β-Naphthoflavone protects mice from aristolochic acid-I-induced acute kidney injury in a CYP1A dependent mechanism

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Aim: The role of CYP1A in the protection of aristolochic acid (AA)I-induced nephrotoxicity has been suggested. In the present study we investigated the effects of β-naphthoflavone (BNF), a non-carcinogen CYP1A inducer, on AAI-induced kidney injury.

Methods: Mice were pretreated with 80 mg/kg BNF by daily intraperitoneal injection (ip) for 3 days followed by a single ip of 10 mg/kg AAI. AAI and its major metabolites in blood, liver and kidney, the expression of CYP1A1 and CYP1A2 in microsomes of liver and kidney, as well as the nephrotoxicity were evaluated.

Results: BNF pretreatment prevented AAI-induced renal damage by facilitating the disposal of AAI in liver. BNF pretreatment induced the expression of CYP1A1 in both liver and kidney; but the induction of CYP1A2 was only observed in liver.

Conclusion: BNF prevents AAI-induced kidney toxicity primarily through CYP1A induction.

Keywords: aristolochic acid; kidney injury; beta-naphthoflavone; biotransformation; CYP1A

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**Materials and methods**

**Chemicals**
AAI was purchased from Sigma Chemical Co (St Louis, MO, USA) and BNF was from Merk & Co (Merck Schuchardt OHG, Hohenbrunn, Germany). Aristolactam I (ALI) was a gift from Dr Ming-hua XU (Shanghai Institute of Materia Medica).

**Animal experiments**
Male C57BL/6 mice (6 weeks old, 18–22 g) were obtained from Shanghai Laboratory Animal Center. All animal experiments were approved by the Shanghai Animal Care and Use Committee [Certificate No.SCXK (Shanghai) 2002-0010]. Animals were divided into three groups (n=15) for different treatments including AAI group (mice receiving corn oil (CO) via intraperitoneal injection (ip) daily for 3 d followed by a single ip of 10 mg/kg AAI prepared in saline) 24 h after the last injection of CO, BNF+AAI group (mice receiving 80 mg/kg BNF prepared in CO daily for ip 3 d followed by a single ip of 10 mg/kg AAI 24 h after the last injection of BNF) and control group (mice receiving CO ip daily for 3 d followed by a single ip of saline 24 h after the last injection of CO). Serum biochemistry and histopathology were performed on d 3, 7, 14 after last injection. Serum urea nitrogen (BUN) and creatinine (CRE) were measured by an automatic HITACHI Clinical Analyzer Model 7080 (Hitachi High-Technologies Corporation, Tokyo, Japan). For the preparation of microsomes and mRNA from tissues, mice were treated with BNF or CO (n=5) via ip daily for 3 d and tissues (liver and kidney) were harvested 24 h after the last injection.

**Histopathology**
Kidneys were collected at indicated time points and fixed in 10% formalin solution before being embedded in paraffin for sections (3 μm thick). Sections were stained with hematoxylin and eosin (H&E) by a standard pathology procedure[23], and then evaluated by a pathologist. Immunostaining of α-SMA was performed by using the anti-mouse α-SMA antibody (1:800, Sigma, St Louis, MO, USA) and avidin-biotin-peroxidase complex (ABC) method described previously[24]. The brownish-color was considered to be evidence of a positive expression of α-SMA in the interstitial cells. Stained vascular cells were considered as positive internal controls. For the morphometric evaluations, three random and nonoverlapping areas (0.125 mm² per area) were chosen and the quantification of area of positively stained cells was carried out using Leica image analyzing software (Leica Qwin). In situ TUNEL assay (Roche Diagnostics, Indianapolis, IN, USA) was performed according to the supplier’s instructions. Briefly, deparaffinized sections were labeled with TdT and biotinylated dUTP, and then were examined under a fluorescence microscope. Approximately, 3000 nuclei in five 200× fields were counted.

**Detection of AAI and its major metabolites in blood, liver and kidney**
For the determination of AAI concentrations, blood samples were collected by tail bleeding at various time points after a single ip of 10 mg/kg AAI. Blood samples (20 μL each) were collected in heparin-coated capillaries and were mixed with an equal volume of saline. The samples were spun at 4000×g for 5 min at 4 °C. Tissue samples were homogenized in saline, the supernatant was removed and spun at 14000×g for 10 min, and then the supernatants were mixed with one-half volume of methanol and spun again at 14000×g for 5 min to remove precipitated proteins. Aliquots of the final supernatants were analyzed and quantified for the levels of AAI and the metabolites AAs and ALI by HPLC.

