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How Uremic Toxins Alter Atorvastatin Disposition: Molecular Mechanisms of Inhibition of the Enzyme CYP3A4

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Background: In uremic patients, the accumulation of gut-derived protein-bound uremic toxins (PBUTs) induces changes in the microenvironment of the patients, leading to changes in the elimination pattern of drugs.

Aims: To assess ways in which PBUTs alter the CYP450 enzymes in hepatocytes as well as the possible effects of specific PBUTs on the metabolism and excretion of atorvastatin (ATV).

Study Design: An experimental study.

Methods: The experimental group was treated with long-term MHD for > 3 months, estimated-glomerular filtration rate (e-GFR) < 15 ml/min, normal Alb level (35.0-55.0 g/l), and no urine; the control group was not treated with hemodialysis, e-GFR < 60 ml/min, normal Alb level, and normal urinary excretion function. A suitable UPLC-MS/MS method was developed for detecting the concentration of 4-hydroxy ATV. Fresh primary hepatocytes were isolated from rats, and the uptake of ATV was tested in the uremic serum (US) group, IS group, and HA group and compared with that in the normal serum group. The metabolic status of ATV in the US group, IS group, and HA group was compared with that in the ATV group. RLM were extracted, and the metabolic experiment of ATV was performed in a human CYP3A4 model. The influence of UTs on pregnane X receptor (PXR)/nuclear factor kappa B (NF-κB) mRNA and the protein expression was also detected.

Results: IS and HA inhibited the ATV metabolism to varying degrees, wherein IS was the most potent inhibitor, producing > 50% inhibition. Meanwhile, the protein expression of CYP3A4 was downregulated after incubation with US, IS, and HA ($p < 0.01$). The excretion of ATV was also inhibited by 59.24% and 71.95% after incubation with IS and HA, respectively. The effects of uremic toxins on PXR/NF-κB mRNA and protein expression elucidated that PBUTs can inhibit ATV uptake and metabolism by exerting inhibitory effects on CYP3A4 through the PXR/NF-κB signaling pathway.

Conclusion: ATV metabolism could be significantly altered in the presence of uremic toxins, suggesting a downregulated effect on the ATV uptake, possibly through Oatp1b1, and also on the activity of CYP3A4 through the PXR/NF-κB signaling pathway.

INTRODUCTION

Chronic kidney disease (CKD), a growing global public health concern, is associated with a significantly high rate of morbidity, mortality, and needless medical costs. It has been estimated that 850 million people globally suffered from CKD in 2017 alone.[1-3] Kidney Disease: Improving Global Outcomes defines CKD as kidney damage or glomerular filtration rate (GFR) of < 60 ml/min/1.73 m^2 for > 3 months upon diagnosis.[4] Research has shown that the incidence of CKD in adults in China is 8.2%, with an estimated 82 million adults with CKD in mainland China from 2018 to 2019.[5] Renal replacement therapies, which are essential interventions to sustain patients with end-stage renal disease (ESRD), include hemodialysis (HD), peritoneal dialysis, and kidney transplantation.[3] The most prevalent type of renal replacement therapy worldwide is HD, which accounts for around 69% of all RRT and 89% of all dialysis cases.[6]

If the condition of CKD patients is not successfully managed, the disease progresses and eventually leads to ESRD. Due to a decrease or loss of GFR function, the renal clearance rate of retention solutes in the blood reduces, prompting these solutes to get stored and deposited in the body. These retention solutes are termed "uremic toxins," which are gut-derived normal by-products of metabolism that become harmful under conditions of uremia, as they are not

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getting eliminated from the body. The existing HD therapy can effectively remove small-molecule water-soluble uremic toxins such as creatinine, but the "photophilic" or the protein-bound uremic toxins (PBUTs) such as indoxyl sulfate (IS) and hippuric acid (HA) are not effectively removed by HD, as these are often closely associated with albumin or other plasma proteins in the circulation.[7] The accumulation of uremic toxins may cause significant alterations in pharmacokinetics and drug response, posing a major concern in terms of drug safety in patients with CKD.

