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Urinary Metabolite Profiling Reveals CYP1A2-mediated Metabolism of Aminoflavone (NSC 686288)

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Abstract

Aminoflavone (AF, NSC 686288), a candidate chemotherapeutic agent, possesses a unique antiproliferative profile against tumor cells. Metabolic bioactivation of AF by drug metabolizing enzymes, especially CYP1A monooxygenases, has been implicated as an underlying mechanism for its selective cytotoxicity in several cell culture-based studies. However, *in vivo* metabolism of AF has not been investigated in detail. In this study, the structural identities of thirteen AF metabolites (twelve of which are novel) in mouse urine or from microsomal incubations, including three monohydroxy-AFs, two dihydroxy-AFs and their sulfate and glucuronide conjugates, as well as one *N*-glucuronide, were determined by accurate mass measurements and LC-MS/MS fragmentation patterns, and a comprehensive map of the AF metabolic pathways was constructed. Significant differences between wild-type and *Cyp1a2*-null mice, with in the relative composition of urinary metabolites of AF, demonstrated that CYP1A2-mediated regioselective oxidation was a major contributor to the metabolism of AF. Comparisons between wild-type and *CYP1A2*-humanized mice further revealed interspecies differences in CYP1A2-mediated catalytic activity. Incubation of AF with liver microsomes from all three mouse lines and with pooled human liver microsomes, confirmed the observations from urinary metabolite profiling. Results from enzyme kinetic analysis further indicated that in addition to CYP1A P450s, CYP2C P450s may also play some role in the metabolism of AF.

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Introduction

The diaminoflavone compound, 5-amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methylchromen-4-one (Aminoflavone; AF; NSC686288) (Fig. 1), is entering phase I clinical trials as a candidate for chemotherapy based on its antiproliferative potency proven in both *in vitro* cell culture and *in vivo* tumor xenograft studies (Akama et al., 1998; Loaiza-Perez et al., 2004; Loaiza-Perez et al., 2004). Results from a comprehensive NCI screening test showed that AF selectively inhibited the growth of diverse types of tumor cells among a panel of 60 widely-used cell lines (Weinstein et al., 1997), and the difference between sensitive and resistant cell lines with regard to the responses to AF treatment was very distinctive and significant (http://www.dtp.nci.nih.gov/docs/dtp_search.html).

Mechanistic studies on AF's cytotoxicity have demonstrated that AF can cause DNA damage through DNA single-strand breaks and DNA-protein crosslinking, and subsequently induce histone H2AX phosphorylation and S-phase arrest (Meng et al., 2005; Pobst and Ames, 2006). As for its unique selectivity, significant effort has focused on the potential bioactivation of AF by the P450, CYP1A1. Recombinant CYP1A1 oxidized AF to multiple metabolites *in vitro* (Kuffel et al., 2002). AF treatment also induced a much higher level of CYP1A1 protein in sensitive cell lines than in resistant cell lines through the aryl hydrocarbon receptor (AhR)-mediated pathway (Kuffel et al., 2002; Loaiza-Perez et al., 2004; Loaiza-Perez et al., 2004). Recently, a relationship between sensitivity to AF treatment and expression level of cytosolic sulfotransferase (SULT) was identified through analyzing the gene expression pattern of AF-sensitive cell lines, and stable transfection of SULT1A1 converted AF-resistant cells into sensitive cells (Meng et al., 2006). Therefore, it is highly possible that multiple xenobiotic-metabolizing phase I and II enzymes, either collectively or separately, contribute to the metabolic activation of AF.

To date, information on the identities of AF metabolites was limited to the detection of one *N*⁴-acetylated metabolite in plasma (Phillips et al., 2000) and one *N*⁴-hydroxylated metabolite after incubations with microsomal membranes (Kuffel et al., 2002). It should also be noted that current knowledge on the bioactivation of AF was mainly derived from *in vitro* incubation and cell line-based experiments. In human and animal studies, liver, kidney, intestine and other extrahepatic tissues will likely participate in the absorption, distribution, metabolism and excretion of AF. Indeed, it is well known that the major circulating forms of chemotherapeutic agents that are destined for uptake into tumors are not the parental compounds in the most cases, and the expression levels of major drug metabolizing enzymes in tumor tissues are significantly different from those in liver. For example, CYP1A1 may be the major AF metabolizing enzyme in tumor cells, but in liver its expression level is far lower than CYP1A2 (Shimada et al., 1996). The literature is replete with examples illustrating significant differences between cell culture models and human and animals studies with regard to the metabolism of specific drugs.

