Shiga-Like Toxin II Impairs Hepatobiliary Transport of Doxorubicin in Rats by Down-Regulation of Hepatic P Glycoprotein and Multidrug Resistance-Associated Protein Mrp2

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We investigated the effect of Shiga-like toxin II (SLT-II), derived from Escherichia coli O157:H7, on the hepatobiliary excretion of doxorubicin, a substrate for P glycoprotein and the multidrug resistance-associated protein Mrp2, and on the expression of P glycoprotein and Mrp2 in rats. Histopathological examination did not show any liver injury in SLT-II-treated rats. A significant delay in the disappearance of doxorubicin from plasma after its intravenous administration (5 mg/kg of body weight) was observed in rats treated 24 h earlier with SLT-II (2 μg/animal). When rats received an infusion of doxorubicin (2.6 μg/min) 24 h after intravenous injection of SLT-II, the steady-state concentration of doxorubicin in plasma increased and the bile flow decreased, whereas the concentration in liver did not alter. SLT-II significantly increased the unbound fraction of doxorubicin in plasma but did not alter the concentration in liver tissue. SLT-II significantly decreased the biliary excretion rate and biliary clearance of doxorubicin based on the total concentration and concentration of the unbound fraction in plasma and liver. Western blot analysis revealed that SLT-II down-regulated P glycoprotein and Mrp2 in the liver, which could explain the observed decrease in the biliary excretion of doxorubicin by SLT-II. A tumor necrosis factor alpha (TNF-α) production inhibitor, pentoxifylline, could not protect SLT-II-induced decreases in the biliary clearance of doxorubicin and down-regulation of both transporters. It is unlikely that TNF-α plays a major role in the SLT-II-induced decrease in the hepatobiliary transport of doxorubicin and the down-regulation of both transporters.

Shiga-like toxin (SLT)-producing Escherichia coli O157:H7 causes damage to capillary blood vessels of the small intestine, kidney, and central nervous system in humans, resulting in colonic ulceration, bloody diarrhea, kidney dysfunction, seizures, and death (24, 35, 36, 44). SLTs are divided into two major subtypes, SLT-I and SLT-II; SLT-II is known to possess potent virulence (19, 27). SLT-II is well known to induce non-specific diarrhea, hemorrhagic colitis, and severe hemolytic-uremic syndrome (HUS), which is the most common complication of E. coli O157:H7 infection and contributes to renal dysfunction and mortality. It has been reported that SLT-II can cause damage to renal tubular cells and renal function in rats (42) and brain capillaries in mice (43). Furthermore, we recently found that SLT-II reduces hepatic cytochrome P450 content and induces down-regulation of CYP3A2 and CYP2C11 (unpublished data). However, no data regarding the effect of SLT-II on liver function are available.

P glycoprotein and the multidrug resistance-associated protein Mrp2, members of the ATP-binding cassette transport proteins, are localized in the bile canalicular membrane of hepatocytes and act as efflux transporter proteins for endogenous and exogenous toxic substances (12, 29, 38). It is thought that liver dysfunction and altered functions of P glycoprotein and Mrp2 in some disease states could exert a large influence on the hepatobiliary excretion of drugs. We previously reported that SLT-II did not alter the expression of P glycoprotein and Mrp2 in the kidneys of rats 24 h after injection (42) but up-regulated P glycoprotein in the brains of mice (43). It is possible that SLT-II induces liver dysfunction and alters the expression of P glycoprotein and Mrp2 in the liver. However, the effects of SLT-II on the P-glycoprotein- and/or Mrp2-mediated hepatobiliary transport system and on the expression of P glycoprotein and/or Mrp2 in the bile canalicular membrane of hepatocytes in humans and animals have not yet been elucidated.

