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Short Communication

Inhibitory effects of uraemic toxins 3-indoxyl sulfate and *p*-cresol on losartan metabolism *in vitro*

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Abstract

Objectives The purpose of this study was to clarify the cause of decreased metabolic clearance of losartan in patients with end-stage renal failure. The influence of serum from haemodialysis patients (uraemic serum) and uraemic toxins on the metabolism of losartan to EXP-3174 was investigated *in vitro*.

Methods The formation of EXP-3174 was estimated using pooled human liver microsomes. 3-Carboxy-4-methyl-5-propyl-2-furanpropanoic acid, hippuric acid, indole-3-acetic acid, 3-indoxyl sulfate and *p*-cresol were used as uraemic toxins.

Key findings Uraemic serum potently decreased the formation of EXP-3174 in pooled human liver microsomes. In addition, 3-indoxyl sulfate and *p*-cresol significantly decreased the formation of EXP-3174 in a concentration-dependent manner. Furthermore, normal serum (10% v/v) with both 3-indoxyl sulfate and *p*-cresol (both 20 μmol/l) significantly decreased the formation of EXP-3174 by 46%, which was similar to the level of inhibition with uraemic serum (10% v/v).

Conclusions These results suggest that decreased the metabolic clearance of losartan in patients with end-stage renal failure is partly due to high concentrations of 3-indoxyl sulfate and *p*-cresol.

Keywords cytochrome P450 2C9; losartan metabolism; uraemic serum; uraemic toxin

Introduction

Drugs that are predominantly cleared by the kidney require dose adjustment in patients with end-stage renal failure (ESRF). It is widely assumed that it is unnecessary to adjust the dose of drugs that are cleared exclusively by hepatic metabolism and transport in these patients. However, accumulated data from animal and human studies do not support this assumption.[1–3]

Angiotensin II receptor antagonists are used to slow the progression of renal disease in patients with type 1 diabetes and in non-diabetic patients who have overt nephropathy. Losartan, an angiotensin II receptor antagonist, is rapidly absorbed after oral administration and undergoes oxidation by cytochrome P450 (CYP)2C9 to form its active metabolite EXP-3174. In patients with normal renal function, renal clearance of losartan accounts for only 12% of systemic clearance.

The pharmacokinetics of losartan and EXP-3174 are altered in patients with ESRF. Both the maximum plasma concentration and area under the plasma concentration–time curve from zero to 24 h of losartan and EXP-3174 are increased in patients with ESRF compared with patients with normal renal function.[2] The metabolic clearance of losartan is reduced in patients with ESRF compared with patients with normal renal function.[2] Therefore, unidentified compounds in uraemic serum may inhibit the metabolism of losartan to EXP-3174 mediated by CYP2C9.

In a previous study, it was demonstrated that the hepatic uptake of digoxin was inhibited by 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) and *p*-cresol.[4] Hanada

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et al.[5] also reported that both indole-3-acetic acid and 3-indoxyl sulfate inhibited the function of CYP1A in a non-competitive manner. However, little is known about whether uraemic toxins such as CMPF, hippuric acid, indole-3-acetic acid, 3-indoxyl sulfate and *p*-cresol affect the formation of EXP-3174 mediated by CYP2C9.

In the present study, the influence of uraemic serum and uraemic toxins on the metabolism of losartan to EXP-3174 was examined in order to clarify the cause of decreased metabolic clearance of losartan in patients with ESRF.

Materials and Methods

Materials

Losartan potassium was purchased from LKT Laboratories, Inc. (St Paul, MN, USA). EXP-3174 (losartan carboxylic acid) was purchased from Toronto Research Chemicals Inc. (North York, Canada). *p*-Cresol, testosterone, $NADP^+$, glucose-6-phospate (G6P), glucose and glucose-6-phospate dehydrogenase (G6PDH) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Indole-3-acetic acid was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). 3-Indoxyl sulfate and hippuric acid were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Pooled human liver microsomes were purchased from BD Biosciences (Woburn, MA, USA). CMPF was synthesized at Kumamoto University (Japan).[6] All other chemicals were commercial reagent- or HPLC-grade products.

Serum samples

Normal human serum was purchased from Wako Pure Chemical Industries, Ltd. Uraemic human serum was obtained by collecting spare serum for biochemical tests from more than 400 patients who had been receiving haemodialysis at Shirasagi Hospital (Osaka, Japan). Serum was frozen at −80°C until use. The Ethical Community of Shirasagi Hospital and Kyoto Pharmaceutical University (Kyoto, Japan) approved this study.

