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RESEARCH PAPER

CYP3A-dependent drug metabolism is reduced in bacterial inflammation in mice

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BACKGROUND AND PURPOSE

Gene expression of Cyp3a11 is reduced by activation of Toll-like receptors (TLRs) by Gram-negative or Gram-positive bacterial components, LPS or lipoteichoic acid (LTA) respectively. The primary adaptor protein in the TLR signalling pathway, TIRAP, plays differential roles in LPS- and LTA-mediated down-regulations of Cyp3a11 mRNA. Here, we have determined the functional relevance of these findings by pharmacokinetic/pharmacodynamic (PK/PD) analysis of the Cyp3a substrate midazolam in mice. Midazolam is also metabolized by Cyp2c in mice.

EXPERIMENTAL APPROACH

Adult male C57BL/6, TIRAP+/+ and TIRAP–/– mice were pretreated with saline, LPS (2 mg·kg–1) or LTA (6 mg·kg–1). Cyp3a11 protein expression, activity and PK/PD studies using midazolam were performed.

KEY RESULTS

Cyp3a11 protein expression in LPS- or LTA-treated mice was reduced by 95% and 60% compared with saline-treated mice. Cyp3a11 activity was reduced by 70% in LPS- or LTA-treated mice. Plasma AUC of midazolam was increased two- to threefold in LPS- and LTA-treated mice. Plasma levels of 1′-OHMDZ decreased significantly only in LTA-treated mice. Both LPS and LTA decreased AUC of 1′-OHMDZ-glucuronide. In the PD study, sleep time was increased by ~2-fold in LPS- and LTA-treated mice. LTA-mediated decrease in Cyp3a11 protein expression and activity was dependent on TIRAP. In PK/PD correlation, AUC of midazolam was increased only in LPS-treated mice compared with saline-treated mice.

CONCLUSIONS AND IMPLICATIONS

LPS or LTA altered PK/PD of midazolam . This is the first study to demonstrate mechanistic differences in regulation of metabolite formation of a clinically relevant drug by Gram-negative or Gram-positive bacterial endotoxins.

Abbreviations

1′-OHMDZ, 1′-hydroxymidazolam; 1′-OHMDZ-gluc, 1′-hydroxymidazolam glucuronide and ICU, Intensive care unit; AUC, area under the curve; CL, clearance; CYP, cytochrome P450; DMEs, drug-metabolizing enzymes; LTA, lipoteichoic acid; PD, pharmacodynamics; PK, pharmacokinetics; TIRAP, Toll-IL-1 receptor domain containing adaptor protein; TLR, Toll-like receptor

Introduction

Inflammation is a complex immunological response elicited during many disease states, making it a critical phenomenon in clinical therapies. An acute inflammatory reaction can be initiated by a wide variety of pathological stimuli, such as bacterial or viral infections, tissue damage or cellular stress, which results in the release of pro-inflammatory cytokines and alteration in the expression of several hepatic proteins. Several studies have shown that inflammation can lead to an impairment of the expression and activity of drug-metabolizing enzymes (DMEs) in rodents and humans (Sewer *et al.*, 1996; Morgan, 1997; Siewert *et al.*, 2000; Giannini *et al.*, 2003). Regulation of DMEs during inflammation is dependent on the type of inflammatory stimuli (Sewer *et al.*, 1996; 1997; Barclay *et al.*, 1999; Chaluvadi *et al.*, 2009). However, it is not known whether reductions in DMEs by different inflammatory stimuli are responsible for reducing the metabolite formation of drugs metabolized by specific DMEs. Reduced metabolite formation during inflammation can lead to increased accumulation of the parent compound in the plasma. This can change the pharmacological activity of clinically relevant medications, leading to reduced efficacy or increased toxicity.

Among the phase I and phase II DMEs, the human CYP3A subfamily is responsible for metabolizing approximately half of currently marketed drugs (Shimada *et al.*, 1994; Thummel *et al.*, 1996; Guengerich, 1999; nomenclature follows Alexander *et al.*, 2011). Studies have shown that the Gram-negative bacterial endotoxin LPS can induce acute phase response in animals, which can lead to decreased expression and activity of key phase I and II DMEs (Morgan, 1989; Renton and Nicholson, 2000), ultimately leading to decreased hepatic drug metabolism (Monshouwer *et al.*, 1996). Also, a clinical study showed that LPS injection led to a significant decrease in the clearance of antipyrine (Shedlofsky *et al.*, 1994). Recently, we showed that LPS-mediated down-regulation of hepatic Cyp3a11 mRNA levels was mediated through activation of the pathogen recognition receptor, the Toll-like receptor 4 (TLR4) (Ghose *et al.*, 2008). In comparison with Gram-negative organisms, Gram-positive bacteria such as *Enterococcus faecalis*, *Staphylococcus aureus* or *Streptococcus pneumoniae* are also highly prevalent. Statistically, infections caused by Gram-positive antigens account for >50% of total organisms causing sepsis (Martin *et al.*, 2003). Gram-positive infections are commonly implicated in producing the toxic shock syndrome, which is an acute, multi-organ illness, typically resulting in shock (Sriskandan and Cohen, 1999). We have shown that activation of TLR2 by the Gram-positive bacterial endotoxin lipoteichoic acid (LTA) reduced the gene expression of hepatic DMEs and transporters (Ghose *et al.*, 2009; 2011). A recent clinical study also showed that LTA treatment elicited a different response in the lungs compared with LPS (Hoogerwerf *et al.*, 2008). However, there is a limited understanding of how alterations in DME genes by LPS or LTA cause changes in metabolism and clearance of clinically relevant medications. It is known that TLR4 or TLR2 activation by LPS or LTA, respectively, is initiated by the primary adaptor protein, Toll-IL-1 receptor domain containing adaptor protein (TIRAP)-dependent pathway. In our previous studies, we have shown that the down-regulation of mRNA levels of key phase I and II DMEs by LTA was mediated by TIRAP, while LPS-mediated effects on these DMEs was not dependent on TIRAP (Ghose *et al.*, 2008; 2011). Thus, Gram-negative and Gram-positive bacteria may regulate drug metabolism by distinct mechanistic pathways.