**HPLC analysis**
The quantification of AAI and its metabolites in the samples was performed on an HPL100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). HPLC/UV analysis was carried out using a Welchrom™ XB-C18 column (5 μm, 4.6×250 mm; Welch Materials, MD, USA) at a flow rate of 0.8 mL/min. An isocratic mobile phase of methanol: 0.1% acetic acid in H2O (7:3) was used for separation. The UV detector was set at 250 nm.

For AAI, the linear ranges of the calibration curves were 0.8–40 μg/mL in serum and 0.1–10 μg/mL in liver and kidney, the regression equation was $y=46478x−15390 \quad (r^2=0.9964)$, $y=48044x−1973.5 \quad (r^2=0.9985)$ and $y=47845x−6422.8 \quad (r^2=0.9956)$, respectively, where $y$ is the peak area and $x$ is the concentration of analyte. The intra and inter-day precisions referred by relative standard deviation (RSD) were less than 5.35% and 12.43% for serum samples, less than 4.71% and 8.61% for liver samples, as well as less than 5.21% and 6.18% for kidney samples. The average percentage recoveries were no less than 70.88%, 84.53%, and 90.01% for serum, liver and kidney samples, respectively.

For ALI, the linear ranges of the calibration curves were 0.1–10 μg/mL in liver and kidney, the regression equation was $y=39991x−936.4 \quad (r^2=0.9986)$ and $y=40231x−6718.2 \quad (r^2=0.9919)$, respectively. The intra and inter-day precisions referred by relative standard deviation (RSD) were less than 7.84% and 14.89% for liver samples, as well as less than 5.07% and 12.32% for kidney samples. The average percentage recoveries were no less than 79.36% and 88.64% for liver and kidney samples, respectively.

In addition, LC-MS/MS analysis was conducted on an Agilent 6300 LC/MSD Trap XCT Ultra (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) to confirm and characterize AAI and its metabolites. The detect conditions refered to Xiao et al[29].

**Real-time RT-PCR**
Total RNA was isolated by using UNIQ-10 column & TRIZOL total RNA isolation kit (Sangon Biotech Co, Shanghai, China). One microgram of total RNA was used for reverse transcription in a reaction volume of 20 μL using Cloned AMV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Two microliters of cDNA was used for real time PCR using TaKaRa Ex Taq R-PCR Version 2.1 kit (TaKaRa Biomedicals Inc, Shiga, Japan). Gene-specific PCR primers for CYP1A1, CYP1A2,
CPR, and β-actin were listed in Table 1 and PCR signal was detected with a DNA Engine Opticon 2 Continuous Fluorescence Detection System (Bio-Rad Laboratories, Inc, CA, USA). PCR was monitored for 45 cycles with annealing temperature at 60 °C. At the end of the PCR cycles, melting curve analysis and 2% agar electrophoresis was performed to assess the purity of the PCR products. Negative control reactions (no template) were routinely included to monitor potential contamination of reagents. Standard curves were generated for CYP1A1, CYP1A2, CPR, and β-actin, respectively. Relative amounts of CYP1A1, CYP1A2, and CPR mRNA were normalized to β-actin mRNA.

| Table 1. Primers for real-time PCR. |
|---|---|---|
| Primer | Sequence (5’–3’) | Product size |
| CYP1A1 Forward | GACCCTTACAAGTATTTGGTCGT | 145 bp |
| Reverse | GGATCCAGACGCTCACTTT | |
| CYP1A2 Forward | CCAGGTGGTGGAATCGGTG | 194 bp |
| Reverse | TCTTAAACCTCTTGAGGGCCG | |
| CPR Forward | ATGGGGGACTCTCACGAAGAC | 169 bp |
| Reverse | TCTTGCTGAACTCCGGTATCTC | |
| β-Actin Forward | GAGATTACTGCTCTGGTGAGAC | 146 bp |
| Reverse | TCATCGTACTCCTGCTTGCTG | |

Western blotting analysis
The concentration of protein extracts from mouse hepatic or renal microsomes was determined using BCA kit (Pierce, Rockford, IL, USA) and 30 μg protein lysates were separated on 10% SDA-PAGE followed by being transferred to nitrocellular membrane. Western-blotting analysis was performed as described[23] and the signal was detected using an ECL system (Amersham, Piscataway, NJ). Antibodies used in this study included monoclonal anti-mouse CYP1A1 (1:100; Santa Cruz, California, USA), sheep anti-rat CYP1A2 (1:1000; Chemicon, Temecula, CA, USA), and polyclonal rabbit anti-mouse cytochrome P450 reductase (1:1000; Abcam, Cambridge, UK).

