EFFECTS OF UREMIC TOXINS ON HEPATIC UPTAKE AND METABOLISM OF ERYTHROMYCIN

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Abbreviations:

Oatp, organic anion transporting polypeptide; ESRD, end stage renal disease; CMPF, 3-carboxy-4-methyl-5-propyl-2-furan-propanoic acid; GSA, guanidinosuccinic acid; IG, indoxyl-β-D-glucuronide.
Abstract:

Hepatic clearance of erythromycin (Ery) is significantly reduced in patients with end stage renal disease (ESRD). Since Ery is primarily eliminated via excretion of unchanged drug in the bile, we suspect that this change could be due to the effect of uremic toxins on hepatic uptake and/or efflux transporters. Using rat hepatocytes and microsomes as model proof of concept systems, we examined six uremic toxins, 3-carboxy-4-methyl-5-propyl-2-furan-propanoic acid (CMPF), indoxyl sulfate (IS), hippuric acid (HA), indole acetic acid (IA), guanidinosuccinic acid (GSA) and indoxyl-β-D-glucuronide (IG), for their effects on Ery uptake and metabolism. Ery and the metabolite N-demethyl-Ery were measured by LC/MS/MS. The uptake of Ery by rat hepatocytes was markedly inhibited by rifampin and digoxin, but not by quinidine, suggesting that Oatp2 plays a major role in the uptake of Ery. At 50 µM, CMPF significantly (p<0.05) reduced hepatocyte accumulation of Ery and N-demethyl-Ery. At higher concentrations (≥200 µM), CMPF appears to also inhibit the enzymatic metabolism of Ery. In contrast, IS did not significantly inhibit the hepatocyte uptake of Ery even at the highest concentration (800 µM) tested, but reduced metabolite generation (p<0.001). The other uremic toxins, HA, IA, IG and GSA, did not affect either hepatic uptake or microsomal metabolism of Ery. CMPF, IS and HA were shown to not inhibit differential P-glycoprotein (P-gp) transport of Ery in cellular systems. Our results suggest that CMPF can directly inhibit the uptake of Ery by inhibiting Oatp2, while IS is more likely to inhibit the enzymatic metabolism of Ery.
Introduction:

More and more evidence suggests that renal failure not only affects the renal disposition of drugs that are cleared by the kidney, but also the non-renal disposition of drugs that are metabolized by the liver (Touchette and Slaughter, 1991; Yuan and Venitz, 2000; Dreisbach and Lertora, 2003). Erythromycin (Ery) exhibits significantly reduced non-renal clearance in patients with end stage renal disease (ESRD) (Welling and Craig, 1978; Kanfer et al., 1987). There have been a number of reports of ototoxicity caused by Ery in ESRD patients and blood levels of Ery were found to be extremely high in such cases (Kroboth et al., 1983). Furthermore, the exposure to Ery was reported to be about 4-5 fold higher in ESRD patients than in healthy controls following a single oral dosing (Kanfer et al., 1987). The markedly increased exposure to Ery in the ESRD patients was most likely due to reduced hepatic clearance since Ery is mainly eliminated by the hepatobiliary system with only a small fraction eliminated unchanged in urine. But the underlying mechanisms of this reduced hepatic clearance are not well understood.