In this study, we determined the metabolism of midazolam as it is a clinically relevant drug, widely used for anaesthesia in surgical and dental procedures. Midazolam is also used as a sedative for acutely agitated patients in intensive care units due to its favourable PK properties. It is considered to be a specific substrate of CYP3A and routinely used as a marker of CYP3A4 activity in humans (von Moltke *et al.*, 1996; Greenblatt *et al.*, 2003). However, *in vivo* studies have shown a fivefold variation (in 98% of study population) (Floyd *et al.*, 2003) and 11-fold variation (in 90% of study population) (He *et al.*, 2005) in the clearance of midazolam. Although midazolam is used to study Cyp3a activity in mice, recent studies in Cyp3a$^{-/-}$ mice showed a significant contribution of Cyp2c enzymes to midazolam metabolism (Perloff *et al.*, 2000; 2003; Warrington *et al.*, 2000; van Waterschoot RA *et al.*, 2008). This compound is not a P-glycoprotein (Pgp) substrate and is therefore mainly cleared by hepatic and/or intestinal metabolism owing to its intermediate extraction ratio ($E = 0.3–0.7$) (Polli *et al.*, 2001; Gorski *et al.*, 2003). In humans, midazolam is oxidized by CYP3A4 to form the primary metabolite, 1′-hydroxymidazolam (1′-OHMDZ) (Heizmann *et al.*, 1983). In mice, Cyp3a and Cyp2c are involved in formation of 1′-OHMDZ and 4′-OHMDZ (van Waterschoot RA *et al.*, 2008). Studies in humans have shown that 1′-OHMDZ further undergoes *o*-glucuronidation to form 1′-hydroxymidazolam-glucuronide (1′-OHMDZ-gluc) by UGT2B4 and 2B7 (Zhu *et al.*, 2008). 1′-OHMDZ has ~50% pharmacological activity as the parent compound, and the affinity of 1′-OHMDZ to the benzodiazepine receptors in the brain is ~60% of that of midazolam. Because of its very short half-life and lower pharmacological activity, 1′-OHMDZ is said to have negligible clinical effects (Bornemann *et al.*, 1985). On the other hand, clinical studies have shown that, accumulation of 1′-OHMDZ-gluc caused prolonged sedation in patients suffering from acute renal failure (Driessen *et al.*, 1991; Bauer *et al.*, 1995; Swart *et al.*, 2005).

To investigate the effects of midazolam and its metabolites on the pharmacological activity in mice, we performed PK/PD correlation using model independent linear regression analysis. We found that the PK and PD profiles of midazolam and its metabolites showed significant differences in LPS- or LTA-treated mice compared to the control. This corresponds to reduced expression and activity of Cyp3a11 in mice. This study provides direct evidence that inflammation reduces the metabolism and clearance of midazolam, which leads to prolonged sedation in mice. This study warrants further investigation of inflammation-mediated alterations in drug metabolism and its effects on the safety and efficacy of therapeutic agents.

Methods

Animals

All animal care and experimental procedures complied strictly with the Institutional Animal Care and Use Commit-

tee guidelines. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). Adult male C57BL/6, TIRAP$^{+/+}$ and TIRAP$^{-/-}$ mice (8–10 weeks) weighing 20–25 g were maintained in a temperature and humidity controlled environment and 12 h light/dark cycle with free access to water and rodent chow *ad libitum*. A total of 75 mice were used in the experiments described here.

Preparation of liver microsomes

Mouse liver microsomes were prepared using a published procedure, with minor modifications as described below (Chen *et al.*, 2003). Mice were injected i.p. with saline, LPS (2 mg·kg^{-1}) or LTA (6 mg kg^{-1}) and, 16h later, the animals were killed by cervical dislocation, the livers perfused with sodium phosphate buffer (pH 7.4) to remove blood and collected for the preparation of microsomes. Livers were homogenized using a motorized homogenizer in ice-cold homogenization buffer [50 mM potassium phosphate buffer (pH 7.4), 250 mM sucrose, 1 mM EDTA] and centrifuged at 18 500× *g* for 15 min at 4°C. The pellet was discarded, and the supernatant was collected and centrifuged again at 85 600× *g* for 60 min at 4°C to yield the microsome pellets. The microsome pellet was resuspended in 250 mM sucrose. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL) using BSA as the standard.

Cyp3a11 protein expression

Protein expression of Cyp3a11 in mouse liver microsomes was determined by immunoblotting analysis as described previously (Ghose *et al.*, 2008; Gandhi *et al.*, 2011), with minor modifications. Hepatic microsomal proteins (10 µg) were separated by SDS-PAGE on a 12% polyacrylamide resolving gel (Bio-Rad, Hercules, CA). Samples were then transferred to nitrocellulose membrane. Membranes were blocked with 5% nonfat milk in TBS-Tween-20 washing buffer for 1 h and then incubated with primary anti-rabbit Cyp3a11 antibody (1:4000) in 5% BSA in TBS-Tween20 overnight at 4°C. Membranes were subsequently washed and probed with a goat anti-rabbit IgG-alkaline phosphatases (IgG-AP) secondary antibody (1:2000) and incubated with Tropix® CDP Star® Nitro block IITM ECL reagent according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Membranes were analysed on FluorChem FC Imaging System (Cell Biosciences, Santa Clara, CA).

Cyp3a11 enzyme activity

Hydroxylation of midazolam was measured using mouse liver microsomes as described in detail previously (He *et al.*, 2006), with minor modifications. The formation of 1′-OHMDZ was used as a specific indicator for mouse Cyp3a11 activity. In brief, incubation mixtures (performed in duplicate) contained 0.05 mg of total microsomal protein, midazolam (0–16 µM), 1.3 mM NADPH and reaction cofactors in 50 mM potassium phosphate buffer (pH 7.4). The reaction mixtures were incubated at 37°C. The reaction was initiated by addition of glucose-6-phosphate dehydrogenase (1 unit·mL^{-1}). After 5 min, the reactions were stopped by the addition of 100 µL of acetonitrile containing phenacetin as the internal standard. The incubation mixture was centrifuged, and the supernatant was analysed by LC-MS/MS analysis. The identity of 1′-OHMDZ and the internal standard was verified by comparing with authenticated standards. The data were fit to the substrate inhibition model and analysed by GraphPad Prism 4.0 software (GraphPad Inc., La Jolla).