HA is typically present in the urine and is widely used as a measure of renal clearance. Its body concentrations rise with the consumption of phenolic chemicals found in wine, fruit, and tea. It can also be produced by intestinal bacteria that metabolize aromatic amino acids. Regardless of their source, phenolic precursors are transformed into benzoic acid, which is then used by hepatic enzymes to produce hippurate. HA can cause free radical generation in kidney tubules by interacting with organic ion transporters. HA also increases systolic blood pressure, altering hemodynamics, and decreasing the GFR. HA damages proximal tubule cells by causing the synthesis of fibrotic proteins via nuclear factor-kappa B (NF-κB) activation. It causes interstitial fibrosis on the proximal tubule, which further accelerates the course of CKD. Finally, HA binds to human serum albumin, inhibiting its interaction with other chemical and inorganic compounds, changing their pharmacokinetics, and causing hazardous effects.[8] Similarly, IS is a well-known gut-derived uremic toxin and a promising therapeutic target. Indole is created by intestinal bacteria as a tryptophan degradation product. It is then absorbed and processed in the liver to form IS, which is the precursor of PBUTs. Increased intestinal concentrations of uremic toxins associated with CKD disturb intestinal barrier integrity and cause translocation of bacterial components and metabolites, thereby triggering intestinal and subsequent systemic inflammation and potentially speeding up the course of CKD.[9,10]

Atorvastatin (ATV) is a commonly used lipid-lowering drug in clinical practice. It is absorbed primarily in the intestine after oral administration and is transported into the liver via the portal vein on the surface of hepatocytes,[11] followed by CYP3A4-mediated metabolic inactivation. Because ATV is largely removed via non-renal clearance and unmodified drug excretion in the bile,[12] this alteration was believed to be caused by the effects of uremic toxins on hepatic uptake and/or efflux transporters. In 2019, the American Heart Association addressed safety concerns and related adverse events of statin medications, indicating that approximately 10% of all patients discontinue statin therapy due to muscle pain.[13] A retrospective cohort analysis reported that patients on high-dose ATV therapy likely have an increased risk of statin-related adverse effects.[14]

Pregnane X receptor (PXR) is an orphan nuclear receptor that is highly expressed throughout the gut, especially in the liver and intestines. It primarily regulates cytochrome *P4503A* gene expression in response to a wide range of ligands.[15] Past studies on the regulatory mechanisms of metabolism have discovered that PXR plays a significant role in controlling the expression of genes encoding drug metabolism enzymes and transporters.[16] Along with PXR, the NF-κB signaling pathway plays a key role in regulating the immune response to inflammation. NF-κB has been reported to be involved in the mechanisms of CYP regulation of transcription.[17] Through its ability to bind to response elements in the gene promoter regions of CYP enzymes, NF-κB can control the stability of CYP proteins.[18]

In this experiment, we explored the effects of two PBUTs (i.e., IS and HA) on the pharmacokinetics of ATV to provide theoretical support for predicting the interaction of PBUTs with CYP3A4. This report is important for establishing safe guidance and efficacious drug therapy in CKD.

MATERIALS AND METHODS

Materials

ATV and HA were purchased from Solarbio Co., Ltd. (Beijing, China). IS, p-hydroxy ATV (p-ATV), and p-hydroxy ATV-d5 (p-ATV-d5) were purchased from Clearsynth Co., Ltd. with > 97% purity. Krebs-Henseleit bicarbonate lyophilized powder, mineral oil, and silicon oil were purchased from Sigma-Aldrich (Shanghai, China). Recombinant human CYP3A4 (0.5 nmol P450 at 1 nmol/ml, 10 mg/ml protein) was purchased from Reid Liver Disease Research Co. (Shanghai, China). The CYP3A4 polyclonal antibody was obtained from Thermo Fisher Scientific (Shanghai, China). PXR polyclonal antibody and NF-κB polyclonal antibody were obtained from Bioss (Beijing, China). The p-p65 NF-κB antibody was procured from California, USA. RNA isolator Total RNA Extraction Reagent, HiScript II Q RT SuperMix, and AceQ quantitative polymerase chain reaction (qPCR) SYBR Green Master Mix were acquired from Vazyme (Nanjing, China). The Cell Counting Kit-8 was purchased from Boster (Wuhan, China).

Serum collection

Blood samples were collected from 60 patients who checked between May 1 and August 31, 2018. The patients signed an informed consent form and ethical approval (approval no.: 2021-GCP-007, date: 22.06.2021) was sought from the Ethics Committee of Sir Run Run Hospital, Nanjing Medical University. The patients were assigned to the following two groups: (1) the uremic group: patients with > 3 months of HD, estimated-glomerular filtration rate (e-GFR) < 15 ml/min/1.73 m^2, normal Alb, and anuria. (2) The control group: patients without HD, with e-GFR ≥ 60 ml/min/1.73 m^2, normal Alb, and normal urinary excretion function.

Exclusion criteria: (1) Patients who declined to sign the informed consent form. (2) Patients who were currently using or had used in the past medications that affected or could affect their CYP3A4 activity. (3) Patients with low Alb levels. (4) Patients with incomplete biochemical data indicators.