In the liver, Ery is partially metabolized by CYP3A4 to N-demethyl-Ery, but is primarily excreted unchanged in the bile as stated by Touchette and Slaughter (1991) although a primary reference was not given. Thus, both uptake and efflux transporters may play an important role in the disposition of Ery. Although studies have demonstrated that CYP3A activity was reduced in chronic renal failure (CRF) rats (Leblond et al., 2000; Guevin et al., 2002), we suspect that metabolism changes can not be the major determinant for a drug where metabolism is not the primary route of elimination. We hypothesize and tested here whether other mechanisms, such as alteration of transporter activities, might contribute to the reduced hepatic clearance of Ery in renal failure.
Hepatic uptake is the first step and can be rate-limiting in the hepatic elimination of xenobiotics (Yamazaki et al., 1996). Transporters located at the basolateral (sinusoidal) membrane of hepatocytes have been found to be involved in the uptake of many drugs (Faber et al., 2003; Hagenbuch and Meier, 2003). Organic anion transport polypeptides (OATP/Oatp; human/rat) are a class of major drug carrier proteins supporting the sodium-independent hepatic uptake of a large number of organic anions, bulky organic cations and neutral compounds (Eckhardt et al., 1999; Konig et al., 2000). Substrate specificity of transporters is broad and considerable overlap has been observed, although unique features of individual transporters have been demonstrated. Many compounds are both substrates and inhibitors of a transporter. Ery is a bulky organic cation with molecular weight of 734. It is an inhibitor of OATP (Cvetkovic et al., 1999). Most likely, its hepatic uptake is also mediated by OATP/Oatps in the liver. Uremic toxins that accumulate in patients with ESRD cause a number of problems. Organic anions such as 3-carboxy-4-methyl-5-propyl-2-furan propanoic acid (CMPF), indoxyl sulfate (IS), and hippuric acid (HA), which are derived from dietary protein and normally excreted into urine, accumulate in the body due to the kidney malfunction. They are significantly elevated in the serum of uremic patients with concentrations approaching 800 µM (IS), 400 µM (CMPF), and 2 mM (HA) (Niwa and Ise, 1994). These substances are well known for their inhibitory effects on serum binding of drugs to albumin (Takamura et al., 1997; Sarnatskaya et al., 2002). They were also found to inhibit the active tubular secretion of organic acids by inhibiting renal organic anion transporters (OATs) (Tsutsumi et al., 2002). As a consequence, the accumulation of these organic acids further stimulates the progression of chronic renal failure (Satoh et al., 2003). These
uremic toxins might also be involved in altering thyroid function in uremic patients. CMPF and indoxyl sulfate, at concentrations normally present in the serum of uremic patients, were found to inhibit the uptake of thyroxin by rat hepatocytes (Lim et al., 1993).

Based on the above evidence, we hypothesize that circulating uremic toxins can inhibit liver uptake transporters, thereby causing reduced hepatic clearance of drugs in renal failure patients. Note that changes in protein binding can not explain the reduction in clearance, since increasing free concentrations would lead to increased clearance. Here, we characterized the effects of uremic toxins on the hepatic uptake and metabolism of the probe drug, Ery, using rat hepatocytes and rat microsomes as a proof of concept system.
Materials and Methods:

Materials: Erythromycin, rifampin, digoxin, quinidine, indoxyl sulfate, hippuric acid, indole acetic acid, guanidinosuccinic acid and indoxyl-β-D-glucuronide, oleandomycin, collagenase and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). CMPF was a generous gift from Professors Masaki Otagiri (Kumamoto University, Japan) and Edward Lindup (University of Liverpool, UK). Demethyl-erythromycin was purchased from U.S. Pharmacopeia (Evansville, IN). [14C]-Erythromycin (48.4 mCi/mmol) was obtained from PerkinElmer (Boston, MA). Liver perfusion buffer and hepatocyte washing buffer were purchased from Invitrogen (Carlsbad, CA). Male Wistar rats (250-300 g, Bantin and Kingman, San Leandro, CA) were housed in the UCSF animal care facility with a 12 hour light/dark cycle and allowed free access to water and food. Approval of the studies reported here was obtained from the Committee on Animal Research, UCSF. The MDR1-MDCK cell line was generously provided by Dr. Ira Pastan of the National Cancer. GG918 (GF120918) was a gift from Glaxo-Wellcome. All cell culture media were obtained from the UCSF Cell Culture Facility (San Francisco, CA). Transwell inserts and six-well plates were from Costar Corp. (Cambridge, MA).