LC-MS/MS

To determine the concentrations of midazolam and its metabolites in microsomal and plasma samples, an API 3200 Qtrap triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX) was used by MRM (multiple reaction monitoring) method in the positive ion mode. A UPLC system, Waters Acquity™ with diode-arrayed detector was used. The UPLC conditions for analysing midazolam, 1′-OHMDZ, 1′-OHMDZ-gluc and phenacetin (internal standard) were as follows: column, Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., 1.7 µm, Waters, Milford, MA); mobile phase A, 0.1% formic acid; mobile phase B, 100% acetonitrile performed in a gradient from 0 to 3 min as follows: 0–0.5 min: 90% A; 0.5–1.0 min: 75% A; 1.0–2.0 min: 60% A and 2.0–2.5 min: held constant at 60% A, 2.5–3.0 min: 90% A respectively. The quantification was performed using MRM method with the transitions of *m/z* 326.1 → *m/z* 291.1 for midazolam, 342.1 → 324.1 for 1′-OHMDZ, 518.1 → 324.1 for 1′-OHMDZ-gluc and 180.0 → 110.0 for phenacetin. The retention times were as follows: midazolam, 1.98 min; 1′-OHMDZ, 1.93 min; 1′-OHMDZ-gluc, 1.72 min and phenacetin, 1.79 min.

Pharmacokinetic (PK) study of midazolam

Mice were injected i.p. with saline, LPS or LTA followed by i.p. injection of midazolam (5 mg·kg^{-1}) 16 h later. Blood samples (10–15 µL) were collected from the tail vein in heparinized tubes at 0, 5, 15, 30, 60, 120, 240, 360 and 480 min following midazolam. For extraction of midazolam and its metabolites, 50 µL of diluted plasma (10-fold dilution) was mixed with 450 µL of acetonitrile and 450 µL of methyl *t*-butyl ether. The samples were vortex-mixed, centrifuged and the organic layer was evaporated under a gentle stream of air and reconstituted in 150 µL of 30% acetonitrile. The lower limits of quantification in this study were 1.95 ng·mL^{-1} for midazolam, 3.91 ng·mL^{-1} for 1′-OHMDZ and 15.63 ng·mL^{-1} for 1′-OHMDZ-gluc. The linear range for midazolam was 1.95–1000 ng·mL^{-1}, for 1′-OHMDZ was 7.81–1000 ng·mL^{-1} and for 1′-OHMDZ-gluc was 62.5–1000 ng·mL^{-1} respectively. The within-day variability did not exceed 15.3% and the between-day variability did not exceed 13.9% respectively. PK parameters such as maximum plasma concentration (C_{max}), time to reach C_{max} (T_{max}), area under the plasma concentration–time curve ($AUC_{0\text{-}8\,h}$), volume of distribution (V), clearance (CL) and half-life ($t_{1/2}$) for midazolam or the metabolites were derived from the plasma concentration-time data by non-compartmental model using WinNonlin 3.3, respectively (Pharsight, Mountain View, CA).

Pharmacodynamic (PD) study of midazolam

For PD studies, mice were pretreated with saline, LPS or LTA followed by i.p. injection of midazolam (80 mg·kg^{-1}) 16 h

later. Sleep time (time between the loss and regaining of righting reflex) in minutes was used as a marker for the PD activity of midazolam.

PK–PD correlation of midazolam and its metabolites with the sleep time

Adult, C57BL/6 mice were divided into three groups ($n = 3–5$ per group) and treated with saline, LPS or LTA. Mice were i.p. injected with a pharmacological dose of midazolam (80 mg·kg^{-1}) 16 h later. Mice were allowed to turn on their back, and blood samples were collected in heparinized tubes at 30, 60, 180 and 300 min respectively. Plasma concentrations of midazolam, 1′-OHMDZ and 1′-OHMDZ-gluc were determined by LC-MS/MS. The AUCs were calculated by the trapezoidal rule. A correlation between the AUC of midazolam and its metabolites with the sleep time was fitted using linear regression analysis.

Statistical analysis

All the experiments were conducted with $n = 3–5$ mice in each group. The numerical data are presented as mean ± SD and analysed by a one-way ANOVA followed by Tukey's *post hoc* analysis, if $P < 0.05$. The enzyme kinetic data with the TIRAP mice was analysed by a two-way ANOVA followed by Bonferroni's multiple comparison test, if $P < 0.05$.

Materials

Midazolam (Cat # 451028) was purchased from BD Biosciences (San Diego, CA). Phenacetin (cat. # 77440) and 1′-OHMDZ (Cat. # UC430) were purchased from Sigma Aldrich (St. Louis, MO). 1′-OHMDZ glucuronide was a kind donation from Dr Gérard Fabre (Sanofi-Aventis, France). Midazolam hydrochloride solution for injections was purchased from Baxter Healthcare Corporation (Deerfield, IL). LPS (*E. coli*) and LTA(*S. aureus*) were purchased from InvivoGen (San Diego, CA) and freshly diluted to 5 mg·mL^{-1} in pyrogen-free 0.9% NaCl solution. The anti-CYP3A antibody was a generous gift from Dr Robert J Edwards, Department of Medicine, Imperial College (London, UK). All solvents were of HPLC grade and were obtained from VWR International, LLC (Suwanee, GA). Unless otherwise noted, all other chemicals were purchased from Sigma-Aldrich.

Results

Regulation of Cyp3a11 expression and activity in inflammation

Our recent studies showed that activation of TLR4 by LPS or TLR2 by LTA down-regulated the gene expression of hepatic Cyp3a11 (Ghose *et al.*, 2008; 2009; 2011). In the present study, we determined the protein expression and activity of Cyp3a11. We observed ~25-fold down-regulation of Cyp3a11 protein expression in LPS-treated mice and ~3-fold in LTA-treated mice compared with saline-treated mice (Figure 1A). We determined Cyp3a11 activity by measuring hydroxylation of the *Cyp3a* substrate, midazolam, as described previously (Thummel *et al.*, 1994). Cyp3a11 activity (V_{max})