Blood samples were collected before MHD for the US group and in the morning after an 8 h fasting period for the control serum (CS) group. The samples were centrifuged for 10 min at 3,000 rpm to separate the serum from the blood and then stored at 80 °C.

Hepatocyte isolation and metabolism study

Fresh rat primary hepatocytes (fRPH) were extracted from male Sprague-Dawley rats by a two-step collagenase perfusion.[19] Once freshly isolated, the cells were kept in an ice bath until the metabolism experiments were performed and the entire process was carried out in < 6 h.

Four groups were prepared as follows: 10% normal serum as a control, 10% normal serum with 20 µm IS or 20 µm HA as PBUT groups and 10% uremic serum (US) group. Reaction buffers were dissolved in Krebs-Heinsleitt buffer and warmed to 37 °C with 100 µm ATV in each group under carbogen bubbling. The cell suspension (2 million cells/ml) was rewarmed to 37 °C and mixed into the same volume of reaction buffer (resulting in 0.5-1 million cells/ml final). After 30 min of incubation under gentle shaking, 0.5 ml of the reaction solution (containing 0.25-0.5 million cells) was removed and centrifuged through an isolation layer with different specific gravity to separate the reaction buffer from the cells and to stop any further reaction. After removing the supernatant layer, ice-cold acetonitrile with an internal standard was applied and centrifuged to induce a protein precipitate for LC-MS/MS analysis.

Microsomal incubations

The rats were anesthetized and the livers were perfused.[20] The digested liver was excised and transferred into the Tris-HCl buffer. The liver was sliced into small pieces, placed in a homogenizer, and added with Tris-HCl buffer (pH 7.4). Homogenization was performed at 0 °C. After low-speed centrifugation at 10,000 xg for approximately 20 min, the supernatant was combined, followed by ultracentrifugation at 100,000 xg for 60 min, and finally, the supernatant was discarded. The resulting precipitate was the rat liver microsomes (RLM). Instructions from the BCA Protein Concentration Assay Kit were used to determine the microsomal protein concentrations.

Briefly, isolated RLM (1.25 mg/ml) was incubated with 20 µm ATV in the absence of PBUTs in phosphate buffer. NADPH (1 mm) was added to start the reaction. After incubation at 37 °C, 200 L of the suspension was transferred into an EP tube containing 100 µl of 1 M dilute hydrochloric acid solution at 0 °C. After 30 s of cortexin, the solution was recentrifuged at 1,000 rpm at 4 °C for 60 and then rested at 0 °C. Aspirate (100 µl of the supernatant) was added into a new EP tube and stored at -80 °C. Isolated RLM were incubated with 20 µm of ATV along with 20 µm of IS, 20 µm of HA, or 10% of US in phosphate buffer.

The incubation of the recombinant human CYP450 enzyme (rhCYP450) was performed similar to that of RLM. For a short period, the enzyme suspensions were incubated with 20 µm of ATV in the absence of UTs in phosphate buffer and later incubated with 20 µm of ATV in the presence of 20 µm IS, 20 µm HA, or 10% US in phosphate buffer in a similar way.

Measurement of ATV and its metabolites

An electrospray-positive ionization mode Triple QuadTM 5,500 LC/MS/MS system (AB Sciex Pte. Ltd., Foster City, CA) was employed. For p-ATV, the multiple reaction monitor was programmed to transition at 575.3-440.3 m/z and p-ATV-d5 at 580.3-445.3 m/z. The collision energy was set at 28 eV for p-ATV and 29 eV for p-ATV-d5, while the ion spray voltage was set at 5,500 eV. An ACQUITY UPLC®BEH C18 (100 × 2.1 mm, 1.7 µm) column served as the analytical column. Acetonitrile (A) and water with 0.1% formic acid (B) constituted the mobile phase. The elution gradients were set as follows: A:B = 85:15 (v/v) in 0.01 min; A:B = 15:85 (v/v) in 0.50 min; A:B = 10:90 (v/v) in 1.80 min; A:B = 85:15 (v/v) in 2.01 min; A:B = 85:15 (v/v) in 2.50 min. The mass system was injected with aliquots (30 µl) at a flow rate of 0.4 ml/min.

RNA extraction, cDNA synthesis, and real-time PCR

The NF-κB and PXR mRNA expressions were measured using real-time qPCR. The Trizol-Based Protein Extraction Method was used to extract the total RNA from the cells. HiScript II Q RT SuperMix was used to prepare cDNA. The qPCR amplification procedures included 40 cycles of annealing and extension at 60 °C for 30 s and denaturing at 95 °C for 15 s. Utilizing this approach, the relative gene expressions were noted. GAPDH mRNA was used for housekeeping to normalize all mRNA levels.