To understand the *in vivo* metabolism of AF and the role of CYP1A2, AF metabolites were identified and their structures elucidated, and urinary metabolites of AF from wild-type, *Cyp1a2*-null and *CYP1A2*-humanized mice were profiled after oral dosing of AF. The regioselectivity of CYP1A2-mediated oxidation and interspecies differences in the metabolism of AF were confirmed after microsomal incubation, and the contribution of major P450s to the metabolism of AF was determined by incubations with recombinant human P450s and enzyme kinetics.

Materials and Methods

Chemicals and reagents

Amino flavone was provided by the Developmental Therapeutics Program (DTP), National Cancer Institute (NCI). α -Naphthoflavone, sulfaphenazole, mephenytoin, HPLC-grade acetonitrile, methanol and formic acid as well as β -nicotinamide adenine dinucleotide (β -NADPH) were purchased from Sigma (St. Louis, MO). Pooled human liver microsomes (HLM) and recombinant human P450s (CYP1A1; 1A2; 1B1; 2A6; 2B6; 2C9; 2C19; 2D6; 2E1; 3A4) from baculovirus-infected insect cells were purchased from BD Biosciences (San Jose, CA).

Animals

Both the *Cyp1a2*-null mouse line (*mCyp1a2*^{-/-}) and *CYP1A2*-humanized mouse line on a *Cyp1a2*-null background (*hCYP1A2*^{+/+}, *mCyp1a2*^{-/-}) were described previously (Pineau et al., 1995; Cheung et al., 2005). Wild-type mice on a 129/Sv strain background were used in this study. All animals were maintained in the NCI's animal facilities under a standard 12 h light/12 h dark cycle with food and water *ad libitum*. Handling and treatment procedures were in accordance with animal study protocols approved by the NCI Animal Care and Use Committee.

In vivo metabolism of AF in mice

Four female mice from each genotype group, age 8-12 weeks, were used for urinary metabolite profiling. Urine samples were collected by housing mice for 24 hours in glass metabolic bowls (Jencons, Leighton Buzzard, U.K.), and stored at -80°C before usage. Control urine samples were collected 2 days before AF treatment, and then a 50 mg/kg dose of AF (suspended in corn oil) was administered by oral gavage. After urine collection, mice were killed and livers were harvested and snap-frozen in liquid nitrogen before storage at -80°C. Samples for UPLC-TOFMS analysis were prepared by mixing 50 μ L of urine with 200 μ L of 50% acetonitrile and centrifuging at 18,000 $\times$ g for 5 min to remove protein and particulates. A 200 μ L aliquot of the supernatant was transferred to an auto-sampler vial for further analysis.

Preparation of mouse liver microsomes

Livers from untreated wild-type (*mCyp1a2*^{+/+}), *Cyp1a2*-null (*mCyp1a2*^{-/-}) and transgenic mice (*hCYP1A2*^{+/+}, *mCyp1a2*^{-/-}) were homogenized and microsomes (MLM) were prepared as described previously (Yu et al., 2003).

In vitro metabolism of AF in MLM and HLM

Microsomal incubations were carried out in 20 mM phosphate-buffered saline (PBS), pH 7.4, containing 0.5 mg/ml microsomal protein, 2 mM MgCl₂, 20 μ M AF and 1 mg/ml freshly prepared β -NADPH in a final volume of 200 μ L. After a 30 min incubation at 37°C, the reaction mix was directly loaded into a pretreated OasisTM column (Waters, Milford, MA) and metabolites eluted with 1 ml methanol. The extract was dried by N₂ flow and reconstituted in 50% aqueous acetonitrile for LC-MS/MS analysis.

In vitro metabolism of AF by recombinant human P450s

Reactions were carried out in 20 mM phosphate-buffered saline (PBS), pH 7.4, containing 20 nM recombinant human P450s (CYP1A1; 1A2; 1B1; 2A6; 2B6; 2C9; 2C19; 2D6; 2E1; 3A4), 2 mM MgCl₂, 20 μ M AF and 1 mg/ml freshly prepared β -NADPH in a final volume of 200 μ L. After 30 min of incubation at 37°C, the reaction mix was directly loaded into a pretreated OasisTM column (Waters, Milford, MA) and metabolites eluted with 1 ml methanol. The extract was dried by N₂ flow and reconstituted in 50% aqueous acetonitrile for LC-MS/MS analysis.

