotics such as OCs through inhibiting their hepatic uptake, without impairing the hepatic uptake of nutrients such as glucose. The effects of YH-439 pretreatment on hepatic transporters other than OCT1 and GLUT2 in terms of expression and function await further investigation.

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