Hepatocyte isolation: Hepatocytes were isolated as described previously by our lab (Lu and Benet, 2004). In brief, male Wistar rats were anesthetized with ketamine/xylazine (12 mg/ml) prior to surgery. The portal vein was cannulated and perfused with oxygenated liver perfusion buffer for 5 minutes followed by perfusion with oxygenated hepatocyte washing buffer supplemented with 2 mM L-glutamine, 10 mM Hepes, and 1.2 U/ml collagenase for 5 minutes at a flow rate of 20 ml/min. The digested liver was excised and
hepatocytes were isolated. The hepatocytes were washed three times with ice cold hepatocyte washing buffer and centrifuged at 50 × g for 2 minutes. Cell viability was determined by trypan blue exclusion. Cells exhibiting a greater than 90% viability were used for the experiments.

**Hepatocyte uptake studies.** Prior to starting the uptake studies, 2 million (in 1 ml solution) hepatocytes were prewarmed for 5 minutes in Kreb-Henseleit buffer (pH 7.4) containing 0.21 g/L of sodium bicarbonate and supplemented with 10 mM glucose. The uptake studies with erythromycin were initiated by adding [14C]-Ery (0.1 μCi, 2 μM) with various inhibitors to the cell suspension. At 5 minutes, the reaction was terminated by transferring 1 million hepatocytes into a centrifuge tube containing 150 μl of 2 N NaOH under a layer of a 500 μl mixture of silicone oil and mineral oil (d 1.015) (Shitara et al., 2003). After immediately centrifuging at 16,000 g for 10 seconds, the samples were left at 65 °C overnight to ensure complete cell lysis. Following removal of the oil layer, 150 μl of 2N HCl and scintillation cocktail were added and radioactivity was measured using a scintillation counter (LS6000TA, Beckman Coulter, Fullerton, CA). For the uptake studies with unlabeled erythromycin, the separation method was modified to allow the samples to be measured by LC/MS/MS. After the reaction, cells were transferred to a centrifuge tube containing only 500 μl mixed oil without 2N NaOH and spun down. After removal of the oil layer, cell pellets were dissolved in 100 μl H2O and precipitated by 200 μl acetonitrile containing the internal standard, oleandomycin. The supernatant was then transferred to a vial for LC/MS/MS measurement.
For the kinetic study of Ery uptake, [14C]-Ery (2 μM) with various concentrations of unlabeled Ery were incubated with rat hepatocytes at 37 °C for 5 minutes. Based on preliminary studies, 5 minutes incubation is within the linear range of Ery uptake by rat hepatocytes. For the inhibition studies, the uptake of unlabeled Ery (2 μM) by rat hepatocytes was measured in the presence or absence of various uremic toxins. The inhibitory effects of CMPF and indoxyl sulfate were further characterized by varying the concentration of these two uremic toxins. Unlabeled Ery was used to allow for measurement of both the parent and metabolite of Ery. Comparing the intracellular ratio of N-demethyl-Ery to Ery allowed us to evaluate which process is affected: uptake, metabolism and/or efflux, as we demonstrated recently (Lau et al., 2003) for liver perfusion studies.

The effects of uremic toxins on the viability of hepatocytes were also examined. Hepatocytes were incubated with uremic toxins at concentrations used for the inhibition study at 37°C for 5 minutes, then the cell viability was assessed by trypan blue exclusion method. Uremic toxins showed no significant effect on cell viability. Under all uremic toxin treated conditions, cells exhibited >85% viability.

Microsomal incubations: Rat liver microsomes were isolated and incubated as described previously by our lab (Salphati and Benet, 1999). In brief, isolated rat microsomes (0.75 mg/ml) were incubated with erythromycin in the presence or absence of various concentrations of the uremic toxins in phosphate buffer. NADPH (1 mM) was added to start the reaction. After incubation at 37°C for 10 mins, the reaction was stopped by protein precipitation by addition of an equal volume of acetonitrile containing the internal
standard, oleandomycin. The supernatants were stored at -80°C for LC/MS/MS measurement.