Enzyme kinetics of recombinant human CYP1A2, CYP2C9, CYP2C19 and CYP3A4

AF ranging from 200 nM to 80 μ M was incubated with recombinant human CYP1A2, CYP2C9, CYP2C19 and CYP3A4 for 20 min under the same condition as described above. Kinetic parameters for three major mono-hydroxylated metabolites, K_m and relative V_{max} (represented as the percentage of the highest activity detected in all reactions), were evaluated by nonlinear regression (Prism™ GraphPad 2.0, San Diego, CA).

Chemical inhibition of HLM-mediate AF metabolism

Different from the aforementioned condition for microsomal incubations, chemical inhibitors of CYP1A2, CYP2C9 and CYP2C19 were pre-incubated with HLM for 5 min before adding AF and NADPH. The reactions were terminated at 20 min and samples processed under the same conditions as described above.

LC-ESI-MS/MS analysis of AF metabolites

For LC-MS/MS analysis, a 5 μ L aliquot of samples from mouse urine or *in vitro* incubation was injected into a Waters UPLC-QTOFMS system (Mildford, MA). An Acquity UPLC™ BEH C₁₈ column (Waters) was used to separate AF and its metabolites at 30°C. Flow rate of mobile phase was 0.6 mL/min with a gradient ranging from water to 95% aqueous acetonitrile containing 0.1% formic acid in a 10-min run. The QTOF Premier™ mass spectrometer was operated in the positive electrospray ionization (ESI) mode. Capillary voltage and cone voltage was maintained at 3 kV and 40 V, respectively. Source temperature and desolvation temperature were set at 120 °C and 350 °C, respectively. Nitrogen was applied as the cone (50 L/hr) and desolvation gas (600 L/hr), and argon as collision gas. For accurate mass measurement, the QTOFMS was calibrated with sodium formate solution (range m/z 100-1000) and monitored by the intermittent injection of the lock mass leucine enkephalin ($[M+H]^+ = 556.2771$ m/z) in real time. Mass chromatograms and mass spectral data were acquired by MassLynx™ software in centroid format, and were further processed by MetaboLynx™ software to screen and identify potential AF metabolites through the mass difference from AF ion (m/z: 321.0851⁺). The structure of each AF metabolite was elucidated by tandem mass spectrometry (MS²) fragmentation with collision energy ranging from 10 to 40 eV.

Statistics

Experimental values are expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed with independent *t*-tests with a P value of <0.01 considered as statistically significant.

Results

Identification and structural elucidation of phase I metabolites of AF by mouse liver microsomes

Previously, a number of potential AF metabolites were identified from HPLC chromatograms of extracted samples derived from microsomal incubations, but only one phase I metabolite of AF was structurally elucidated (Kuffel et al., 2002). To obtain comprehensive structural information on the phase I metabolites of AF and set a basis for further *in vivo* metabolism studies, AF was incubated with MLM from untreated mice. Extracted samples containing AF and its metabolites were separated by the ultra-performance liquid chromatography (UPLC) system, and their accurate masses measured by a TOF mass spectrometer. By comparing chromatographic differences between control (without NADPH) and normal (with NADPH) samples, and by examining the mass difference between AF and new ions generated by the MLM incubation, three monohydroxylated and two dihydroxylated metabolites were identified (Table 1). Their structures were further elucidated by MS² fragmentation (Fig. 2).

As the reference compound for the structural elucidation of AF metabolites, the fragmentation pattern of the parent AF ion (**I**) was examined first to show the formation of major fragment ions (186^+ and 136^+) via a retro Diels-Alder (rDA) reaction in C-ring (Fig. 2A). Further detachment of the carbonyl group ($-C=O$) from ion 186^+ generated ion 158^+ . In addition, the breaking of the C-C bond between the B-ring and C-ring yielded ion 110^+ ($C_6H_6NF^+$ by accurate mass measurement) from the B-ring.

