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Amentoflavone is a potent broad-spectrum inhibitor of human UDP-glucuronosyltransferases

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ABSTRACT

Amentoflavone (AMF), an abundant natural biflavonoid found in many medicinal plants, displays various beneficial effects including anti-inflammatory, anti-oxidative and anti-cancer. Despite the extensive studies on pharmacological activities, the toxicity or undesirable effects of AMF are rarely reported. In this study, the inhibitory effects of AMF on human UDP-glucuronosyltransferases (UGTs) were carefully investigated. AMF displayed strong inhibition towards most of human UGTs including UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4 and 2B17, with the IC_{50} values ranging from 0.12 μM to 16.81 μM. Inhibition constants (K_i) of AMF against various human UGTs varied from 0.29 μM to 11.51 μM. Further investigation demonstrated that AMF was a noncompetitive inhibitor of UGT1A1 mediated NCHN-*O*-glucuronidation but functioned as a competitive inhibitor of UGT1A1 mediated 4-MU-*O*-glucuronidation. In addition, AMF was a competitive inhibitor of UGT1A4 mediated TFP-*N*-glucuronidation in both UGT1A4 and human liver microsomes, while functioned as a competitive inhibitor of UGT1A9 mediated propofol or 4-MU-*O*-glucuronidation. These findings demonstrated that AMF was a strong and broad-spectrum natural inhibitor of most human UGTs, which might bring potential risks of herb-drug interactions (HDIs) *via* UGT inhibition. Additionally, this study provided novel insights into the underlying mechanism of AMF-associated toxicity from the perspective of UGT inhibition.

1. Introduction

Amentoflavone (AMF), a natural biflavonoid compound distributed in *Selaginella tamariscina, Cupressus funebris*, *Ginkgo biloba* and *Hypericum perforatum,* possesses a variety of biological activities including anxiolytic, anti-inflammatory, anti-oxidative, anti-cancer, and neuroprotective effects [1–5]. In contrast to the extensive studies on its pharmacological effects, the toxicity and side effects of AMF are rarely reported. Ten years ago, a case report has reported that oral administration of *C. funebris* extract would trigger acute hepatic failure and acute renal failure, while AMF was the major biflavonoid in this extract [6]. This finding suggests that constitutes from *C. funebris* may bring some toxic effects, but the underlying mechanism of *C. funebris* extract induced acute hepatic/renal failure has not been revealed yet. In addition, several studies have reported that AMF can strongly inhibit the catalytic activities of many human cytochrome P450 enzymes (CYPs) including CYP2C9, 2C19, 2D6 and 3A4 [7,8]. These studies suggest that AMF can affect the metabolic elimination of many CYP-substrate drugs or endogenous compounds, which may bring undesirable effects, such as adverse herb-drug interactions (HDIs).

As the most important class of detoxification enzymes, UDP-glucuronosyltransferases (UGTs) play pivotal roles in the metabolic elimination and detoxification of a wide variety of endogenous (e.g., bilirubin and steroids) and xenobiotic (e.g., etoposide, propofol and diethylstilbestrol) compounds. To date, more than twenty human UGTs have been identified, which fall into two major families, i.e., UGT1 and UGT2. Human UGTs are highly expressed in metabolic organs including liver, intestine and kidney, which together constitute the most

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important part of the metabolic defense system of human body [20–23]. Strong inhibition on human UGTs by xenobiotics may trigger some undesirable effects, including adverse herb/drug-drug interactions (H/DDIs), and some metabolic disorders (such as hyperbilirubinemia and liver disorder) [24,25]. To avoid the potential risks of UGTs-mediated H/DDIs and the occurrence of UGTs-inhibition associated undesirable effects, it is necessary to evaluate the inhibitory effects of drug candidates or major constituents in herbs on UGTs. Taking into account that AMF is an abundant constituent from various medicinal plants, and the inhibitory effects of AMF on human phase II drug metabolizing enzymes (such as UGTs) have not been well-characterized, it is necessary to evaluate the potential inhibitory effects of AMF on UGTs *in vitro* and to explore AMF-associated toxicity from the perspective of UGTs inhibition.

In the present study, the inhibitory effects of AMF on human UGTs were fully investigated. A panel of commercially available human UGTs was used to screen the inhibition potential of AMF on the glucuronidation activities of each UGT isoform *in vitro*, using a nonspecific probe substrate (4-methylumbelliferone, 4-MU) for all tested UGTs except for UGT1A4 and 2B10, a specific probe substrate (trifluoperazine, TFP) for UGT1A4, a specific probe substrate (cotinine) for UGT2B10, a specific probe for UGT1A1 (N-3-carboxy propyl-4-hydroxy-1,8-naphthalimide, NCHN) and a specific probe for UGT1A9 (propofol). Furthermore, human liver microsomes (HLMs) were utilized as the enzyme source to investigate the inhibitory effects of AMF on three important human UGTs (UGT1A1, 1A4 and 1A9), which played key roles in glucuronidation of both endogenous compounds and therapeutic drugs. The inhibition constant (K_i) and inhibition mechanisms of AMF against each UGT isoform were also comprehensively characterized.