Statistical analysis
All Data are expressed as mean±SD. Statistical differences were determined by Student’s t-test and one-way ANOVA followed by a Tukey post-hoc test. The criterion for statistical significance was P<0.05.

Results
BNF protects mouse from AAI-induced renal damages
The conventional renal function index, such as the serum BUN and CRE, increased markedly at day 7 and then decreased to normal level at day 14 in the AAI group. BNF pretreatment significantly reduced the AAI-induced increase of BUN and CRE with no difference compared to those in control group (Figure 1).

The progress of the lesions in kidney after AAI administration was observed by histopathological examinations. Slight tubular dilation in kidney was seen after 3 days of AAI injection. Then, lesions of extensive tubular necrosis, tubular dilation and massive granular and hyaline cast occurred after 7 days of AAI administration. Lesions characterized by tubular dilation (atrophy) along with discrete tubular necrosis and slight interstitial fibrosis were seen in kidney after 14 days of AAI injection. Simultaneously, kidneys from mice of BNF+AAI group displayed much less lesions (Figure 2).

Further analysis by using TUNEL assay indicated that the renal damage was associated with induction of apoptosis, an early event after AAI administration (Figure 3A and 3C). BNF pretreatment greatly inhibited AAI-induced apoptosis in tubular epithelium cells. In addition, immunohistochemistry analysis revealed that BNF prevented AAI-induced tubulointerstitial fibrosis by inhibiting AAI-induced α-SMA expression, a phenotypic marker of myofibroblasts (Figure 3B and 3D). Together, these results demonstrate that BNF protects mice against AAI-induced renal damage.

AAI was rapidly metabolized in BNF pretreated mice.
We next studied the pharmacokinetics of AAI and its metabolites in vivo. BNF pretreatment markedly decreased the exposure level of AAI in mice as indicated by much lower value of pharmacokinetic parameters such as Cmax, tmax and AUC of AAI in BNF+AAI group than that in AAI group following a single ip of AAI at 10 mg/kg (Figure 4A, Table 2). Simulta-
neously, the level of AAIa, the major metabolite of AAI, was higher in mice in the BNF+AAI group than that in AAI group (Figure 4B). These results suggest that BNF-pretreatment can enhance the metabolism of AAI in vivo.

Figure 2. Kidney histological features of mice with AAI injection. Mice were sacrificed at indicated time after AAI administration. Kidneys were collected to perform H&E staining on (A) day 3, (B) day 7 and (C) day 14. Arrowheads, tubular dilation; stars, tubular necrosis and granular cast; pound sign, hyaline cast; arrows, mineralization. Scale bar, 50 μm.

Figure 3. Immunohistochemical staining of kidney from AAI-treated mice. (A) In situ TUNEL staining after 3 days of AAI injection. Green fluorescences (arrows) were observed as positive staining of apoptotic cells. Magnification, ×200. (B) Immunostaining of α-SMA after 7 days of AAI injection. Expression of α-SMA in tubulointerstitium (arrows) was indicated, and staining on vessels (arrowheads) was shown as an internal positive control, scale bar, 50 μm. (C) Scoring of apoptotic cells. (D) Scoring of expression of α-SMA. Data are expressed as mean±SD. n=5. *P>0.05, **P<0.05, ***P<0.01 vs control group; †P<0.01 vs AAI group by ANOVA test.
BNF reduced the accumulation of AAI in kidney

To examine whether the change in pharmacokinetics of AAI upon BNF pretreatment was due to the change in the tissue distribution of AAI, AAI and its major metabolites in liver and kidney was measured by HPLC (Figure 5). Thirty minutes after a single ip of AAI at 10 mg/kg, the level of AAI in kidney was about 3-fold higher than that in liver and BNF pretreatment resulted in a 3-fold reduction in the level of AAI in both liver and kidney (Figure 5A). The level of AAIa was higher in liver than that in kidney and was not significantly affected by BNF pretreatment (Figure 5B). Another metabolite, ALI, was about 4-fold higher in kidney than that in liver and BNF pretreatment decreased ALI formation in both liver and kidney (Figure 5C). Together, these results suggest that BNF pretreatment decreases the accumulation of AAI in kidney of mice.