The present study aimed to evaluate the effect of SLT-II on the hepatobiliary disposition of the P-glycoprotein substrate doxorubicin. It would be a suitable model drug, as it is also a substrate for Mrp2 (10, 21) and its predominant pathway for elimination is biliary and renal excretion (31, 34, 39). We also studied the effect of SLT-II on the expression of P glycoprotein and Mrp2 in the liver and examined the role of a cytokine, tumor necrosis factor alpha (TNF-α), in SLT-II-induced changes in P-glycoprotein-mediated hepatobiliary transport, since TNF-α appears to be a determinant of the pathological changes in HUS induced by SLT-producing E. coli infection (17, 36, 37, 40).
MATERIALS AND METHODS

Chemicals. Doxorubicin hydrochloride, daunorubicin hydrochloride, and pentoxifylline (PTX) were purchased from Sigma Chemical Co., Ltd. (St. Louis, Mo.). Doxorubicin hydrochloride in the form of a commercial preparation for injection used in the in vivo experiment was purchased from Kyowa Hakko Kogyo (Tokyo, Japan). All other reagents are commercially available and were of analytical grade. All reagents used in this study were used without further purification.

Preparation of SLT-II. SLT-II was prepared from a clinically isolated E. coli O157:H7 strain, NGY12, according to previously reported methods (42, 43). The concentration of SLT-II in the crude preparation was determined to be 20 μg/ml by using a reverse passive latex agglutination kit (VTEC-RPLA; Denka Seiken Co., Tokyo, Japan).

Animals. Male Wistar rats (Nippon SLC, Hamamatsu, Japan), weighing 270 to 280 g, were used in this study. The rats were housed under controlled environmental conditions (temperature, 23 ± 1°C, and humidity, 55% ± 5%) with food (a commercial diet) and water freely available. All animal experiments were carried out in accordance with the guidelines of the Nagoya University School of Medicine for the care and use of laboratory animals.

Histopathological examinations. Rats under light anesthesia with sodium pentobarbital (25 mg/kg) were killed by exsanguination 24 h after intravenous injection of SLT-II (2 μg/animal) or saline. The liver was immediately removed. For light microscopy, small pieces of liver tissue were fixed in 10% formaldehyde in neutral phosphate-buffered saline (PBS). Fixed tissue specimens were dehydrated through a graded series of ethanol treatments and were usually embedded in paraffin wax. Sections of 4 to 6 μm each were treated with hematoxylin and eosin stain and periodic acid-Schiff reagent. For electron microscopy, small pieces of liver tissue were fixed in phosphate-buffered 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C. After being rinsed in the buffer, the specimens were postfixed in 1% osmium tetroxide and embedded in Epon 812 resin. Ultrathin sections were cut on a Reichert Ultracut-U ultramicrotome with a diamond knife, double stained with uranyl acetate and lead citrate, and examined in a Hitachi H-7000 electron microscope. Semithin sections were studied with toluidine blue and examined with a light microscope. Veterinary pathologists performed the histopathological examinations.

In vivo biliary clearance experiments. To determine the effect of SLT-II on the systemic pharmacokinetics of doxorubicin, 1 day before the experiments, rats were anesthetized with sodium pentobarbital (25 mg/kg) were killed by exsanguination 24 h after intravenous injection of SLT-II (2 μg/animal) or saline. The liver was immediately removed.

For light microscopy, small pieces of liver tissue were fixed in 10% formaldehyde in neutral phosphate-buffered saline (PBS). Fixed tissue specimens were dehydrated through a graded series of ethanol treatments and were usually embedded in paraffin wax. Sections of 4 to 6 μm each were treated with hematoxylin and eosin stain and periodic acid-Schiff reagent. For electron microscopy, small pieces of liver tissue were fixed in phosphate-buffered 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C. After being rinsed in the buffer, the specimens were postfixed in 1% osmium tetroxide and embedded in Epon 812 resin. Ultrathin sections were cut on a Reichert Ultracut-U ultramicrotome with a diamond knife, double stained with uranyl acetate and lead citrate, and examined in a Hitachi H-7000 electron microscope. Semithin sections were studied with toluidine blue and examined with a light microscope. Veterinary pathologists performed the histopathological examinations.