Inhibitory experiments

Inhibition experiments were performed following the method reported by Nishikawa *et al*.[7] with slight modifications.

The reactions were performed with an NADPH-generating system. The reaction mixture (final volume 300 μl) contained 0.6 mmol/l $NADP^+$, 6 mmol/l G6P, 2 unit/ml G6PDH and 2 μmol/l losartan in the presence of normal or uraemic serum (1–10% v/v), or uraemic toxins (CMPF, hippuric acid, indole-3-acetic acid and *p*-cresol (all at 3–300 μmol/l) or 3-indoxyl sulfate 3–1000 μmol/l; 0.5% DMSO was used as the negative control) in 100 mol/l sodium phosphate buffer (pH 7.4). The reaction mixture was incubated at 37°C for 5 min, and the reaction was started by the addition of pooled human liver microsomes (0.1 mg protein/ml). Whole mixtures were incubated at 37°C for 45 min; the reaction was terminated by adding 5 mol/l phosphoric acid. It has been reported that the maximum concentration of losartan in ESRF patients is 1.4–5.6 μmol/l.[2] A losartan concentration of 2 μmol/l was therefore chosen for this study.

HPLC analysis

EXP-3174 was measured by HPLC. To 1 ml of sample was added 100 μl methanol, 2 μg testosterone (internal standard), 1 ml 0.3 mol/l sodium phosphate buffer (pH 3.5) and 5 ml *t*-butyl methyl ether, and the mixture shaken at 280 strokes/min for 15 min. After centrifugation at 1630*g* for 20 min, the upper layer was collected and evaporated to dryness at 40°C under a stream of nitrogen. The residue was then dissolved in mobile phase (50 mmol/l sodium phosphate buffer, pH 2.3 and acetonitrile; 66 : 34) and the upper layer injected onto the HPLC column. Absorbance was measured at 250 nm using a UV detector (SPD-6A, Shimadzu, Kyoto, Japan). The HPLC apparatus consisted of a Shimadzu SIL-20A auto injector; Shimadzu LC-10AS pump and an Inertsil ODS-III column (5 μm, 250 × 4 mm i.d.; GL Science, Tokyo, Japan). The column temperature was 35°C and the mobile phase was delivered at a flow rate of 1.0 ml/min. The retention times for testosterone (internal standard) and EXP-3174 were approximately 30 and 38 min, respectively. The calibration curves for EXP-3174 (5–100 ng/ml) showed good linearity ($R^2 > 0.999$).

Determination of kinetic parameters

Kinetic parameters for the metabolism of losartan *in vitro*, such as the Michaelis constant (K_m, μmol/l) and maximum metabolic rate (V_{max}, pmol/min per mg protein) were calculated by fitting the equation $v = (V_{max} \times [S])/(K_m + [S])$ to the results of a concentration-dependency experiment by a non-linear least-squares method using a non-linear least-squares program (MULTI program).[8]

Data analysis

The significance of differences between the mean values was determined by Dunn's test; $P < 0.05$ was considered significant.

Results

Time profile and Michaelis–Menten plot of EXP-3174 formation

EXP-3174 formation from 5 μmol/l losartan in pooled human liver microsomes increased linearly up to 90 min and was concentration dependent. K_m and V_{max} values were 3.59 ± 0.87 μmol/l and 32.0 ± 2.2 pmol/min per mg protein, respectively. The formation of EXP-3174 was therefore determined using 2 μmol/l losartan and an incubation time of 45 min.

Effects of uraemic serum

Normal serum (up to 10% v/v) caused negligible inhibition of EXP-3174 formation in pooled human liver microsomes whereas uraemic serum potently decreased the formation of EXP-3174 in a concentration-dependent manner (Figure 1).

Effects of uraemic toxins

3-Indoxyl sulfate and *p*-cresol significantly decreased the formation of EXP-3174 in a concentration-dependent manner. CMPF, hippuric acid and indole-3-acetic acid (up

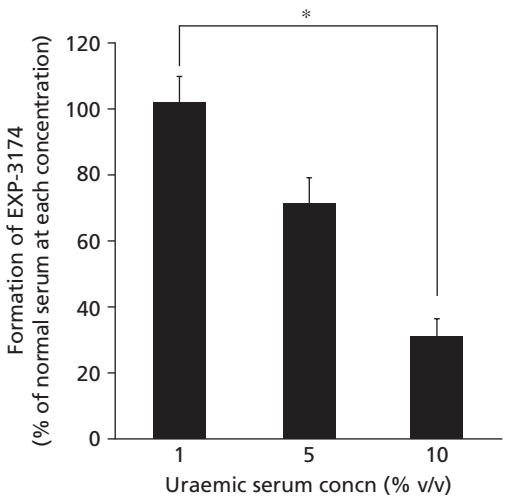

Figure 1 Effects of uraemic serum on the formation of EXP-3174 from losartan in pooled human liver microsomes. Bars show the formation of EXP-3174 as a percentage of that in normal serum at each concentration; means ± SE of 3 or 4 experiments. $^{*}P < 0.05$.

to 300 μmol/l) did not inhibit the formation of EXP-3174 (Figure 2).