Western blotting

A protease inhibitor (PMSF)-containing RIPA lysate was used to lyse fRPH. After the combination with 5× loading buffer, the isolated protein samples were boiled for 5 min. The SDS-PAGE technique was used to separate the proteins, which were then moved onto a nitrocellulose membrane. After blocking the membranes for 1.5 h with 5% free-fat milk in Tris-Buffered Saline and TWEEN® 20 (TBST) buffer, the membranes were incubated at 4 °C overnight with the addition of suitable primary antibodies. The membranes were then incubated with secondary antibodies for 1.5 h after rinsing thrice with the TBST buffer. An ECL detection kit was used to identify and visualize the immunoreactive proteins.

Statistical analysis

The data were displayed as the mean ± standard deviation values, and every experiment was conducted at least thrice. Statistical significance was assessed using SPSS 24.0 software (SPSS, Chicago, IL). The differences between the groups were compared through a One-Way analysis of variance (ANOVA); if the ANOVA revealed statistical significance, a t-test was later conducted. It was determined that $p < 0.05$ or 0.01 were statistically significant. The line graphs were created with GraphPad Prism 6.01.

RESULTS

Hepatocyte metabolism study and uremic toxins inhibited the metabolism of ATV

To determine the effect of PBUTs on the production of ATV metabolites, we incubated fRPH with 100 µm of ATV along with 10% US, 10% CS, 20 µm IS, and 20 µm HA for designated time points (1, 2, 5, 10, 20, and 30 min). According to Figure 1, US, IS, and HA inhibited the metabolism of ATV in hepatocytes. The p-ATV metabolite

was reduced to varying degrees after treatment with UTs when compared with the CS group. At 2, 5, 10, and 30 min, UTs showed a statistically significant inhibitory effect on ATV metabolism, with the highest inhibition occurring at 30 min. The concentrations of p-ATV in the IS and HA groups were 59.42% and 71.95% for those in the control group, respectively.

Microsomal study and uremic toxins inhibition on ATV metabolism

To further study the degree of CYP3A4 inhibition by UTs, we incubated RLM with 20 μm of ATV in the reaction buffers of 10% US, 20 μm IS, and 20 μm HA. The results revealed that US, IS, and HA inhibited the metabolism of ATV, and the concentration of p-ATV was quite lower than that of the control group, as shown in Figure 2a. After 5 min, the concentration of p-ATV in the IS group significantly decreased when compared with that in the control group, as depicted in Figure 2b. After 10 min, the concentration of metabolites in the HA group significantly decreased, similar to the IS group (Figure 2c). After incubation for 30 and 60 min, the concentrations of p-ATV in the US, IS, and HA groups further decreased as illustrated (Figure 2d, e). The concentrations of p-ATV in the IS and HA groups decreased at 10, 20, and 60 min, indicating that both IS and HA have an inhibitory effect on the metabolism of ATV ($p < 0.01$). Considering that differences between human and rat species can produce different results, we explored the influence of US along with IS and HA on rhCYP3A4 to confirm the inhibition of CYP3A4. Figure 3a depicts that the concentration of ATV metabolites in rhCYP3A4 increased with incubation time. As shown in Figure 3b-e, after incubation with US, IS, and HA for 5, 15, 30, and 60 min, the concentrations of p-ATV in the US, IS, and HA groups were reduced to different degrees compared with the CS group. IS inhibited ATV metabolism more than HA, demonstrating that uremic toxins had the propensity to inhibit the rhCYP3A4 activity. From this data, we assumed that PBUT monomers may competitively inhibit ATV metabolism.

Protein expressions of CYP3A4 on hepatocytes

To validate the obtained effects of US and UTs on CYP3A4, we assessed the protein expressions of CYP3A4 on fRPH treated with US, IS, and HA. As described in Figure 4, the protein expression of CYP3A4 was reduced by 38.00% ($p < 0.01$) after incubation with US when compared with the CS group. In the presence of HA, CYP3A4 protein expression was reduced by 45.81% ($p < 0.01$), and, in the presence of IS, it was reduced by 8.15% ($p < 0.01$). According to this result, HA appears to inhibit CYP3A4 expression more strongly in fRPH.