Biochemical and coagulation tests. Twenty-four hours after intravenous injection of SLT-II or saline in rats, blood samples were collected by exsanguinations from the abdominal aorta of rats under light anesthesia with pentobarbital (25 mg/kg). Serum was obtained by centrifugation of blood, whereas plasma was obtained by centrifugation with 3.13% sodium citrate as an anticoagulant. Concentrations of albumin and total protein in serum were determined by using the well-known bromcresol green and biuret reactions, respectively. Values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, and cholinesterase in the serum were determined with assay kits (Wako Pure Chemical Industries, Ltd., Japan). Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured with the instruments in Orienta Technika Co., Tokyo, Japan). Plasma protein binding experiments. To estimate the effect of SLT-II on the protein binding of doxorubicin, the protein binding experiment was done by ultrafiltration with an Ultrafree-MC ultrafiltration device (Amicon Corp., Bedford, Mass.). Each plasma sample (approximately 200 μl) obtained from biliary clearance experiments was poured into the device and was centrifuged at 2,000 × g for 40 min at 25°C. The ultrafiltrate was then assayed for measurement of the unbound fraction of doxorubicin. Adsorption of doxorubicin to the device was negligible. The total and unbound (ultrafiltrate) concentrations of doxorubicin were measured by high-performance liquid chromatography (HPLC).

Tissue binding experiments. Livers were obtained from control rats (saline treated) and rats treated 24 h earlier with SLT-II (2 μg/animal). After the livers were washed with a sufficient volume of ice-cold saline, liver homogenate samples with a concentration of 40% were prepared from the portions of each liver (approximately 2 g) with a tight homogenizer (20 strokes up and down) in PBS (pH 7.4) at 4°C. The homogenate samples were diluted serially with PBS (40, 20, 10, and 5%). A designated amount of doxorubicin (2 μg), which was determined based on in vivo clearance experiments, was added to each homogenate sample, and samples were incubated at 37°C for 5 min. After incubation, the samples (approximately 500 μl) were centrifuged at 6,000 × g for 10 min at 25°C. The supernatant was poured into an Ultrafree-MC device and was centrifuged again at 2,000 × g for 60 min at 25°C. The concentrations of the homogenate and ultrafiltrate were assayed for measurement of the unbound fraction of doxorubicin in liver tissue (fs). The fs at 100% homogeneity was extrapolated.

Drug analysis. Concentrations of doxorubicin in plasma, bile, and liver were determined by HPLC. Bile samples were properly diluted in distilled water, and liver was added to 5 volumes of PBS and homogenized. The liver homogenate was further diluted with 20 volumes of PBS. Briefly, 50 μl of each sample and 200 μl of a solution of methanol and 40% ZnSO4 (1.1, vol/vol) containing an internal standard of daunomycin (0.1 μg/ml) were mixed and centrifuged at 6,000 × g for 5 min. After centrifugation, the supernatant (120 μl) was subjected directly to HPLC. The apparatus used for HPLC was an LC-6A system (Shimazu, Kyoto, Japan) equipped with a fluorescence detector (RF-10AXL; Shimadzu) (excitation, 480 nm; emission, 560 nm) consisting of an LC-6A liquid chromatography system and an SIL-6A autoinjector. The conditions were as follows: column, a Cosmosil SC-18 (4.6 by 150 mm; Nacalai Tesque, Kyoto, Japan); mobile phase, 0.5% phosphoric acid-methanol (1:1, vol/vol); column temperature (OTC-6A; Shimadzu), 50°C; flow rate, 1.2 ml/min. This assay was shown to be linear for the concentrations studied, with a correlation coefficient of 0.999. No interference with the peak of doxorubicin was observed in any samples. The detection limit was approximately 0.02 μg/ml for plasma and bile samples and 0.05 μg/g of tissue for liver homogenate. The within-day and between-day coefficients of variation for this assay were less than 8%.

Data analysis. Plasma concentration-time data for doxorubicin after a single intravenous administration were analyzed by a noncompartmental method. The area under the curve (AUC) and the area under the first-moment curve (AUMC) were calculated by the trapezoidal rule with extrapolation to infinity. The systemic clearance (CLsys) was determined as dose/AUC. The mean residence time (MRT) was calculated as AUMC/AUC. The volume of distribution at steady state was calculated as CLsys × MRT. Each parameter was calculated with the nonlinear least-squares regression program WinNonlin (version 2.1; Pharsight Co., Mountain View, Calif.).