Effects of normal serum with 3-indoxyl sulfate and/or *p*-cresol

The formation of EXP-3174 in normal serum with 3-indoxyl sulfate or *p*-cresol (both at 20 μmol/l) was decreased by 31% (Figure 3). When 3-indoxyl sulfate and *p*-cresol (both 20 μmol/l) were added together, the formation of EXP-3174 in normal serum (10% v/v) was decreased by 46% compared with normal serum. This level was similar to the formation of EXP-3174 in uraemic serum.

Kinetic analysis of the inhibitory effect of 3-indoxyl sulfate and *p*-cresol

The K_m values of EXP-3174 formation in the presence of 3-indoxyl sulfate and *p*-cresol were 3.23 ± 0.76 and 3.63 ± 0.72 μmol/l, respectively. These values were similar to those in the absence of uraemic toxins (3.23 ± 0.85 μmol/l). V_{max} values for EXP-3174 formation in the presence of 3-indoxyl sulfate and *p*-cresol were 20.7 ± 1.3 and 20.8 ± 1.1 pmol/min mg protein, respectively. These values were lower than that in the absence of uraemic toxins (30.5 ± 2.2 pmol/min per mg protein). These results suggest that 3-indoxyl sulfate and *p*-cresol inhibited EXP-3174 formation in a non-competitive manner (Figure 4).

Discussion

This study showed that both 3-indoxyl sulfate and *p*-cresol, which were used at concentrations equivalent to the estimated unbound concentration in patients with ESRF (ca. 20 μmol/l[4,9–11]), inhibited the conversion of losartan to EXP-3174. In addition, normal serum with both 3-indoxyl sulfate and *p*-cresol decreased the formation of EXP-3174 by 46%, which was a similar level of inhibition as with uraemic serum. This suggests that, because at concentrations found in clinical studies 3-indoxyl sulfate and *p*-cresol inhibit EXP-3174 formation *in vitro*, these uraemic toxins inhibit EXP-3174 formation in patients. It is not clear whether these uraemic toxins are substrates of the hepatic uptake transporter, but *p*-cresol has been shown to inhibit the hepatic uptake of digoxin.[4] Therefore, concentrations of these uraemic toxins might be higher in hepatocytes and may inhibit more strongly in clinical situations. In contrast, CMPF, hippuric acid and indole-3-acetic acid, which were used at concentrations equivalent to the estimated unbound concentration in ESRF patients (ca. 2, 100 and 3 μmol/l, respectively[4,9–11]), did not inhibit EXP-3174 formation. This suggests that CMPF, hippuric acid and indole-3-acetic acid do not inhibit EXP-3174 formation in clinical situations. These results suggest that uraemic serum inhibits the conversion of losartan to EXP-3174, and that the inhibitory compounds may be 3-indoxyl sulfate and *p*-cresol.

Uraemic serum used in this study was obtained by collecting spare serum for biochemical tests from more than 400 patients. This serum will have contained drugs taken by the patients. However, it is thought that this would have minimal influence in this study because of the low concentrations, as barely observed by HPLC-UV methods.

Dreisbach *et al.*[12,13] suggested that the detoxification of the anticoagulant warfarin, which is an important drug in clinical use, by CYP2C9 was decreased to 50% in patients with ESRF. This phenomenon can be partly explained by the inhibitory effect of 3-indoxyl sulfate and *p*-cresol. Therefore, substrates of CYP2C9 should be used with care in thse patients. Similar results have been reported for CYP3A. Nolin *et al.*[14] reported that erythromycin N-demethylation (human CYP3A activity) was decreased in patients with ESRF, suggesting that the decrease was related to uraemic toxins. In addition, Sun *et al.*[15] reported that 3-indoxyl sulfate significantly inhibited the metabolism of erythromycin in rat hepatocytes (rat CYP3A activity), and Hanada *et al.*[5] reported that 3-indoxyl sulfate significantly inhibited testosterone 6β-hydroxylation (human CYP3A activity). Thus, uraemic toxins might affect the metabolism of various drugs. The present report shows that 3-indoxyl sulfate and *p*-cresol inhibit the function of CYP2C9 in a non-competitive manner (Figure 4). Hanada *et al.*[5] reported that 3-indoxyl sulfate and indole-3-acetic acid inhibit the function of CYP1A in a non-competitive manner, and 3-indoxyl sulfate inhibited the function of UDP glucuronosyltransferase UGT2B. Therefore, uraemic toxins such as 3-indoxyl sulfate may inhibit various functional proteins, including others of the CYP family, in a non-specific manner.