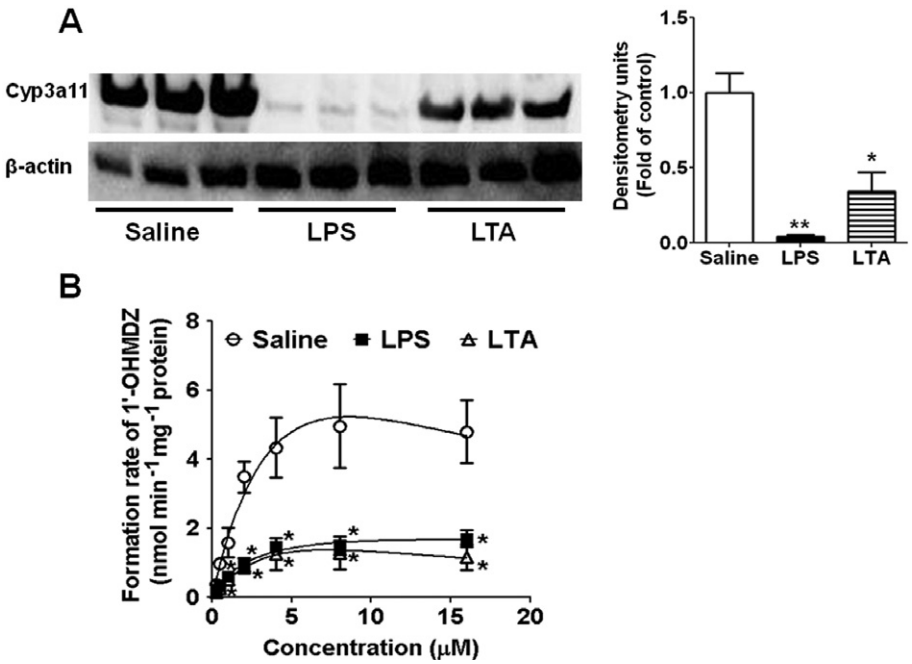

Figure 1

Regulation of Cyp3a11 protein expression and activity: Mice were injected i.p. with saline, LPS (2 mg·kg^{-1}) or LTA (6 mg·kg^{-1}), and 16h later livers were collected and analysed ($n = 4$ per group). Microsomes were prepared as described. (A) Cyp3a11 expression was analysed by Western blot analysis of microsomal fractions (10 μg protein·per well) using anti-rabbit Cyp3a11 antibody. *$P < 0.05$ and **$P < 0.01$. compared with saline. (B) Hydroxylation of midazolam was used as a marker of Cyp3a11 activity. Data are shown as mean ± SD. * $P < 0.05$, significantly different from saline.

Table 1

Enzyme kinetic parameters of midazolam

Parameters	Saline	LPS	LTA
V_{max} (nmol·min^{-1}·mg^{-1})	14.14 ± 4.15	2.88 ± 0.41*	3.42 ± 2.46*
K_M (μM)	6.58 ± 1.44	3.24 ± 0.29*	4.85 ± 2.87
CL_{int} (mL·min^{-1}·mg^{-1})	2.14 ± 0.27	0.89 ± 0.07*	0.67 ± 0.08*
K_i (μM)	9.65 ± 2.09	24.75 ± 12.99	13.51 ± 6.54

*P < 0.05 when compared to the respective parameter in the saline group.

Table 2

PK parameters of (A) midazolam, (B) 1′-OHMDZ and (C) 1′-OHMDZ-gluc

Parameter	Saline	LPS	LTA
(A) midazolam			
$AUC_{0\to 8\ h}$ (μmol.min·L^{-1})	13.69 ± 3.36	37.24 ± 14.13*	24.63 ± 6.94*
C_{max} (μmol·L^{-1})	0.24 ± 0.02	0.41 ± 0.11*	0.40 ± 0.18
T_{max} (min)	10.00 ± 5.77	15.00 ± 0.00	12.5 ± 5.00
$T_{1/2}$ (min)	31.52 ± 17.23	54.92 ± 13.36	99.91 ± 46.67*
CL (L·min^{-1}·kg^{-1})	1.13 ± 0.22	0.46 ± 0.18*	0.65 ± 0.19*
V_d (L·kg^{-1})	47.57 ± 16.05	35.36 ± 13.59	92.52 ± 35.58*
(B) 1′-OHMDZ			
$AUC_{0\to 8\ h}$ (μmol.min·L^{-1})	216.42 ± 56.78	168.09 ± 36.98	117.60 ± 26.52*
T_{max} (min)	29.54 ± 6.98	36.46 ± 7.67	34.74 ± 8.78
C_{max} (μmol L^{-1})	1.56 ± 0.49	1.03 ± 0.16	0.84 ± 0.27*
(C) 1′-OHMDZ-gluc			
$AUC_{0\to 8\ h}$ (μmol.min·L^{-1})	24.50 ± 3.06	18.13 ± 2.44*	16.20 ± 0.50*
T_{max} (min)	29.42 ± 4.67	49.63 ± 6.80*	33.31 ± 12.08
C_{max} (μmol·L^{-1})	0.20 ± 0.02	0.08 ± 0.01*	0.12 ± 0.01*

*P < 0.05 when compared to the respective parameter in the saline group.

decreased significantly (~4–5-fold) in both, LPS- and LTA-treated mice compared with saline-treated mice (Figure 1B). There was no significant difference in the K_M values between saline and LTA-treated mice. However, the K_M was significantly lower in LPS-treated mice, compared with saline-treated mice (Table 1). The intrinsic clearance (CL_{int}) was significantly lower in LPS- or LTA-treated mice (~2–3-fold reduction in CL_{int}) compared with saline-treated mice. The substrate inhibition constant (K_i) did not change significantly amongst the three groups (Table 1).

PK of midazolam

As hepatic Cyp3a11 protein expression and activity were reduced by LPS or LTA, we determined the plasma PK profiles of midazolam. The results demonstrated a significant increase in the AUC of midazolam in LPS- or LTA-treated mice (~2–3-fold) compared with saline-treated mice (Figure 2, Table 2A). Similarly, there was a significant decrease in the clearance of midazolam in LPS or LTA groups (~2–3-fold) compared with the saline group (Table 2A). The PK parameters for 1′-OHMDZ showed an interesting outcome in LPS- or LTA-treated mice. There was a significant decrease in the C_{max} of 1′-OHMDZ with a corresponding decrease in AUC (~2-fold reduction) in LTA-treated mice compared with saline-treated mice (Figure 2, Table 2B). These parameters were unchanged in LPS-treated mice. On the other hand, AUC of the secondary metabolite, 1′-OHMDZ-gluc, was significantly decreased (~1.5-fold reduction) in LPS- or LTA-treated mice compared with saline-treated mice (Figure 2, Table 2C).