For biliary clearance experiments, the biliary clearance based on concentration in plasma (CLb/p) was calculated by dividing the biliary excretion rates by the steady-state concentration of doxorubicin in plasma (Css) determined for that collection period. The biliary clearance based on concentration in liver (CLb/l) was calculated by dividing the biliary excretion rate by the concentration in liver (Csl) determined for that collection period. The CLb/l was calculated as the clearance of doxorubicin in bile (AUCb) divided by the bile flow rate (fR). The AUCb was extrapolated to infinity. The CLb/p for unbound drug (CLb/p,U) was calculated as CLb/p × fs, where fs represents the unbound plasma fraction. The CLb/l for unbound drug
(CL_{\text{SYS}}) was calculated as \(\text{CL}_{\text{SYS}} = \frac{f_t}{K_t}\). Each parameter was calculated by using the mean value for three datum points during 60 min. \(K_t\) is the ratio of \(C_t\) to \(C_{\text{SS}}\).

**Western blot analysis.** Livers were obtained from control rats (saline) and rats that had been treated 24 h earlier with SLT-II. The livers were washed with a sufficient volume of ice-cold saline. Each liver was suspended in 10 volumes of 10 mM Tris-HCl buffer (pH 8.0) containing complete protease inhibitor, 1.5 \(\mu\)g of aprotinin/ml, and 1 mM phenylmethylsulfonyl fluoride (Sigma Chemicals). The suspension was homogenized with a tight homogenizer (20 strokes up and down) and centrifuged at 2,000 \(\times\) g for 15 min at 4 °C. The supernatant was centrifuged at 80,000 \(\times\) g for 60 min at 4 °C. The pellet was dissolved in Laemmli buffer and incubated at 37°C for 15 min.

The protein concentration in the solution was measured with a protein assay (Bio-Rad Laboratories, Richmond, Calif.) using bovine serum albumin (Sigma Chemical) as a standard. The protein (40 \(\mu\)g) was separated by electrophoresis on a sodium dodecyl sulfate–8% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked in PBS containing 0.1% Tween 20 and 5% nonfat dry milk and detected by C219 mouse monoclonal antibody to P glycoprotein (Dako A/S, Glostrup, Denmark) and human monoclonal antibody to Mrp2 (Alexis Biochemicals, San Diego, Calif.). To quantify the relative levels of P glycoprotein and Mrp2 in each gel, the intensity of the stained bands was measured with the NIH Image program (National Institutes of Health, Bethesda, Md.).

**Statistical analysis.** Data are expressed as the means ± standard deviations for the indicated number of rats. Statistical comparisons were assessed by one-way analysis of variance. When the \(F\) ratios were significant (\(P < 0.05\)), Scheffe’s post-hoc tests were done. Statistical comparisons of biochemical data were analyzed by rank nonparametric tests (Mann-Whitney U test). StatView software (version 4.54; Abacus Concepts Inc., Berkeley, Calif.) was used for the analysis. \(P\) values of < 0.05 were considered significant.

**RESULTS**

Diarrhea was the first definite sign of an *E. coli* O157:H7 infection and was experienced in most of SLT-II-treated rats; all rats died within 3 to 4 days after injection of SLT-II. SLT-II significantly decreased the body weight of liver weights of rats by approximately 10 and 20%, respectively. Data from the biochemical and coagulation tests are summarized in Table 1. The biochemical parameters showed that the dose of SLT-II used in this study significantly decreased concentrations of albumin and ALT in serum. However, there were no significant differences in values for total protein, AST, total cholesterol, cholinesterase, PT, APTT, and Normotest between SLT-II-treated and untreated rats. Light micrographs of semithin sections stained with toluidine blue are shown in Fig. 1. Histopathological examination revealed that hepatocytes of SLT-II-treated rats had no evidence of a massive necrotic and apoptotic area, while a number of fatty droplets were observed. In the quantitative evaluation of liver histopathological changes, a reduction in the size of hepatocytes and vacuolization were observed in SLT-II-treated rats: approximately 50% of hepatocytes of SLT-II-treated rats had small and multiple vacuoles, but only 1% of hepatocytes of untreated rats had one or two vacuoles. It is likely that SLT-II somewhat reduces the liver function.