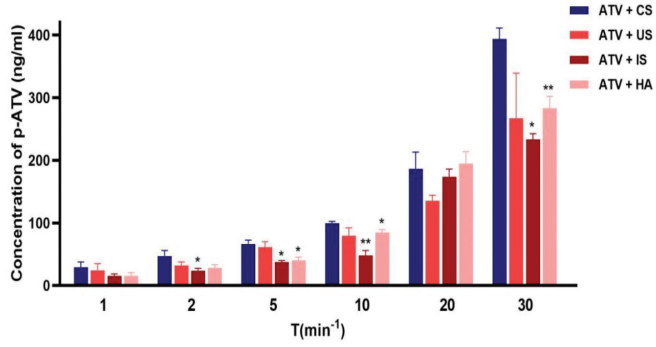

FIG. 1. Uremic toxins reduced the concentration of p-ATV in primary rat hepatocytes. After administration to fresh rat primary hepatocytes for 1, 2, 5, 10, 20, and 30 min at 37 °C, the cells were separated by oil filtration and the supernatant was used to detect the metabolites.

*p-ATV, p-hydroxy-atorvastatin; CS, control serum; US, uremic serum; IS, indoxyl sulfate; HA, hippuric acid; average ± standard deviation. *p < 0.05, **p < 0.01.*

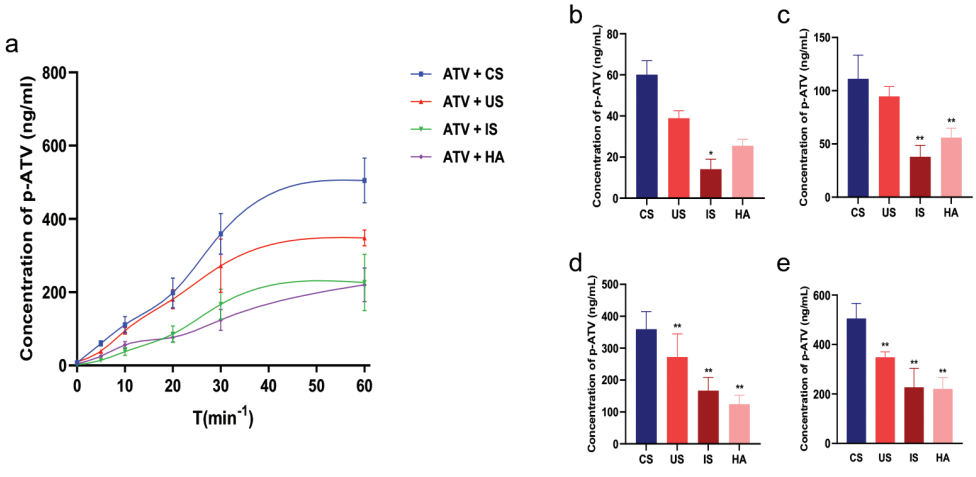

FIG. 2. Uremic toxins inhibited ATV metabolism in microsomes (a). Microsomes were treated with 10% US, 20 μm IS, 20 μm HA, and 20 μm ATV. After 5 min (b), 10 min (c), 30 min (d), and 60 min (e) of treatment, the metabolic reaction tended to terminate and the concentrations of p-ATV were significantly reduced in the IS, HA, and US groups (b).

*ATV, atorvastatin; CS, control serum; US, uremic serum; IS, indoxyl sulfate; HA, hippuric acid; average ± standard deviation, *p < 0.05, **p < 0.01.*

Effect of US, IS, and HA on the expression of PXR and NF-κB p65 genes in primary rat hepatocytes

PXR and NF-κB p65 mRNA expressions in fRPH exposed to US, IS, and HA revealed that PXR mRNA was downregulated following US, IS, and HA stimulation (Figure 5a). Conversely, IS and US increased the NF-κB p65 mRNA levels although HA had no discernible effect (Figure 5b).

Effect of US, IS, and HA on the expression of PXR and p-NF-κB p65/NF-κB p65 proteins

The influence of US, IS, and HA on PXR and p-NF-κB p65/NF-κB p65 protein expression was investigated. The cells were incubated with 10% US, 200 μm IS, and 200 μm HA. As shown in Figure 6a, US, IS, and HA significantly decreased PXR expression compared with the CS group ($p < 0.01$). In the presence of 200 μm IS, the protein expression of PXR was 51% ($p < 0.01$) of the control. The p-NF-κB p65/NF-κB p65 expression was upregulated after 10% US, 200 μm IS, and 200 μm HA incubation, with more than a 5-fold increase in the IS group (Figure 6b).