PD of midazolam

Being a short-acting benzodiazepine, midazolam is commonly used in surgical and dental procedures for mild anaesthesia. Therefore, we used sleep time as a marker for the pharmacological activity of midazolam. We observed an almost 2-fold increase in the sleep time in LPS- (288.75 min) or LTA- (220.0 min) treated mice compared with saline-treated mice (151.33 min).

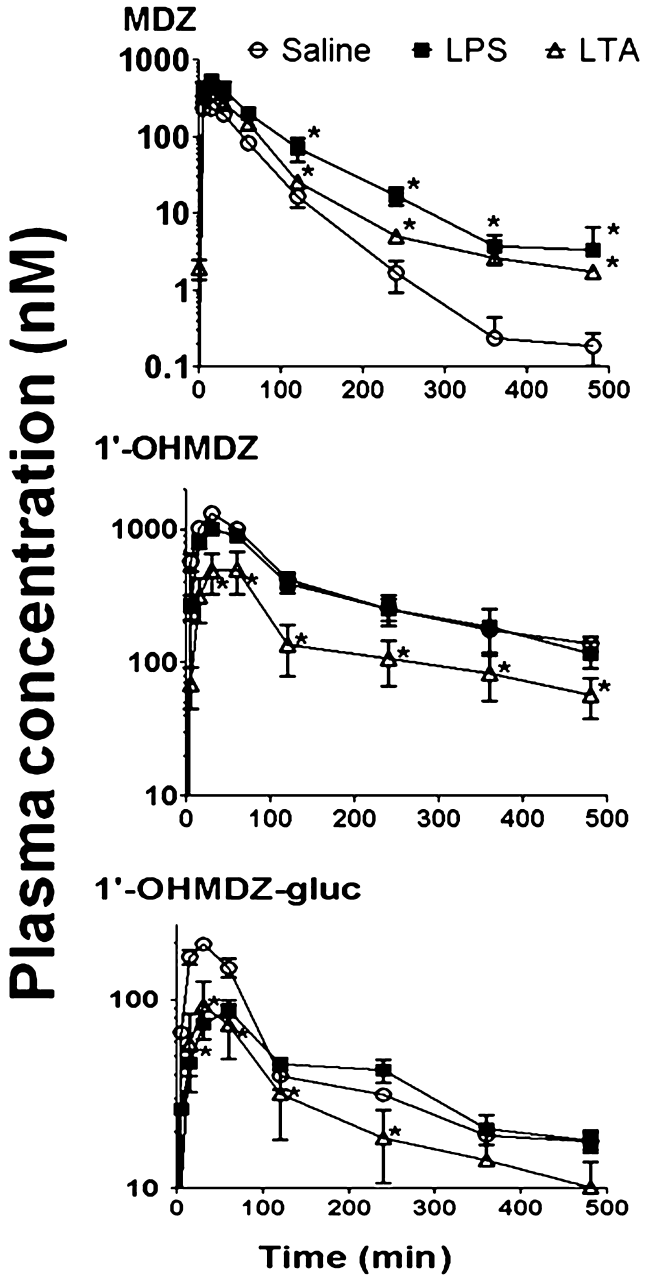

Figure 2

PK of midazolam in LPS- or LTA-induced inflammation. Plasma concentration versus time profiles from 0 to 8 h for midazolam, 1′-OHMDZ and 1′-OHMDZ-gluc in saline-, LPS- (2 mg·kg^{-1}) or LTA- (6 mg·kg^{-1}) treated mice followed by i.p. administration of midazolam (5 mg·kg^{-1}) are shown. Plasma samples were processed as described. n = 4–5. Data are shown as mean ± SD. *P < 0.05, significantly different from saline.

PK–PD correlation of midazolam and its metabolites with the sleep time

Due to the formation of an active metabolite of midazolam, another goal of this study was to determine a correlation between the plasma levels of midazolam or its metabolites to the pharmacological activity of midazolam in mice. The pharmacological potency of 1′-OHMDZ compared with the parent compound remains controversial (Mandema *et al.*, 1992; Reed *et al.*, 2001). Therefore, in this study, we examined whether the sedative–hypnotic activity of midazolam is related to plasma concentrations of either midazolam, or its metabolites following the administration of a single i.p. dose of midazolam (80 mg·kg^{-1}) in mice using conventional model-independent analysis. This dose of midazolam was shown to induce sleep in mice. Plasma concentration–time profiles of midazolam, 1′-OHMDZ or 1′-OHMDZ-gluc in saline, LPS- or LTA-treated mice are shown in Figure 3A. We observed a significant increase in the plasma levels of midazolam in the LPS group at 3 and 5 h compared with the saline group, whereas plasma levels of midazolam in the LTA group were significantly different only at 5 h compared with the saline group (Figure 3A). We also observed significantly reduced plasma levels of 1′-OHMDZ in the LPS group at 30 and 60 min compared with the saline group. Plasma level of 1′-OHMDZ was lower in the LTA group only at 30 min. On the other hand, we also observed significantly reduced plasma levels of 1′-OHMDZ-gluc in the LPS group at 30 and 60 min. Surprisingly, the plasma levels of 1′-OHMDZ-gluc increased at 5 h in the LPS group compared with the saline group (Figure 3A). However, in the LTA group, the plasma levels of 1′-OHMDZ-gluc were significantly reduced only at 30 min (Figure 3A).

The next aim was to determine the correlation between AUCs of midazolam and the metabolites with the sleep time. Our results demonstrated that higher AUC of midazolam in the LPS group corresponded with the increased sleep time in mice compared with the saline group (Figure 3B). However, AUC of midazolam in the LTA group did not show a significant increase compared with the saline group. On the other hand, the AUC of 1′-OHMDZ or 1′-OHMDZ-gluc in the LPS or LTA group did not show a significant difference compared with the saline group (Figure 3B). Thus, increased sleep time of mice correlated with increased AUC of midazolam. Furthermore, there was an inverse correlation between sleep time and AUC of metabolites. This study therefore confirms that the increase in the pharmacological activity of midazolam is associated with the concentrations of the parent compound in LPS-treated mice, and not to any of the metabolites studied.