Table 2. Comparison of pharmacokinetic parameters between mice in AAI group and that in BNF+AAI group. n=5. Values are expressed as mean±SD.  ①P<0.05, ②P<0.01 vs AAI group.

| Groups         | Cmax (μg/mL) | tmax (min) | AUC (min·μg/mL) | t1/2 (min) |
|----------------|--------------|------------|-----------------|------------|
| AAI            | 8.73±1.57    | 16.00±5.48 | 418.37±126.09   | 27.55±3.37 |
| AAI+BNF        | 3.08 ±0.61   | 6.25±2.50  | 149.84±20.30    | 31.46±8.80 |

The expressions of CYP1A1 and CYP1A2 were different in liver from that in kidney from BNF-pretreated mice

To investigate the discrepancy of liver and kidney in metabolizing AAI, we then examined if BNF regulated the expression of CYP1A1 and CYP1A2 differentially in mice. After BNF treatment, the mRNA levels of CYP1A1, CYP1A2, and CPR in liver was increased about 4-fold, 16-fold, and 3-fold, respectively, compared to vehicle-treated group. However, in kidney, only CYP1A1 mRNA levels showed a 2-fold increase after BNF treatment (Figure 6A, 6B and 6C). Similarly, Western-blotting analysis revealed that protein levels of CYP1A1, CYP1A2 and CPR in liver was greatly upregulated after BNF treatment, but only CYP1A1 protein was induced in kidney (Figure 6D). These results suggest that BNF induces the expressions of both CYP1A1 and CYP1A2 in liver, but only CYP1A1 in kidney.

Discussion

AAN is a unique disease characterized by prominent tubu-
lar atrophy and interstitial fibrosis\cite{26, 27}. Up to now, therapeutic strategies have been mainly conservative when renal insufficiency is detected\cite{10}. Angiotensin-converting enzyme inhibitors did not modify the evolution of the renal disease\cite{28}. Previous studies have investigated possible mechanisms of AA-induced nephrotoxicity both in vitro and in vivo and have suggested the role of AAI-induced apoptosis in renal tubular cells through either the endoplasmic reticulum (ER) stress pathway or the mitochondrial cell death pathway\cite{29, 30}. In this study, BNF pretreatment prevented the tubular apoptosis, tubular necrosis and tubulointerstitial fibrosis, along with improved renal function by increasing the metabolism of AAI so as to decrease its accumulation in kidney. Our results also suggest that CYP1A enzyme is critical during this process.

AAIa and ALI were the major metabolites of AAI formed in liver and in kidney, respectively. After BNF treatment, both AAI and ALI were decreased, but AAIa was unchanged in either liver or kidney, compared to the vehicle-treated mice. The lack of increase in AAIa raises the possibility that AAIa might have undergone an extensively phase II conjugation and was readily eliminated from the body\cite{31}. Furthermore, considering AAIa was much less cytotoxic and mutagenic than AAI\cite{14, 32}, we speculate that the formation of AAIa by CYP1A plays a role in detoxification of AAI in vivo in contrast to its toxicity in vitro. The discrepant roles of CYP1A in vivo and in vitro have been shown before\cite{33–35} and our study supports that CYP1A is cytoprotective rather than cytotoxic in vivo\cite{36}.

In summary, BNF can increase the metabolism of AAI to AAIa by inducing CYP1A to decrease the accumulation of AAI in kidney, and subsequently protect AAI-induced nephrotoxicity.

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**Author contribution**

Ying XIAO, Xiang XUE, Li-kun GONG, and Jin REN designed the experiment; Xiang XUE, Ying XIAO, Yuan-feng WU and Guo-zhen XIN performed research; Yong QIAN and Tian-pei XIE contributed new analytical tools and reagents; Xiang XUE and Ying XIAO analyzed data; Xiang XUE and Ying XIAO wrote the manuscript. Li-kun GONG and Jin REN revised the manuscript.

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**Figure 6.** CYP1A1, CYP1A2, and CPR mRNA and protein levels in liver and kidney. Tissues were collected at 24 h after the last injection from mice of CO and BNF group. Microsomes were prepared and RNA was extracted. (A) CYP1A1, (B) CYP1A2 and (C) CPR mRNA by real-time PCR analysis are shown. n=5. Data are expressed as mean±SD. *P<0.05, **P<0.01 vs control group. ***P<0.01 vs the level of mRNA in liver. (D) Western blotting analysis of CYP1A1, CYP1A2, and CPR proteins.
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