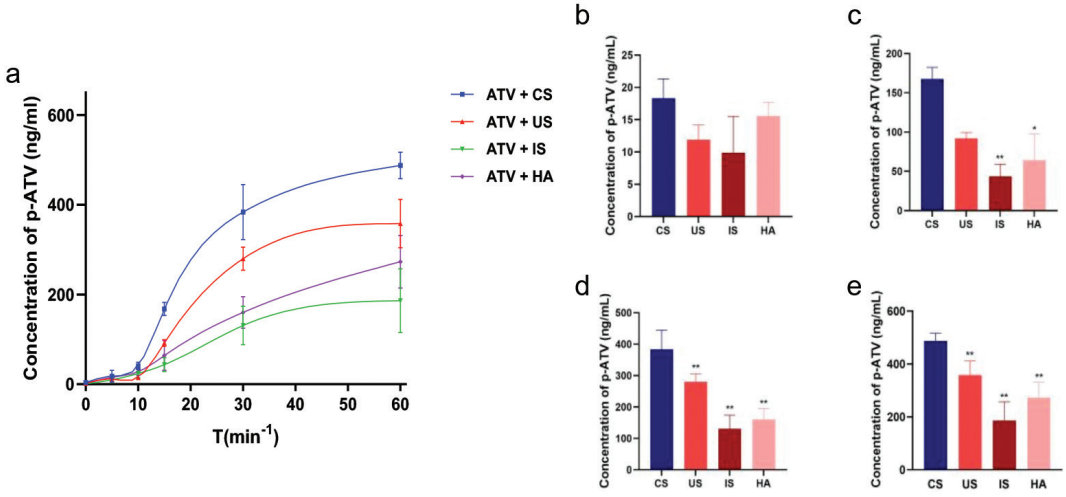

FIG. 3. ATV metabolism was inhibited after incubation with uremic toxins in rhCYP3A4 (a). After treatment with 10% US, 20 μm IS, 20 μm HA, and 20 μm ATV for 5 min (b), 15 min (c), 30 min (d), and 60 min (e), the metabolic reaction tended to terminate and the concentrations of p-ATV were significantly reduced in IS, HA, and US groups.

*ATV, atorvastatin; p-ATV, p-hydroxy-atorvastatin; CS, control serum; US, uremic serum; IS, indoxyl sulfate; HA, hippuric acid; average ± standard deviation, *p < 0.05, **p < 0.01.*

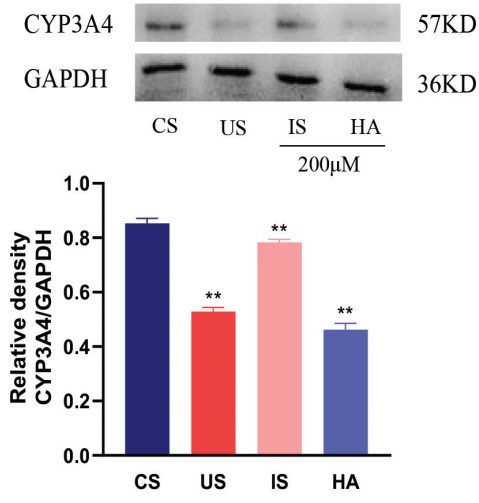

FIG. 4. Uremic toxins downregulated the protein expression of CYP3A4. Total protein was extracted from primary rat hepatocytes after administration for 24 h, and the protein expression of CYP3A4 in cell lysates was assessed by Western blotting. Protein expressions were quantified by ImageJ. Data were normalized with GAPDH.

*CS, control serum; US, uremic serum; IS, indoxyl sulfate; HA, hippuric acid; average ± standard deviation, **p < 0.01.*

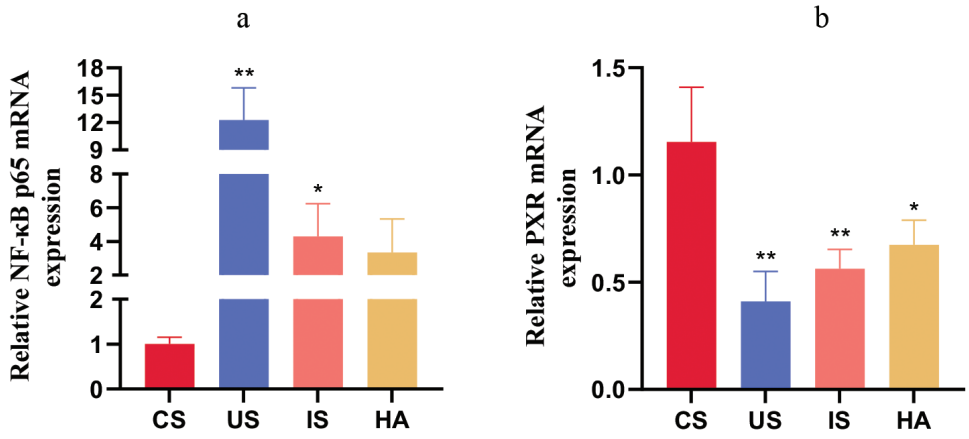

FIG. 5. Effects of US and uremic toxins on NF-κB p65 and PXR mRNA. Total RNA was extracted from primary rat hepatocytes after 24 h of administration, and the cDNA was synthesized for amplification. All mRNA levels were normalized to GAPDH mRNA.