The formation of EXP-3174 was decreased by approximately 40% in patients with ESRF compared with patients with normal renal function,[2] and the decrease in intrinsic clearance was estimated as approximately 60%.[16–18] In this study, 3-indoxyl sulfate and *p*-cresol, at concentrations equivalent to unbound concentrations found in patients with ESRF, decreased the formation of EXP-3174 by 46%. In addition, it was demonstrated that hepatic uptake of

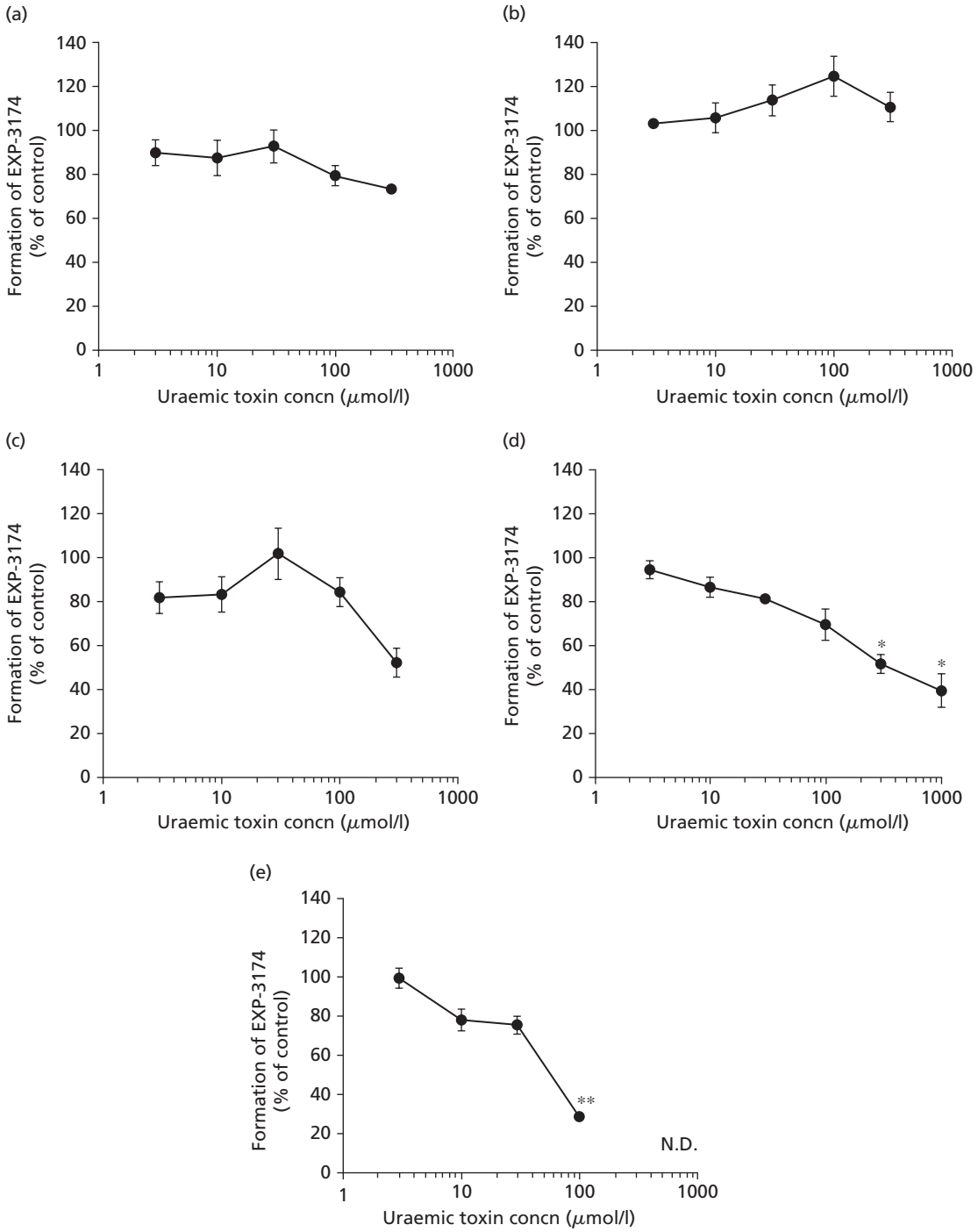

Figure 2 Effects of uraemic toxins on the formation of EXP-3174 in pooled human liver microsomes. (a) CMPF; (b) hippuric acid; (c) indole-3-acetic acid; (d) 3-indoxyl sulfate; (e) *p*-cersol. Values are means ± SE of 3 or 4 experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs control. ND, not detected.