Role of TIRAP in Cyp3a11 expression and activity

Our previous studies showed that TIRAP played a differential role in regulating the gene expression of various phase I and phase II enzymes in LPS- or LTA-induced inflammation (Ghose *et al.*, 2008; 2009; 2011). Therefore, we sought to determine whether TIRAP also played a role in midazolam metabolism upon LPS or LTA treatment. We observed a 2–3-fold reduction in Cyp3a11 protein expression in liver microsomes from LPS- or LTA-treated TIRAP$^{+/+}$ mice compared with saline-treated mice (Figure 4). We also observed a 2–3-fold decrease in Cyp3a11 activity (V_{max}) and CL_{int} in LPS- or LTA-treated TIRAP$^{+/+}$ mice compared with saline-treated mice (Figure 5). However, this reduction in Cyp3a11 protein expression and activity was attenuated only in the LTA-treated TIRAP$^{-/-}$ mice (Figures 4 and 5, Table 3). Cyp3a11 expression and activity were comparable in saline-treated

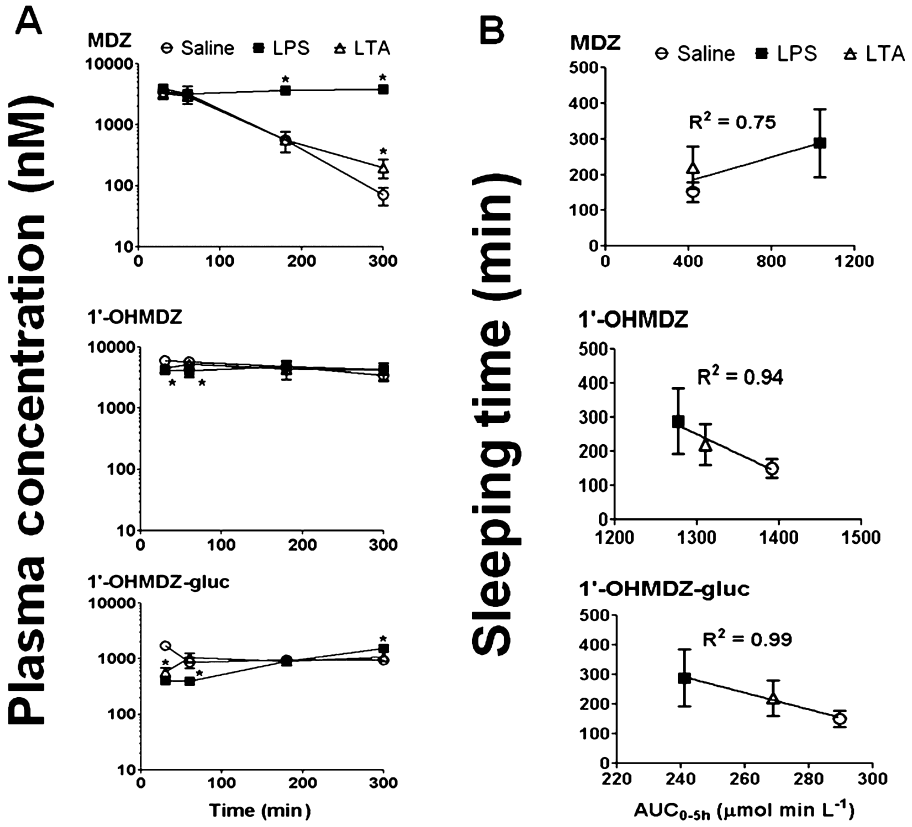

Figure 3

PK–PD correlation of midazolam and its metabolites with the sleep time: Mice were injected i.p. with saline, LPS (2 mg·kg^{-1}) or LTA (6 mg·kg^{-1}) followed by midazolam (80 mg·kg^{-1}) 16 h later. Blood samples were collected at 30 and 60 min and 3 and 5 h after midazolam. (A) PK profiles of midazolam, 1′-OHMDZ and 1′-OHMDZ-gluc. (B) Correlation of plasma AUC of midazolam and its metabolites with the sleep time was calculated by linear regression analysis. Data are shown as mean ± SD. *$P < 0.05$, significantly different from saline.

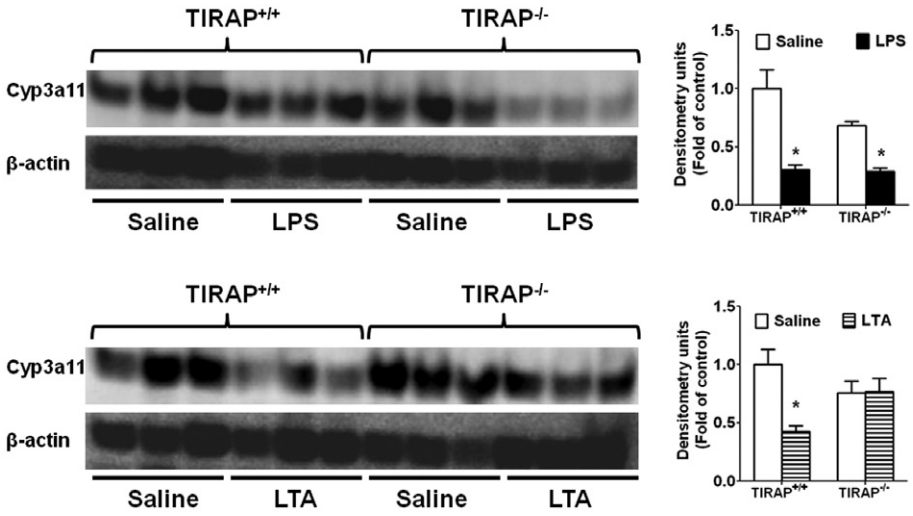

Figure 4

Role of TIRAP in Cyp3a11 protein expression: Mice were injected i.p. with saline, LPS (2 mg·kg^{-1}) or LTA (6 mg·kg^{-1}), and 16h later livers were collected and analysed ($n = 4$ per group). Microsomes were prepared as described. Cyp3a11 expression was analysed by Western blot analysis of microsomal fractions (10 μg protein·per well) using anti-rabbit Cyp3a11 antibody. Data are shown as mean ± SD. *$P < 0.05$, significantly different from saline.