*CS, control serum; US, uremic serum; IS, indoxyl sulfate; HA, hippuric acid; NF-κB, nuclear factor kappa B; PXR, pregnane X receptor; average ± standard deviation, *p < 0.05, **p < 0.01.*

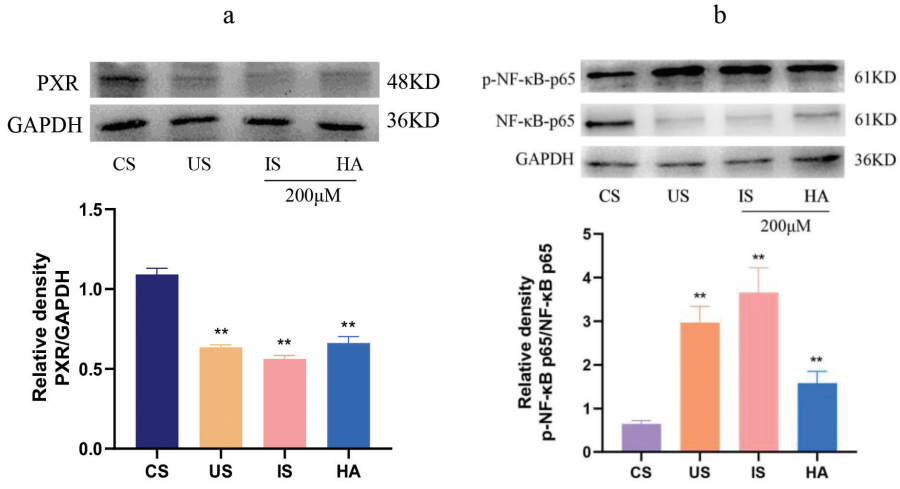

FIG. 6. Uremic toxins downregulated the protein expression of PXR and p-NF-κB p65/NF-κB p65. Total protein was extracted from primary rat hepatocytes after 24 h of administration, and the protein expression of PXR, p-NF-κB, and NF-κB in cell lysates was assessed by Western blotting. Protein expressions were quantified by ImageJ. Data were normalized with GAPDH.

*CS, control serum; US, uremic serum; IS, indoxyl sulfate; HA, hippuric acid; NF-κB, nuclear factor kappa B; PXR, pregnane X receptor; average ± standard deviation, **p < 0.01.*

DISCUSSION

As a global health problem, CKD has attracted a great deal of attention. When this disease progresses to ESRD, some patients require maintenance hemodialysis (MHD). Uremic toxins are often present in ESRD patients, with water-soluble small-molecule solutes and middle-molecules being almost eliminated by MHD,[21] whereas PBUTs were retained.[22] PBUTs accumulate in the circulation and tissues, which can increase the risk of accelerated CKD progression and cardiovascular diseases.[23] Currently, the pharmacokinetics of drugs in the body are most likely to vary, increasing the incidence of drug-related adverse reactions. Therefore, studying the variables influencing the in vivo administration of drugs to patients with MHD seems interesting.

Several experimental studies have shown that the clearance process of non-really cleared drugs is somewhat altered in patients with CKD.[24] Most of these drugs act as substrates for drug-metabolizing enzymes or drug transporters associated with non-renal clearance. It has been shown that people with CKD have altered non-renal clearance of over 75 routinely used medicines, the majority of which are removed by oxidative metabolism mediated by the CYP450 enzyme.[25] Previously, digoxin and erythromycin, both of which are excreted in the bile, have been demonstrated to have their uptake,

transport, and metabolism altered by uremic toxins.[26,27] Similarly, since ATV undergoes unchanged excretion in the bile and is a substrate for CYP450 enzymes like the abovementioned two drugs, it was probable to assume how the disposition of ATV could be altered in the presence of retention solutes. IS and HA can strongly bind to plasma proteins and are frequently studied in the context of the alteration of drug metabolism. Previously, both are and HA were revealed to alter the disposition and elimination of digoxin and erythromycin. With the richness of data in the literature on these two UTs, we included these UTs in our study as ATV probably reflects a change in its pharmacokinetics given the context of its enzyme metabolism profile.[26,27]