digoxin was inhibited by uraemic serum.[4] Furthermore, Leblond *et al*.[19–21] reported the down-regulation of CYP proteins, including CYP2C11 and CYP3A1, in a rat model of chronic renal failure. Therefore, other factors, such as hepatic uptake and the down-regulation of human CYP proteins, might be involved in decreasing the non-renal clearance of losartan.

In conclusion, this report suggests that decreased metabolic clearance of losartan in patients with ESRF is partly induced by high concentrations of 3-indoxyl sulfate and *p*-cresol. As losartan is widely used in patients with ESRF, it is necessary to investigate the effects of uraemic serum and uraemic toxins on the hepatic uptake of losartan and the down-regulation of human CYP proteins.

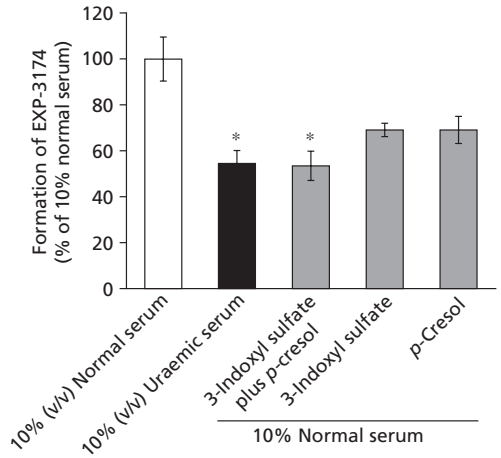

Figure 3 Effects of normal serum (10% v/v) with 3-indoxyl sulfate or *p*-cresol (both 20 μmol/l) on the formation of EXP-3174 in pooled human liver microsomes. Bars represent means ± SE of 3 or 4 experiments. $^{*}P < 0.05$ vs 10% normal serum.

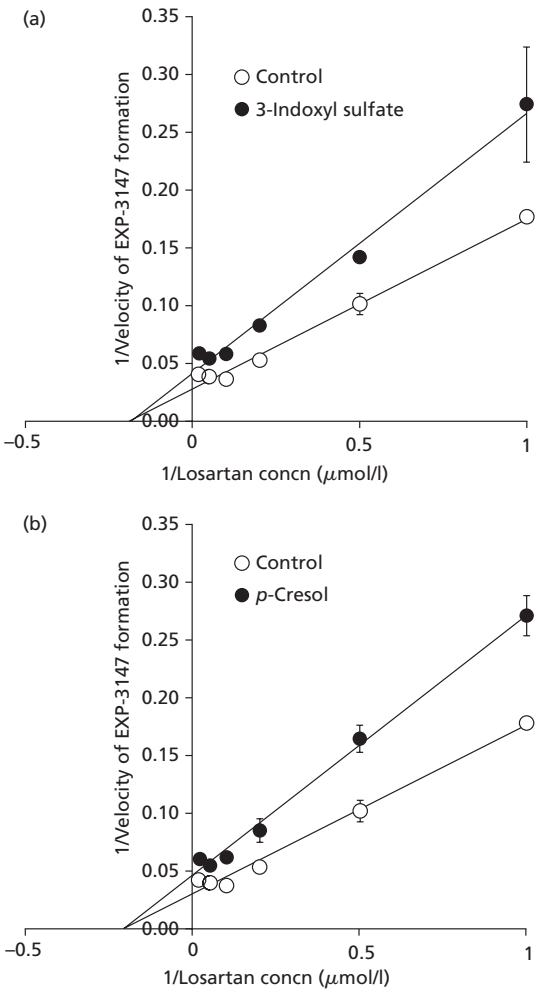

Figure 4 Lineweaver–Burk plots for the inhibitory effect of (a) 3-indoxyl sulfate (500 μmol/l) and (b) *p*-cresol (50 μmol/l) on formation of EXP-3174 from losartan (1, 2, 5, 10, 20 or 50 μmol/l) in pooled human liver microsomes. Points represent means ± SE of 3 or 4 experiments.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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