2. Materials and methods

2.1. Chemicals and reagents

Amentoflavone (AMF), magnolol and nilotinib were purchased from Chengdu Pufei De Biotech Co., Ltd (Chengdu, Sichuan, China). 4-Methylumbelliferone (4-MU), 4-MU-*β*-D-glucuronide (4-MUG), TFP (dihydrochloride salt), propofol, propofol glucuronide (PG), androstrone, and uridine-5′-diphosphoglucuronic acid (UDPGA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cotinine was purchased from Alfa Aesar (Ward Hill, MA). NCHN and NCHN-*O*-glucuronide (NCHNG) were synthesized by the authors as previously reported [26]. The purity of each compound was above 98% according to LC-UV analysis. Pooled HLMs (n = 50) were purchased from Celsis *in vitro* Technology (Baltimore, MD, USA). A panel of 13 recombinant human UGT isoforms (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15 and 2B17) were stably expressed in baculovirus-infected insect cells and purchased from Corning Life Sciences (Lowell, MA, USA). Solvents and other reagents were of analytical reagent grade.

2.2. 4-MU-O-glucuronidation based inhibition assays

4-MU is typically used as a non-selective probe substrate for all UGT isoforms except for UGT1A4 and 2B10. In brief, the 200 μL reaction mixture contained 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 4 mM UDPGA, AMF (0–100 μM), and 4-MU (10–2000 μM depending on the UGT isoforms). After 3 min preincubation at 37 °C, the reaction was initiated by the addition of 10 μL of UDPGA. Incubation conditions for each recombinant UGT isoforms were the same as those previously reported [27,28]. The reactions were then terminated by adding 200 μL ice-cold acetonitrile followed by centrifugation at 20,000 × *g* for 20 min. The aliquots of supernatant were then taken for ultra-fast liquid chromatography-ultraviolet spectrometry (UFLC-UV) analysis. UGT isoforms inhibitors nilotinib, magnolol, and androsterone were used as positive controls for UGT1A1, 1A9, and 2B7, respectively [28–30]. Quantification of 4-MUG was accomplished using a standard curve for 4-MUG.

2.3. TFP-N-glucuronidation based inhibition assays

TFP-*N*-glucuronidation was commonly used as a marker reaction for UGT1A4 in the inhibition studies [30]. The inhibitory effects of AMF on UGT1A4-mediated TFP-*N*-glucuronidation were quantified in both HLM and recombinant UGT1A4. Briefly, the incubation mixture of 200 μL contained AMF (0–100 μM), TFP (15–120 μM), 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 4 mM UDPGA and recombinant UGT1A4 (0.1 mg/mL) or HLM (0.3 mg/mL). After incubation at 37 °C for 30 min (recombinant UGT1A4) or 120 min (HLM), the reactions were terminated by the addition of 200 μL ice-cold acetonitrile followed by centrifugation at 20,000 × *g* for 20 min. Then, 10 μL aliquots of the supernatants were taken for HPLC-UV analysis. Since standard TFP-*N*-glucuronide was not commercially available, the standard curve of the substrate was used to quantify the formation of the glucuronide.

2.4. NCHN-O-glucuronidation based inhibition assays

The inhibitory effects of AMF on UGT1A1-mediated NCHN-*O*-glucuronidation were quantified in both HLM and recombinant UGT1A1. The incubations were performed as we described previously [26]. Reaction mixtures contained 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, and 4 mM UDPGA, AMF (0–100 μM), NCHN (15–100 μM) as well as HLM or recombinant UGT1A1. Then the screening experiments were performed with HLM (0.2 mg/mL) at 37 °C for 30 min, or with recombinant UGT1A1 (0.06 mg/mL) at 37 °C for 90 min. Notably, HLM was firstly activated with Brij58 (0.1 mg/mg protein) on ice for 20 min before incubation. The reactions were treated and terminated as described above. Lastly, 200 μL supernatant was transferred into a 96-well plate for fluorescence measurement using a multi-mode microplate reader. The NCHNG concentration of incubation samples was determined using the NCHNG standard curve.

2.5. Propofol-O-glucuronidation based inhibition assays

The inhibitory effects of AMF on UGT1A9 were also quantified in both HLM and recombinant UGT1A9. Briefly, AMF (0–100 μM) and propofol (30–200 μM) was incubated with HLM (0.05 mg/mL) or recombinant UGT1A9 (0.05 mg/mL) at 37 °C for 20 min. Notably, HLM was pre-incubated with Brij58 (0.1 mg/mg protein) on ice for 20 min before incubation. Then, the reactions were initiated by the addition of UDPGA and were terminated by adding acetonitrile with 1.5 μM 4-MUG as the internal standard. The samples were treated as described above, and the aliquots of supernatant were taken for LC-MS/MS analysis. The calibration curve was constructed by a peak area ratio (PG/4-MUG) over a concentration range of 20–5000 ng/mL PG. Quantitation of PG was achieved by comparing peak area ratio to that of standard curve.

2.6. Cotinine-N-glucuronidation based inhibition assays

Cotinine-*N*-glucuronidation was used as a marker reaction for UGT2B10 in the inhibition studies [31,32]. The inhibitory effects of AMF on UGT2B10-mediated cotinine-*N*-glucuronidation were quantified in both HLM and UGT2B10. Briefly, the incubation mixture of 200 μL contained AMF (0–100 μM), cotinine (1–4 mM), 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 4 mM UDPGA and recombinant UGT2B10 (0.5 mg/mL) or HLM (0.5 mg/mL). After incubation at 37 °C for 90 min, the reactions were terminated by the addition of 200 μL ice-cold acetonitrile followed by centrifugation at 20,000 × *g* for 20 min. Then, 10 μL aliquots of the supernatants were taken for HPLC-UV analysis. The standard curve of cotinine was used to quantify the formation of cotinine-*N*-glucuronide.

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