**Transport Experiments:** The transport experiments were adapted with modifications from Zhang and Benet (1998). Briefly, the cells were washed once and pre-incubated for 20 minutes at 37°C in 5% CO2 in Hank’s Balanced Salt Solution with 22.5 mM HEPES (HBSS-H). For measuring drug secretion (B→A), 2.5 ml of HBSS-H solution containing the drug was put into the basolateral (B) side and 1.5 ml of HBSS-H was put into the apical (A) side. At selected times, 200 µl samples were taken from the A side and replaced with fresh HBSS-H. For measuring drug absorption (A→B), the drug solution was put into the A side and samples were taken from the B side. For inhibition studies, the inhibitor was put in both the A and B sides. During the studies, the cells were incubated in a shaking incubator (Boekel Scientific, Feasterville, PA).

**LC/MS/MS measurement of erythromycin:** A micromass Quattro Ultima instrument (Waters Corp., Milford, MA) with electrospray/positive ionization was used. The Multiple Reaction Monitor (MRM) was set at 734.3 – 576.3 m/z for Ery, 720.3 – 562.2 m/z for N-demethly-Ery, and 688.5 – 544.3 m/z for the internal standard, oleandomycin. The cone voltage and collision energy were set at 40V and 20 eV, respectively. The analytical column was a Keystone BDS C18 (4.6 x 50 mm, 5µ particle size) column (Keystone Scientific, Inc., Bellefonte, PA). The mobile phase consisted of 30% acetonitrile containing 0.1 % formic acid. Five microliter aliquots were injected and the flow rate set at 0.8 ml/min with ¼ split into the mass system. Prior analyses demonstrated that linearity (n=2
at each level) was observed from 0.078 to 10 µM with a $r^2$ of 0.9977. The detection limit was estimated to be 2 nM. Inter- and intra-day coefficients of variation were estimated as 7.6% and 5%, respectively.

**Data Analysis:** The kinetic parameters for the uptake of $^{[14C]}$-Ery were calculated using the following equation:

$$V_0 = \frac{V_{\text{max}} \times S}{K_m + S} + P_{\text{diff}} \times S$$

where $V_0$ is the initial uptake rate (pmol/min/million cells), $S$ is the substrate concentration (µM), $K_m$ is the Michaelis-Menten constant (µM), $V_{\text{max}}$ is the maximum uptake rate and $P_{\text{diff}}$ is the nonsaturable diffusion process (µl/min/million cells). To obtain the kinetic parameters, the data was fitted to the above equation using a nonlinear least-squares method by SigmaPlot2001 (Version 7.1, SPSS, Chicago, IL). The input data were weighted as the reciprocal of the observed values. Student t-test was used to analyze differences between two groups. Analysis of variance was used to analyze differences among more than two groups, and the significance of difference between two means in these groups was evaluated using Bonferroni multiple comparison test. The P value for statistical significance was set at <0.05.
Results:

Uptake of erythromycin by rat hepatocytes: [\textsuperscript{14}C]-Ery (2 µM) with various concentrations of unlabeled Ery were incubated with 2 million rat hepatocytes for 5 minutes to determine the kinetic parameters. The Eadie-Hofstee plot of the data is shown in Fig. 1. Both a saturable component and a nonsaturable component were observed. The fitted kinetic parameters were 72.0 ± 20.4 µM for $K_m$, 276.0 ± 51.8 pmol/min/million cells for $V_{\text{max}}$, and 3.3 ± 0.1 µl/min/million cells for $P_{\text{diff}}$. The saturable component estimated by $V_{\text{max}} / K_m$ accounts for about 55% of the total uptake.

To determine whether uptake of Ery was mediated by Oatp, Ery was incubated with rat hepatocytes in the presence of various Oatp inhibitors. Quinidine and digoxin are relatively specific inhibitors of Oatp1 and Oatp2, respectively, while rifampin is an inhibitor of both Oatp1 and Oatp2 (Shitara et al., 2002). At concentrations of 100 µM, both rifampin and digoxin significantly inhibited the uptake of Ery (Fig. 2), whereas quinidine did not have any effect. This is even more obvious when only the active transport of Ery is compared as depicted in Figure 2B. The inhibition profiles suggest that Ery uptake is mediated by Oatp2 in rat hepatocytes.