![FIG. 1. Light micrographs of liver cells of control (a) and SLT-II-treated (b) rats. Magnification, \(\times\)100.](http://aac.asm.org/)
rats (1.34 ± 0.49 and 1.32 ± 0.21 liters/kg). We then performed in vivo biliary clearance experiments with the continuous infusion method. Pharmacokinetic parameters for the biliary excretion of doxorubicin in SLT-II-treated and untreated rats are summarized in Table 2. The C_{SS} in SLT-II-treated rats was significantly higher than that in untreated rats (0.21 versus 0.15 μg/ml). No significant difference in the concentration of doxorubicin in liver measured at the end of the clearance experiment was observed between SLT-II-treated and untreated rats (4.3 and 3.9 μg/g of liver). In addition, it significantly decreased the CL B/P (5.0 to 2.3 ml/min), a biliary excretion rate divided by the C_{L}, and the bile flow rate (29.9 to 18.2 μl/min). SLT-II significantly increased the f_{p} but did not alter the f_{u}, in spite of its strong binding to liver cells. On the other hand, a significant decrease in the liver-to-plasma concentration ratio (K_{u}) was observed in SLT-II-treated rats (26.6 and 19.9). To exclude the effect of plasma protein binding on the biliary excretion of doxorubicin, the CL B/P, U was calculated (Table 3). The value of CL B/P, U for doxorubicin in SLT-II-treated rats significantly decreased to 40% of the value obtained with untreated rats.

To investigate the role of TNF-α in the SLT-II-induced reduction of the biliary excretion of doxorubicin, the protective effect of PTX against SLT-II-induced decrease in the CL B/P of doxorubicin was examined. Pretreatment with PTX could not ameliorate SLT-II-induced decrease in the CL B/P of doxorubicin.

We investigated the effect of SLT-II on the expression levels of Mrp2 protein and P glycoprotein in liver tissue by Western blot analysis. Figure 3A shows changes in the levels of Mrp2 and P glycoprotein as a function of SLT-II. As shown in Fig. 3B, SLT-II significantly reduced the protein levels of P glycoprotein and Mrp2. We also investigated the protective effect of PTX against SLT-induced decreases in the levels of both transporters. PTX did not show a protective effect.

**DISCUSSION**

The present study aimed to prove our hypothesis that SLT-II could alter the hepatobiliary disposition of doxorubicin by inducing liver damage and/or impairing the function and/or activity of P glycoprotein and Mrp2, which are located on the bile canalicular surfaces of hepatocytes. Some in vitro studies using multidrug-resistant cells expressing P glycoprotein showed that P glycoprotein elevated the sensitivity to SLT and the biosynthesis of globotriaosylceramide (Gb₃), a functional receptor of SLT (13, 23), and P-glycoprotein inhibitors protected the increased Gb₃ and SLT sensitivity (22). These findings suggest the possibility that SLT-II can cause damage to the tissues expressing P glycoprotein. However, histopathological and biochemical examination revealed that SLT-II did not cause severe liver cell injury, although there were reduction in the size of hepatocytes, vacuolization, and decreases in animal body and liver weights. This discrepancy may be due to difference between in vivo and in vitro experiments.

When doxorubicin was administered intravenously to rats treated with SLT-II, a significant delay in the disappearance of doxorubicin from plasma was observed, suggesting that SLT-II modifies the pharmacokinetics of doxorubicin. We investigated whether SLT-II could alter the hepatobiliary disposition of doxorubicin, since doxorubicin is rapidly distributed into the liver by passive diffusion (9, 30, 32) and is primarily eliminated into the bile (31, 34, 39). The in vivo biliary clearance experiment revealed that SLT-II significantly decreases the hepatobiliary excretion of doxorubicin and induces a decrease in its biliary excretion rate, but with no change in the amount accumulated in the liver. Our results are supported by a report that GF120918, a P-glycoprotein inhibitor, significantly decreased the biliary excretion of doxorubicin without alteration in the liver concentration in perfusion model of isolated liver (6).

It is thought that SLT-II-induced changes in the protein binding of doxorubicin might influence its uptake into the hepatocytes from blood and biliary excretion of doxorubicin. The observed decreases in total K_{u} and the K_{u} for the unbound fraction of doxorubicin (K_{u, p1}) in SLT-II-treated rats may be due, in part, to decrease in the biliary excretion of doxorubicin and/or its efflux into blood from hepatocytes. On the other hand, SLT-II did not alter the f_{p} of doxorubicin in spite of high

TABLE 2. Steady-state pharmacokinetic parameters of total doxorubicin in control and SLT-II-treated rats