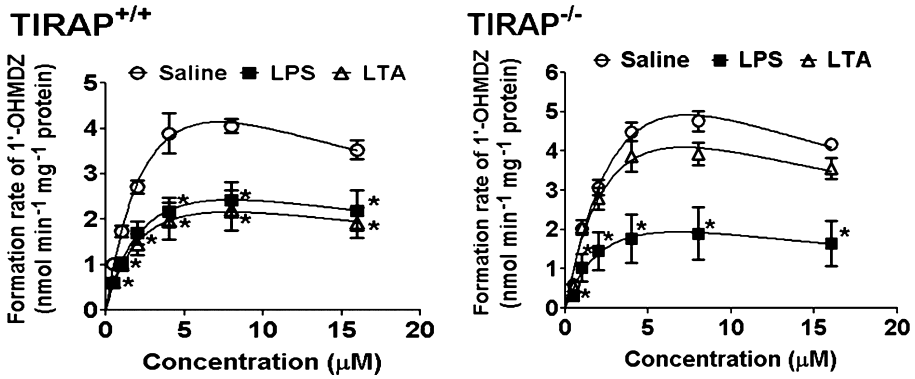

Figure 5

Role of TIRAP in Cyp3a11 activity: Mice were injected i.p. with saline, LPS (2 mg·kg^{-1}) or LTA (6 mg·kg^{-1}) , and 16h later livers were collected and analysed ($n = 4$ per group). Microsomes were prepared as described. Hydroxylation of midazolam was determined as a marker of Cyp3a11 activity in microsomes. Data are shown as mean ± SD. *$P < 0.05$, significantly different from saline.

Table 3

Enzyme kinetic parameters of midazolam in TIRAP mice

	TIRAP$^{+/+}$			TIRAP$^{-/-}$		
Parameters	**Saline**	**LPS**	**LTA**	**Saline**	**LPS**	**LTA**
V_{max} (nmol·min^{-1}·mg^{-1})	8.88 ± 1.83	4.32 ± 0.86*	4.02 ± 2.50*	13.72 ± 3.78	5.18 ± 1.59*	9.17 ± 3.54
K_M (μM)	4.04 ± 1.00	3.06 ± 0.82	3.05 ± 2.33	6.50 ± 2.47	3.24 ± 1.19*	4.19 ± 2.31
CL_{int} (mL·min^{-1}·mg^{-1})	2.22 ± 0.18	1.45 ± 0.27*	1.41 ± 0.24*	2.17 ± 0.22	1.64 ± 0.20*	3.12 ± 1.35
K_i (μM)	13.47 ± 3.91	23.89 ± 14.55	63.02 ± 64.55	9.29 ± 3.66	14.26 ± 3.25	15.42 ± 7.57

*$P < 0.05$ when compared to the respective parameter in the saline group.

TIRAP$^{+/+}$ and TIRAP$^{-/-}$ mice. There was no significant difference in the K_M values between saline- and LTA-treated mice; however, the K_M was significantly lower in LPS-treated mice compared with saline-treated mice.

Discussion

In this study, we report that inflammation induced by the bacterial endotoxins can significantly alter Cyp3a11 enzyme activity and PK/PD of the Cyp3a11 specific substrate, midazolam. The regulation of midazolam metabolism by Gram-negative or Gram-positive bacterial components occurs by distinct mechanistic pathways.

We observed a significant reduction in Cyp3a11 protein expression as well as activity in LPS- or LTA-induced inflammation compared with saline pretreatment (Figure 1A). Also, the reduction in Cyp3a11 protein expression and activity (as shown by reduced formation of 1′-OHMDZ (Figure 1B), in mouse liver microsomes by LPS was comparable to previous studies (Barclay *et al.*, 1999). To further extend the significance of this finding, our next goal was to study the effect of inflammation on PK/PD of midazolam in mice.

In the PK studies, we observed a significant increase in the plasma exposure ($AUC_{0-8\,h}$) and a significant decrease in the CL of midazolam in LPS- as well as LTA-treated mice (Figure 2; Table 2A). Changes in the PK profiles of midazolam by LPS or LTA can have serious implications on the efficacy and safety of midazolam. Several studies have shown clinically relevant drug–drug interactions with midazolam in animals as well as humans (Palkama *et al.*, 1999; Kotegawa *et al.*, 2002). Increased midazolam plasma levels can lead to respiratory depression, further leading to a requirement for intubation and ventilatory support. Our results can serve as a paradigm for midazolam dosage optimization in patients with underlying bacterial infections. Although LPS did not have a significant effect on the reduction in plasma levels of 1′-OHMDZ, LTA treatment showed a significant effect on reduced 1′-OHMDZ plasma levels compared with saline pretreatment (Figure 2). There can be several possible explanations for this intriguing observation. First, although LPS was shown to down-regulate Cyp3a11 protein expression and activity, regulation of other CYPs might be involved in hepatic metabolism of midazolam. A recent study showed that midazolam was metabolized even in the *Cyp3a*$^{-/-}$ mouse model (van Waterschoot RA *et al.*, 2008). The authors reported that absence of Cyp3a11 was compensated by up-regulation of other CYP enzymes, such as CYP2C. Although LPS has been known to suppress CYP2C enzymes at the transcriptional level (Morgan, 1997), the role of LPS on CYP2C activity still remains unknown. In addition, a recent study showed a slight, but significant tendency of increase in

CYP2C activity in rats treated with LPS isolated from *E. coli* Nissle 1917 (Matuskova *et al.*, 2009). Midazolam is primarily metabolized by Cyp3a11; however, there is a significant contribution from Cyp2c11, which was studied using anti-Cyp2c11 antibody, in mouse liver microsomes (Perloff *et al.*, 2000; Warrington *et al.*, 2000). Thus, the reduction in midazolam metabolism by LPS or LTA in mice may also be due to reduced expression and activity of both Cyp3a11 and Cyp2c enzymes. It was recently shown that LPS treatment induced serum β-glucuronidase activity in rats (Shimoi *et al.*, 2001). β-glucuronidases are responsible for hydrolyzing hydrophilic conjugates such as luteolin glucuronide. β-glucuronidases can thereby play an essential role in either detoxification of reactive metabolites or in reducing the pharmacological action of active metabolites. During inflammation, β-glucuronidases, which are present in immune cells such as neutrophils and eosinophils, are released in blood (Marshall *et al.*, 1988). This leads us to suggest that the glucuronide conjugate of 1′-OHMDZ might be hydrolysed back to 1′-OHMDZ by the serum β-glucuronidases. However, in contrast to Shimoi *et al.*, (2001), we did not observe any change in serum β-glucuronidase activity in LPS-treated mice up to 8 h compared with saline-treated mice (data not shown). This can be possibly a species-dependent effect (rat vs. mouse) or the route of administration of LPS (i.v. vs. i.p.), which needs further evaluations.