Inhibitory effects of uremic toxins on hepatocyte uptake of erythromycin: To determine the inhibitory effects of uremic toxins on the uptake of Ery, rat hepatocytes were incubated with unlabeled Ery (2 µM) in the presence of CMPF 400 µM, IS 800 µM, HA 2 mM, indole acetic acid (IA) 80 µM, guanidinosuccinic acid (GSA) 320 µM and indoxyl-β-D-glucuronide (IG) 500 µM at 37°C for 5 mins. The chosen concentration of each uremic toxin represents the highest measured value reported in uremic patients (Vanholder et al.,
2003). Among the six toxins tested, CMPF was the only one exhibiting significant inhibition on the uptake of Ery (Fig. 3A) as determined by measuring intracellular Ery concentrations. In the CMPF treated hepatocytes, the intracellular concentration of Ery was 45.5 ± 4.1% (p<0.001) of the control. Since 55% of Ery hepatic uptake is estimated to be mediated by transporters (Fig.1) as discussed above, CMPF at the concentration (400 µM) tested completely blocked the Oatp2-mediated uptake. The intracellular metabolite of Ery showed a comparable reduction (34.1 ± 5.3%, p<0.001) (Fig. 3B). IS, IA, HA, IG and GSA had no effect on the hepatic uptake of Ery (Fig. 3A). On the other hand, although IS did not inhibit the uptake of Ery, it significantly reduced the intracellular concentration of N-demethyl-Ery (Fig. 3B). In the presence of 800 µM IS, intracellular N-demethyl-Ery was decreased by 36% (63.9 ± 4.1%) suggesting an inhibitory effect on Ery metabolism.

To further characterize the inhibitory effects of CMPF and IS on the uptake and metabolism of Ery, 2 µM Ery was incubated with rat hepatocytes in the presence of various concentrations of CMPF and IS. CMPF at concentrations of 25 µM (and higher) significantly reduced the intracellular accumulation of Ery (25.3 ± 1.8 vs 33.1 ± 0.8 pmol/million cells per 5 mins, p<0.05) (Fig. 4A) and at 50 µM and greater, decreased the intracellular N-demethyl-Ery (8.9 ± 0.6 vs 10.8 ± 0.5 pmol/million cells per 5 mins, p<0.05) (Fig. 4B). The inhibition was dose-dependent. The estimated IC₅₀ for CMPF on 2 µM Ery was 40.0 ± 1.2 µM. Ratios of intracellular N-demethyl-Ery to Ery were not significantly different from the control at CMPF concentrations of 200 µM and lower (Table 1), indicating that the reduced metabolite generation was due to limited uptake, not due to inhibited enzymatic metabolism. However, at the highest concentration of CMPF (400 µM), the ratio was significantly lower than that of control suggesting the inhibited
enzymatic metabolism was the primary effect since the transporter-mediated uptake was already completely blocked at even lower concentrations of CMPF (Table 1).

In contrast, IS did not significantly affect the uptake of Ery (Fig. 5A), but it significantly reduced the intracellular levels of N-demethyl-Ery at higher concentrations (≥200 µM) (Fig. 5B). The intracellular metabolite to parent ratios of Ery were also significantly reduced by IS at concentrations of 50 µM and higher (Table 1), which suggested an inhibitory effect of IS on the enzymatic metabolism of Ery.

**Effects of indoxyl sulfate and CMPF on microsomal metabolism of Ery:** To further examine whether CMPF and IS can directly inhibit the enzymatic metabolism of Ery, microsomal incubations of Ery (2 µM) in the presence of various concentrations of CMPF and IS were conducted. Both CMPF and IS could inhibit the metabolite formation of Ery at higher concentrations (≥ 200 µM for CMPF and ≥ 100 µM for IS) (Table 2). IS tended to have a stronger inhibitory effect on the enzymatic metabolism of Ery than CMPF. At concentrations of 100 µM and greater (Table 2), IS significantly inhibited the metabolite formation by 31%.