<table>
<thead>
<tr>
<th>Rat group</th>
<th>C_{ss} (μg/ml)</th>
<th>C_{L} (μg/g of liver)</th>
<th>CL_B/P (ml/min)</th>
<th>CL_B/P (g of liver/min)</th>
<th>K_{u} (ml/g of liver)</th>
<th>f_{p}</th>
<th>f_{u}</th>
</tr>
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<tr>
<td>Control</td>
<td>0.148 ± 0.013</td>
<td>3.94 ± 0.51</td>
<td>5.03 ± 0.65</td>
<td>0.190 ± 0.024</td>
<td>26.6 ± 3.40</td>
<td>0.277 ± 0.005</td>
<td>0.0035 ± 0.0010</td>
</tr>
<tr>
<td>SLT-II treated</td>
<td>0.214 ± 0.018b</td>
<td>4.26 ± 0.62</td>
<td>2.31 ± 0.52b</td>
<td>0.118 ± 0.033b</td>
<td>19.9 ± 2.26b</td>
<td>0.335 ± 0.024b</td>
<td>0.0034 ± 0.0001</td>
</tr>
</tbody>
</table>

a Values are means ± standard deviations (n = 7).

b Significantly different from the value for control rats (P < 0.05).
liver tissue binding potency (>99.5%). It has been reported that doxorubicin strongly binds to the nucleus in hepatocytes (26). We therefore presume that doxorubicin distribution in liver tissue might be dominated by the binding to the hepatocytes nucleus and that the observed insignificant difference in the liver concentration between SLT-II-treated and untreated rats is due to its sequestration in the liver. Some reports suggested that Mrp3 plays a significant role in the basolateral export of Mrp3 substrates under conditions in which Mrp2 is absent or down-regulated (1, 11, 18, 28, 29). As SLT-II could down-regulate Mrp2 expression in the canalicular membrane of hepatocytes in this study, therefore, it could be inferred that SLT-II may up-regulate Mrp3 or an Mrp3-like protein in the basolateral membrane in hepatocytes to enhance efflux into the blood, so as to protect liver from the damage caused by the accumulation of doxorubicin.

The ratio of the concentration in bile to the concentration of unbound doxorubicin in liver was very high in both SLT-II-treated and untreated rats (1,700), suggesting that doxorubicin is concentratively excreted into the bile by some drug transporters, including P glycoprotein. The CLB/L,U of doxorubicin in SLT-II-treated rats was also significantly lower than that in untreated rats (33.3 versus 53.8 g of liver/min). These results suggest that SLT-II decreases the rate of membrane penetration of doxorubicin through the bile canalicular membrane by decreasing the function and/or activity of hepatic P glycoprotein and/or Mrp2. We therefore studied whether SLT-II could reduce the expression of P glycoprotein and Mrp2 in relation to the biliary excretion of doxorubicin by Western blot analysis. As expected, SLT-II down-regulated both transporters in the liver. The observed decreases in the expression of both transporters could, at least in part, explain the SLT-II-induced decrease in the hepatobiliary transport of doxorubicin. Otherwise, because the efflux pumping function of both drug transporters is ATP dependent, altered ATP content in the liver could significantly influence the biliary excretion of doxorubicin. Considering that SLT-II does not alter ATP content in the liver and brain (unpublished data), the observed decrease in the hepatobiliary transport of doxorubicin in SLT-II-treated rats might be due to reduction in the activity of hepatic P glycoprotein and/or Mrp2. Moreover, the observed decrease in the bile flow in SLT-II-treated rats may be due, in part, to decrease in the expression of the canalicular bile salt export pump (BSEP), which is called a sister of P glycoprotein (15), in the liver, since bile salts are known to be transported by BSEP and BSEP can be stained with a monoclonal antibody, C219, used in this study (8).

It has been reported that doxorubicin is metabolized by aldo-keto reductase, cytochrome P450 (CYP2B1), and glucuronide-conjugating enzymes (3, 4, 16, 39, 41) and that its major metabolites, 7-deoxydoxorubicinolone and doxorubicinol, are substrates of P glycoprotein (6, 39). We have found that SLT-II decreases hepatic P450-dependent drug-metabolizing enzyme activity in rats (K. Kitaichi, C. S. Hui, Y. Nishio, M. Nadai, K. Takagi, E. Shibata, K. Takagi, M. Ito, M. Ohta, H. Yoshizumi, and T. Hasegawa, presented at the Millennial World Congress.