The effect of UTs on ATV was investigated at the subcellular level in RLM and fRPH as model proof-of-concept systems. Owing to the scarcity, high costs, and limited supply of human primary hepatocytes for research, fRPH and RLM are often employed in research to compensate for the lack of relevant liver data. Humans have a non-lobated liver, which makes tissue excision difficult, while rodents have lobated livers, with individual lobes easily ligated and removed.[28] Moreover, human primary hepatocytes cannot be cultivated in vitro.[29] In addition, the functionality and microarchitecture of the rat liver are very similar to that of humans. Our results showed that the US and UT monomers, IS and HA, inhibited ATV metabolism in RLM. Because ATV is a substrate of CYP3A4,[30] uremic toxins may inhibit CYP3A4 activity, resulting in metabolic inhibition. Although human liver microsomes and RLM are similar, there are undeniable differences that could lead to differences in drug metabolites and metabolic pathways between these two species. This study used an in vitro model of rhCYP3A4 metabolism to reduce errors induced by species differences. The results revealed the inhibition of drug metabolism, demonstrating the inhibition of CYP3A4 by uremic toxins, especially PBUTs. Furthermore, Western blotting results showed that US, IS, and HA inhibited CYP3A4 protein expression. The results obtained in our study were fairly similar to those of studies conducted on digoxin and erythromycin, suggesting a possible effect of UTs on CYP enzymes.

PXR has previously been known to be involved in ATV metabolism because ATV metabolites are ligands of PXR.[31] In addition, we evaluated the effects of uremic toxins on PXR and NF-κB P65 expressions. The results suggested that US, IS, and HA can inhibit the PXR mRNA and protein expression. The NF-κB p65 mRNA and p-NF-κB p65/NF-κB p65 protein expression was upregulated after treatment with 10% US, 200 μm IS, and 200 μm HA. PXR is a nuclear receptor that can regulate phase I metabolic enzymes, phase II metabolizing enzymes, MDR1, and MRP2.[32] PXR can be activated by a wide range of exogenous ligands and it binds to the transcriptional binding sites of drug-metabolizing enzymes and transporters so as to regulate the expression of these two proteins. In primary cultures of endothelial cells obtained from epileptic brain tissues, PXR expression significantly increased and resulted in some submissions of CYP overexpression as well as PXR silencing, which resulted in the specific downregulation of CYP3A4 expression.[33]

In recent years, several scholars have suggested that inflammation can affect changes in metabolic enzymes and transporters in the drug body and that there is a relationship between NF-κB and nuclear receptor proteins.[34,35] Moreover, ATV is known to have the ability to reduce inflammation by inhibiting both NF-B activity and chemokine gene expression.[36] CKD is often accompanied by persistent local and systemic inflammation, as characterized by an increased production of multiple inflammatory mediators.[37] In the context of uremic toxins, the alteration of the expression of NF-κB levels can lead to increased inflammation and worsening of CKD as well as CVD conditions.

Our study is limited by the fact that the extraction of human hepatocytes, being a robust process, was reserved for future studies on this subject. With a plethora of PBUTs, this study observed the possible effects of only two uremic toxins, IS and HA, with the convenience of previous evidence on their activity. A deeper understanding of the molecular processes through which uremic toxins possibly alter the pharmacokinetics and, ultimately, the response of medications in patients with CKD would be possible with additional research that assimilates the studies conducted in human extracted hepatocytes or human hepatocyte cell lines. Another important aspect that can be considered in future studies is the measurement of the levels of uremic toxin concentrations in the US. It is expected to provide further data into the mechanisms of uremic toxin-to-drug interactions. The US used in this study came from patients with long-term MHD. The PBUTs are difficult to clear or isolate through the existing laboratory methods or MHD. As stated earlier, IS and HA are the main PBUTs; therefore, this study specifically studied how IS and HA impacted ATV drug metabolism, which is why we tested them separately.

Since specific drug literature is scarce in terms of the influence of uremic toxins on the pharmacokinetics of drugs, the present assessed the elimination profile of ATV in the context of UTs. Moreover, the gene and protein expression of PXR was downregulated, but the NF-κB p65 mRNA, NF-κB, and p65 protein expression were upregulated, suggesting an inhibitory effect of uremic toxins on the activity of CYP3A4 through the PXR/NF-κB signal pathway. Understanding how uremic toxins may impact the drug metabolism of specific drugs, specifically the non-renal elimination drugs, is another useful strategy for adjusting treatment strategies and improving the treatment outcomes in CKD patients.

Ethics Committee Approval: This study was approved by the Ethics Committee of Sir Run Run Hospital, Nanjing Medical University (approval number: 2021-GCP-007, date: 22.06.2021).

Informed Consent: Informed consent was obtained.

Data Sharing Statement: The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

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