**Effects of uremic toxins on Ery fluxes in MDR1-MDCK cells:** Ery transport in the B→A direction was larger than the A→B direction in MDR1-MDCK cells (B→A/A→B ratio = 64). The differential transport was significantly decreased in the presence of the P-gp inhibitor, GG918 (B→A/A→B ratio =29). These results confirmed that Ery is a P-gp substrate. However, the uremic toxins, CMPF (500 µM and 1 mM), IS (1 mM) and HA (2 mM) had no significant effects on either the B→A or the A→B flux of Ery.
Discussion:

In the present study, we investigated the effects of six uremic toxins, CMPF, indoxyl sulfate, hippuric acid, indole acetic acid, indoxyl-β-D-glucuronide and guanidinosuccinic acid, on the uptake of Ery using rat hepatocytes, and their effects on Ery metabolism using rat microsomes. Our results demonstrate that CMPF significantly inhibits the uptake of Ery by hepatocytes, mainly by inhibiting the liver uptake transporter Oatp, while at higher concentrations (≥200 µM), it appears to also inhibit the enzymatic metabolism of Ery. In contrast, indoxyl sulfate is more likely to inhibit the enzymatic metabolism of Ery and has no effect on hepatic uptake. In cellular bi-directional transport studies with Ery, we showed that neither CMPF nor indoxyl sulfate, the two uremic toxins affecting hepatocyte kinetics of Ery, could affect P-glycoprotein transport of Ery, a known P-gp substrate.

It has been previously reported that renal failure could alter hepatic clearance of drugs in many cases, although the underlying mechanisms remain unclear. Studies with the major metabolic enzyme system, CYP, showed that specific isoforms were down-regulated in CRF rats such as CYP2C11, CYP3A1 and CYP3A2, but other isoforms such as CYP1A2 and CYP2D were unaffected (Leblond et al., 2000; Guevin et al., 2002). A detailed review of metabolic alterations in renal failure was recently published (Pichette and Leblond, 2003). Although the mechanisms responsible for the reduced metabolism are not known, studies suggest that uremic toxins may affect CYP promoters (Pichette and Leblond, 2003) and thereby cause down-regulation of certain CYP isoforms. Here, we show that uremic toxins can also directly affect CYP activity. Indoxyl sulfate and CMPF (at high concentrations) directly inhibit the N-demethylation of Ery mediated by
CYP3A. This is consistent with previous findings of uremia’s effects on enzymatic metabolism (Yoshitani et al., 2002). For example, indoxyl sulfate was found to be able to inhibit losartan metabolism mediated by rat hepatic microsomes (Yoshitani et al., 2002). CMPF was shown to inhibit both phase I (O-demethylation) and phase II (glutathione conjugation and glucuronidation) pathways of drug metabolism in rabbit liver homogenates (Walters et al., 1995).

However, down-regulation or inhibition of the CYP system was not present or correlated with reduced hepatic clearance in many cases (Fillastre et al., 1980; Hori et al., 1985). We propose an alternative mechanism for the reduced hepatic clearance of drugs due to renal failure, that is, impaired hepatic uptake mediated by uptake transporters. With increasing awareness of the effects of hepatic transporters on drug disposition, our laboratory has investigated their importance in drug metabolism. Recent studies from our laboratory showed that decreased digoxin metabolism was found in the isolated perfused rat liver and rat hepatocytes during Oatp2 inhibition (Lau et al., 2004; Lu and Benet, 2004). The effect of uremic toxins on hepatic transporters had not previously been investigated, and although our studies were on rat hepatocytes, we believe that this work serves as a proof of concept example of the potential consequences of uremic toxins on hepatic drug disposition. In the present study, we showed that CMPF potently inhibited the uptake of Ery by rat hepatocytes, and consequently reduced the metabolism of Ery. The effect of CMPF on Ery uptake appeared to be due to inhibition of Oatp2. Since transporters play an important role in the elimination of Ery, we expect that limited access of Ery to hepatocytes in the presence of CMPF would lead to less metabolism and less elimination into bile as well. We therefore suspect that inhibition of the hepatic
uptake of Ery by circulating CMPF could at least in part account for the reduced hepatic clearance of Ery in ESRD patients. A recent study using the Ery breath test (EBT) to measure metabolic activity in patients with renal failure found that the baseline metabolic activity was significantly lower than that of healthy volunteers (Dowling et al., 2003). We hypothesize that the reduced CO₂ generation measured by the EBT reflects decreased hepatic uptake of Ery.