<table>
<thead>
<tr>
<th>Rat group</th>
<th>C_{SS,U} (µg/ml)</th>
<th>C_{L,U} (µg/g of liver)</th>
<th>CL_{B/P,U} (ml/min)</th>
<th>CL_{B/L,U} (g of liver/min)</th>
<th>( K_{p,U} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.041 ± 0.003</td>
<td>0.014 ± 0.002</td>
<td>18.15 ± 2.34</td>
<td>53.80 ± 6.69</td>
<td>0.340 ± 0.044</td>
</tr>
<tr>
<td>SLT-II</td>
<td>0.072 ± 0.006a</td>
<td>0.015 ± 0.002</td>
<td>6.88 ± 1.55b</td>
<td>33.32 ± 9.41b</td>
<td>0.203 ± 0.023b</td>
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* Values are means ± standard deviations (n = 6).
* Significantly different from the value for control rats (P < 0.05).

**TABLE 3. Steady-state pharmacokinetic parameters of unbound doxorubicin in control and SLT-II-treated rats**

![FIG. 3. (A) P-glycoprotein and Mrp2 expression in the livers of rats treated with SLT-II and untreated rats (control); (B) relative staining intensities for P glycoprotein and Mrp2 in control and SLT-II-treated rats. Liver samples were taken 24 h after intravenous injection of SLT-II (2 µg/animal). Each bar shows the intensity ratio compared to the value for the control rats; values are means ± standard deviations (n = 3). “a” indicates values that are significantly different from the control value (P < 0.05).**
of Pharmaceutical Sciences, San Francisco, Calif., April 2000). Most recently, we found that SLT-II reduces the total hepatic cytochrome P450 content and down-regulates the expression of the P450 isozymes CYP3A2 and CYP2C11 in rats (unpublished data). These findings suggest the possibility that SLT-II decreases the metabolism of doxorubicin, leading to increases in the amount of unchanged doxorubicin in the liver. Therefore, SLT-II-induced decrease in the hepatic metabolism of doxorubicin could influence significantly the disposition of this drug. Considering that doxorubicin is pooled deeply in the nucleus in liver cells and that SLT-II did not alter the amount of doxorubicin in the liver, the influence of the metabolic activity on the hepatobiliary excretion of doxorubicin might be very small (26).

It is generally accepted that SLTs causes HUS in humans and animals through damage to the vascular endothelial vessels by cytokines (5, 14), due to up-regulation of the expression of Gb3 receptor in endothelial cells (20), suggesting that cytokines play a central role in SLT-II-induced histopathological lesions. On the other hand, cytokines such as TNF-α or interleukin-1β (IL-1β) and IL-6 play an important role in the regulation of hepatic transporters (2, 33). It was recently reported that SLT-II overproduces TNF-α in plasma and that PTX partly ameliorates SLT-II-induced reductions in kidney and brain functions (42, 43). Therefore, we focused on TNF-α and presumed that the inhibition of the production of TNF-α and/or the activity of macrophages could protect SLT-II-induced impairment of drug transporter function. Unexpectedly, PTX could not protect SLT-II-induced decreases in the hepatobiliary excretion of doxorubicin and in the protein levels of both P-glycoprotein and Mrp2. The present results are not consistent with the report that IL-1 and TNF-α decreased Mrp2 expression and that decreased expression of Mrp2 was restored by anti-IL-1 or anti-TNF-α antibody (25). The reason for the discrepancy is not clear at present, but it may be related to the different diseases and/or differences between the rat strains. It is unlikely that TNF-α plays a major role in the SLT-II-induced decrease in the hepatobiliary excretion of doxorubicin and the regulation of the hepatic drug-transporting P-glycoprotein and Mrp2. Other cytokines must be taken into consideration.

In conclusion, the present study is the first to report that pretreatment of rats with SLT-II results in decreases in the hepatobiliary excretion of doxorubicin mainly due to decreases in P-glycoprotein and Mrp2 expression and bile flow rate. It is unlikely that TNF-α is involved in these decreases. The results reported here, at least, should provide further evidence regarding the hepatobiliary disposition of P-glycoprotein substrates in the animal model of E. coli O157:H7 infection.

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