AF metabolite **II** was a mono-hydroxy derivative of AF based on accurate mass measurement and the loss of free radical $HO\cdot$ (-17). The presence of ion 202^+ instead of ion 186^+ indicated that the oxidation had occurred in the A-ring (Fig. 2B). Therefore, the identity of this metabolite was assigned as N^5 -hydroxy-AF (N^5 -OH-AF). However, it is recognized that hydroxylation might also have occurred on the 7-methyl group, but this was deemed unlikely due to the ready loss of $HO\cdot$ from the pseudomolecular ion, a known characteristic of *N*-hydroxy compounds (Saito et al., 1985).

The structure of AF metabolite **III** was previously reported as $N^{4'}$ -hydroxy-AF ($N^{4'}$ -OH-AF) (Kuffel et al., 2002). As shown in Fig. 2C, both the presence of ions 186^+ and 158^+ provided evidence that the oxidation did not occur in the A-ring and loss of the $-CH_2NO$ group from the parent ion (-44 from 337^+ to 293^+), supported this structure.

Generation of ion 186^+ and ion 152^+ by fragmenting the AF metabolite **IV** ion excluded the possibility of oxidation in the A-ring (Fig. 2D). The high abundance of fragment ion 110^+ ($C_6H_6NF^+$) as a result of breaking of the C-C bond between the B-ring and C-ring also excluded the presence of a hydroxyl group in the B-ring (inlaid spectrum in Fig. 2D). Therefore, the only possible oxidation site is the carbon-3 in the C-ring, which confirms the structure of metabolite **IV** as 3-hydroxy-AF (3-OH-AF). In this connection, it should be noted that both quercetin and kaempferol, the two most abundant natural flavonols in human diet, have the 3-hydroxy group in their structure (Havsteen, 2002).

Both AF metabolites **V** and **VI** are the dihydroxylated AFs based on their accurate masses (Table 1). Similar to metabolite **II**, the fragmentation of metabolite **V** yielded the characteristic ion 202^+ , which indicated a N^5 -hydroxylation reaction. Moreover, neutral loss of mass 44, similar to metabolite **III**, proved the existence of $N^{4'}$ -hydroxy group. (Fig. 2E). By combining the aforementioned evidence, the structure of metabolite **V** was designated as $N^5, N^{4'}$ -dihydroxy-AF. As for metabolite **VI**, the presence of ion 202^+ and ion 152^+ resembled the MS^2 spectra of metabolites **II** and **IV** (Fig. 2F). Therefore, the structure of metabolite **VI** was designated as 3, N^5 -dihydroxy-AF.

Identification and structural elucidation of urinary metabolites of AF

The observation of five major phase I metabolites after MLM incubation suggested that AF might also be extensively metabolized *in vivo*. To test this hypothesis, urine samples from the AF-treated mice (50 mg/kg) were analyzed under the same chromatographic and spectroscopic conditions as extracts from *in vitro* incubation. To achieve an exhaustive detection and comprehensive coverage of major urinary metabolites, a software-assisted metabolite screening and detection (as described in the *Materials and Methods*) was conducted based on the data from accurate mass measurements of all urinary ions and the theoretical mass changes caused by the common phase I and II metabolizing reactions. The results revealed the presence of AF parent ion (**I**), N^5 -OH-AF (**II**) and eight new conjugated metabolites (**VII-XIV**) in mouse urine samples (Table 2). Structural identities of those urinary metabolites were further elucidated by MS^2 analysis. Their major fragment ions are shown in Table 2, and MS^2 spectra of the six most abundant phase II conjugates (**VIII, IX, X, XI, XII, and XIV**) presented in Fig. 3A-F.

AF metabolite **VII** as a sulfate conjugate was confirmed by the neutral loss of mass 80 (SO_3). Appearance of fragment ions 202^+ and 136^+ matched the fragmentation pattern of N^5 -OH-AF (**II**) (Table 2). Therefore, it was designated as N^5 -hydroxy-AF sulfate.

Metabolite **VIII** was also a sulfate conjugate of monohydroxylated AF because of its accurate mass and neutral loss of mass 80. Fragment ions 186^+ and 152^+ indicated its resemblance to 3-OH-AF (**III**) (Fig. 3A), so its chemical identity was designated as 3-hydroxy-AF sulfate (**VIII**).