The effects of renal failure on hepatic uptake of organic anions were documented in early studies (Yates et al., 1983; Bowmer and Yates, 1984). Studies showed that the hepatic uptake and initial biliary excretion of bromosulfophthalein were decreased in rats with glycerol-induced acute renal failure (Bowmer and Yates, 1984). A similar phenomenon was observed for another organic anion, indocyanine green (Yates et al., 1983). In addition, the kinetic changes of these organic anions were a consequence of renal failure, rather than a direct hepato-toxic effect of glycerol. Similar to erythromycin, the uptakes of BSP and indocyanine green are mediated by the hepatic transporter Oatp (Jacquemin et al., 1994), and we believe that the reduced hepatic clearance of these organic anions is most likely the result of impaired uptake activity. Our results also may suggest a mechanism for the uremic effects on the hepatic uptake of thyroxin (Lim et al., 1993). CMPF and indoxyl sulfate, at concentrations normally present in uremic patients, were found to inhibit the uptake of thyroxin by rat hepatocytes (Lim et al., 1993). Oatps are the major transporters responsible for the uptake of thyroxin in liver (Fujiwara et al., 2001), therefore, the reduced hepatic uptake of thyroxin, BSP and erythromycin are most likely due to inhibition of these uptake transporters by uremic toxins such as CMPF.
These findings may be helpful in understanding the reduced hepatic clearance of other drugs in patients with renal failure. Studies with several statins all showed various degrees of reduced clearance in renal impairment (product information Mevinacor, Cranoc and Zocor and data in Querin et al. (1991) that can not be properly interpreted on the basis of decreased creatinine clearance alone. For instance, investigation of the pharmacokinetics of cerivastatin in renal impairment (Vormfelde et al., 1999) showed a significant increase of the unbound fraction ($f_u$) of the drug, but a doubling of its total area under the curve (AUC). The increased $f_u$ should lead to increased clearance of cerivastatin, but the opposite was found. The reduced clearance of cerivastatin was speculated to be due to impaired hepatic uptake since 70-80% of cerivatatin uptake was mediated by OATP in the liver (Shitara et al., 2003). The OATP-mediated uptake has also been demonstrated for other statins (Yamazaki et al., 1996; Hsiang et al., 1999), and animal experiments also showed that uptake into liver cells is the rate-limiting step in the hepatic clearance of pravastatin (Yamazaki et al., 1996). It was therefore proposed that progression of renal disease will reduce the expression and function of the uptake transporters in the liver and that this transporter has a low overall capacity. On the basis of the present study, we suggest that CMPF can significantly inhibit hepatic uptake transporters. Since CMPF has also been found to inhibit drug binding to albumin (Takamura et al., 1997), thereby increasing $f_u$, inhibition of hepatic uptake by CMPF may be even greater than the observed reduction of fractional hepatic clearance of the statins.

In chronic renal failure, circulating uremic toxins may occupy the liver uptake transporters and accumulate in the liver to a large extent, thereby suppressing the uptake
transporters directly and indirectly (down-regulation). Further research is required to clarify the in vivo effects of these uremic toxins on hepatic drug metabolism and elimination.

In conclusion, our present studies show that CMPF can directly inhibit the Oatp mediated uptake of erythromycin by rat hepatocytes, while indoxyl sulfate was more likely to inhibit the enzymatic metabolism of erythromycin. Therefore, both of these uremic toxins might be expected to contribute to the reduced hepatic clearance of erythromycin in patients with ESRD.
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References


Footnotes

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Figure legends

**Figure 1:** Eadie-Hofstee plot of the uptake of $[^{14}C]$-Ery in rat hepatocytes. $[^{14}C]$-Ery (2 µM) with various amount of unlabeled Ery were incubated with rat hepatocytes at 37°C for 5 minutes. Each symbol represents the mean ± SEM (n=3). The solid line represents the best fit, and the R-squared value for the regression is 0.99.