Mostly, phase II metabolites are considered less active due to their hydrophilic properties, which leads to increased elimination. However, a clinical study reported that accumulation of 1′-OHMDZ-gluc led to increased sedation in patients with renal impairment (Bauer *et al.*, 1995). Therefore, we evaluated the PK profiles of 1′-OHMDZ-gluc in LPS- or LTA-treated mice. Interestingly, we observed a significant reduction in the C_{max} and AUC of 1′-OHMDZ-gluc in LPS- as well as LTA-treated mice (Figure 2) compared with saline-treated mice. Thus, further work will be required to elucidate the pharmacological potency of 1′-OHMDZ-gluc across species. As LPS-induced inflammation down-regulated the gene expression of several hepatic and renal UGT isoforms (Richardson *et al.*, 2006), the decreased AUC of 1′-OHMDZ-gluc could be attributed to down-regulation of hepatic UGTs.

In the PD studies, we observed ~2-fold increase in the sleep time in LPS-treated mice compared with saline-treated mice. Similar to the LPS study, compared with saline, the sleep time in LTA-treated mice was increased by ~1.5-fold. LPS treatment in mice was shown to be associated with decreased plasma protein content (Hartmann *et al.*, 2005). As midazolam is >97% protein-bound drug and only the unbound drug is pharmacologically active, the reduced protein binding of midazolam in LPS-treated mice can contribute to increased distribution and retention of midazolam in the brain. It is not known whether the decrease in the protein content in LTA-treated mice is responsible for the increased V_d of midazolam, suggesting a higher unbound fraction of the drug.

The fact that many psychotropic drugs are converted into active metabolites has major implications for PK/PD modelling. Usually, the metabolites of midazolam are considered less active due to their hydrophilic nature and consequent rapid elimination by the kidneys. However, several studies have also shown that 1′-OHMDZ is about 50% active as midazolam in producing the sedative–hypnotic effects in healthy volunteers (Ziegler *et al.*, 1983; Mandema *et al.*, 1992). Another clinical study also showed that prolonged sedation in ICU patients suffering with renal failure was correlated with significantly increased plasma levels of 1′-OHMDZ-gluc (Bauer *et al.*, 1995). Due to its short half-life and rapid conversion to the glucuronide conjugate, we examined the relationship between the plasma concentrations of 1′-OHMDZ and its glucuronide conjugate with the sleep time induced by midazolam administration in mice. The pharmacological effect of midazolam or its metabolites is determined by their ability to penetrate the blood–brain barrier, which in turn is dependent on their plasma protein binding and lipophilicity. As mentioned earlier, midazolam is >97% plasma protein bound and has an intermediate-to-high hepatic extraction ratio drug (ER = 0.3 to 0.7). Therefore, its metabolism is dependent on liver blood flow as well as protein binding. Previous *in vitro* and *in vivo* studies have shown that LPS treatment can decrease albumin synthesis by prevention of albumin transcription (Ruot *et al.*, 2000; Wang *et al.*, 2005). Since only the plasma unbound fractions of midazolam and its metabolites can penetrate the brain, any differences between plasma protein binding of midazolam and its metabolites can affect their relative *in vivo* potency. Midazolam is more lipophilic than its metabolites. We can therefore postulate that concentration ratio of midazolam/total metabolites in the brain, in the absence of any differences in plasma protein binding, is even greater than that found in plasma. This further supports the proposal that midazolam is the major contributor to the overall sedative activity observed. Also, our data show overall higher levels of plasma midazolam concentration in LPS groups as compared to saline. In the PK study with the lower dose (5 $mg \cdot kg^{-1}$), we observed very high levels of midazolam plasma concentrations in LTA-treated mice. Although we observed a significant increase in the PD effect of midazolam with LTA treatment, the AUC of midazolam in LTA group did not differ significantly from saline-treated mice at the higher dose (80 $mg \cdot kg^{-1}$). However, we observed a significant increase in the V_d and $t_{1/2}$ of midazolam in the LTA group compared with the saline group, which can possibly lead to prolonged sedation in mice. The increase in V_d and $t_{1/2}$ can be attributed to an increased unbound fraction of drug.

A key adaptor of the TLR signalling pathway, namely TIRAP, is known to regulate the inflammatory responses of LPS or LTA on cell surfaces of immune cells (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001; 2002). We recently showed that the gene expression of Cyp3a11 in mice was dependent on TIRAP in LTA-induced inflammation (Ghose *et al.*, 2011). However, TIRAP was not involved in regulating gene expression of Cyp3a11 in LPS-induced inflammation (Ghose *et al.*, 2008). In agreement with our previous studies, our results showed that TIRAP played a significant role only in LTA-, and not in LPS-mediated, down-regulation of Cyp3a11 protein expression as well as activity (Figures 4 and 5). This differential role of TIRAP in regulating LPS- or LTA-induced inflammation provides new approaches to counteract changes in drug metabolism during infection and inflammation.

In conclusion, we observed significant effects of the two inflammatory mediators (Gram-positive and Gram-negative bacterial endotoxins) on metabolic profiles of midazolam. Both LPS and LTA significantly altered the PK profiles of

midazolam and a differential role in regulating the PK profiles of 1′-OHMDZ. The plasma levels of 1′-OHMDZ-gluc were significantly reduced in LPS- as well as LTA-treated mice. In the PD study, both LPS and LTA played significant roles in increasing the pharmacological activity of midazolam. This is the first study demonstrating a detailed analysis of midazolam and its metabolites (phase I and phase II) during Gram-negative and Gram-positive acute phase response on the PK/PD of midazolam. We also showed that TIRAP played a significant role in the regulation of *in vitro* metabolism of midazolam by LTA. As about 50% of cases of sepsis and septic shock are caused by Gram-positive bacteria, examination of Cyp3a11 regulation by LTA may prove to be a useful tool to study the PK/PD of clinically relevant medications. These studies show that activation of TLR signalling pathways affect drug metabolism in mice. However, further studies are needed to investigate the effect of these signalling pathways on drug disposition in human diseases.

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Conflict of interest

The authors state no conflict of interest.

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