**Figure 2:** Effects of various Oatp inhibitors on the A) total and B) active uptake of $[^{14}C]$-Ery by rat hepatocytes. $[^{14}C]$-Ery (2 µM) was incubated with 100 µM rifampin (Rif), quinidine (Qin) and digoxin (Dig). The total uptake of Ery in the absence of inhibitors was 29.4 ± 5.9 pmol/5 mins/million cells, of which active uptake was estimated to be 55%. Uptake of Ery in the presence of inhibitors was expressed as the percent of the respective controls (in the absence of inhibitors). Each column and bar represents the mean ± SEM (n=3). * p<0.05.

**Figure 3:** Inhibition of Ery uptake of by uremic toxins. The uptake of Ery (2 µM) by rat hepatocytes was measured in the presence of CMPF (400 µM), IS (800 µM), HA (2 mM), GSA (320 µM), IA (80 µM) and IG (500 µM). The amount of intracellular Ery (A) and N-demethyl-Ery (B) in the presence of uremic toxins was expressed as the percent of control (in the absence of uremic toxins). Each column and bar represents the mean ± SEM (n=3). *** p<0.001.

**Figure 4:** Dose-dependend inhibition of CMPF on Ery uptake by rat hepatocytes. The amount of intracellular Ery (A) and N-demethyl-Ery (B) in the presence of CMPF was
expressed as the percent of control (in the absence of CMPF). Each column and bar represents the mean ± SEM (n=3). * P<0.05, ** P<0.01, *** P<0.001.

**Figure 5:** Effects of various concentrations of indoxyl sulfate on intracellular accumulation of erythromycin and N-demethyl-Ery. The amount of intracellular Ery (A) and N-demethyl-Ery (B) in the presence of IS was expressed as the percent of control (in the absence of IS). Each column and bar represents the mean ± SEM (n=3). * P<0.05, ** P<0.01, *** P<0.001.
Hepatocytes were incubated with Ery (2 µM) in the presence of various concentrations of CMPF and indoxyl sulfate. The intracellular amount of Ery and N-demethyl-Ery was measured in each group.

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</tbody>
</table>

a each value represents the mean ± SEM.

b p< 0.05, significantly different from controls.

c not determined.
TABLE 2

*Effects of CMPF and indoxyl sulfate on the microsomal metabolism of Ery.*

<table>
<thead>
<tr>
<th>Concentrations (µM)</th>
<th>N-demethyl-Ery (% of control)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMPF</td>
</tr>
<tr>
<td>0</td>
<td>100.0 ± 2.4</td>
</tr>
<tr>
<td>25</td>
<td>99.2 ± 1.0</td>
</tr>
<tr>
<td>50</td>
<td>99.0 ± 1.2</td>
</tr>
<tr>
<td>100</td>
<td>92.0 ± 3.1</td>
</tr>
<tr>
<td>200</td>
<td>85.1 ± 3.7 (^b)</td>
</tr>
<tr>
<td>400</td>
<td>80.5 ± 3.1 (^b)</td>
</tr>
<tr>
<td>800</td>
<td>72.6 ± 5.1 (^b)</td>
</tr>
</tbody>
</table>

\(^a\) each value represents mean ± SEM.

\(^b\) \(p<0.05\), significantly different from control.
Figure 1.
Figure 2

A

B
Figure 3

A

B

N-demethyl-Ery (% of control)

Ery (% of control)
Figure 4

A

B
Figure 5

A

![Graph showing Ery (pmol/5min/million cells) versus indoxyl sulfate (µM)]

B

![Graph showing demethyl-Ery (pmol/5min/million cells) versus indoxyl sulfate (µM)]