Formation of ion 353^+ after neutral loss of sulfate group (-80) from metabolite **IX** indicated a dihydroxylated AF moiety (Fig. 3B). The presence of ion 202^+ and 152^+ validated the structure as 3, N^5 -dihydroxy-AF sulfate. Since the retention time (RT) shift caused by sulfation at the 3-OH group (0.19 min) was far less than that derived from sulfation at N^5 -OH group (0.90 min) (Table 1 and 2), and RT shift of metabolite **IX** from its parent compound 3, N^5 -dihydroxy-AF (**V**) was 0.11 min, the sulfate group of metabolite **IX** was putatively conjugated to the 3-OH group.

Metabolite **X** was a direct glucuronidation product of AF because of neutral loss of mass 176 (glucuronic acid moiety) and characteristic fragment ions of 186^+ and 136^+ from deconjugated AF (Fig. 3C). Furthermore, the presence of ion 363^+ from potential rDA reaction revealed that glucuronidation occurred on the A-ring, in which the N^5 atom is the only possible conjugation site. Therefore, metabolite **X** was designated as AF- N^5 -glucuronide.

Formation of ion 337^+ after neutral loss of the glucuronic acid group (-176) from metabolite **XI** indicated a monohydroxylated AF moiety (Fig. 3D). Ions 320^+ and 291^+ were generated by consecutive loss of hydroxyl free radical ($\text{HO}\cdot$) and carbonyl group (HCO) from the deconjugated molecule. It was noted that the deconjugated ion (337^+) and parent ion (513^+) coeluted at RT 4.74 min because of in-source fragmentation under the described MS condition, so a MS^2 scan was performed on ion 337^+ to obtain further structural information. As shown in the inlaid spectrum of Fig. 3D, the deconjugated ion possessed a very similar fragmentation pattern as N^5 -OH-AF (**II**). In addition, since it is known that glucuronidation of N -hydroxylamine mainly occurs at the nitrogen rather than oxygen atom (Kadlubar et al., 1977; Kaderlik et al., 1994), metabolite **XI** was putatively designated as N^5 -OH-AF- N^5 -glucuronide.

Ions 513^+ , 337^+ (loss of glucuronic acid) and 320^+ (loss of $\text{HO}\cdot$) in MS^2 spectra of metabolite **XII** indicated that it was another glucuronide of monohydroxylated AF (Fig. 3E). The presence of ion 293^+ and ion 186^+ was consistent with the fragmentation pattern of $N^{4'}$ -OH-AF (**III**). Therefore, metabolite **XII** was putatively designated as $N^{4'}$ -OH-AF- $N^{4'}$ -glucuronide.

Formation of ion 353^+ after neutral loss of glucuronic acid group (-176) from metabolite **XIII** indicated a dihydroxylated AF moiety (Table 2). The presence of ions 309^+ and 202^+ , which were the characteristic fragments of N^5 , $N^{4'}$ -diOH-AF (**V**), indicated metabolite **XIII** as N^5 , $N^{4'}$ -diOH-AF glucuronide.

Ions 529^+ , 353^+ (loss of glucuronic acid) and 336^+ (loss of $\text{HO}\cdot$) in MS^2 spectra of metabolite **XIV** indicated it was another glucuronide of dihydroxylated AF (Fig. 3F). The presence of ion 202^+ and ion 152^+ resembled to the fragmentation pattern of 3, N^5 -diOH-AF (**VI**). Since glucuronidation was the preferred conjugation reaction for N^5 -OH-AF and sulfation for 3-OH-AF, as proven by the high abundance of N^5 -OH-AF- N^5 -glucuronide (**XI**) and 3-OH-AF sulfate (**VIII**), metabolite **XIV** was putatively designated as 3, N^5 -diOH-AF- N^5 -glucuronide.

Relative composition of urinary AF metabolites in wild-type, *Cyp1a2*-null and *CYP1A2*-humanized mice

Previous studies on the metabolism of AF by using tumor cell lines have shown that CYP1A1 and CYP1A2 contributed to the metabolic bioactivation of AF through an AhR-mediated pathway (Kuffel et al., 2002; Loaiza-Perez et al., 2004; Loaiza-Perez et al., 2004). However, it is well known that CYP1A2 plays a much more prominent role in xenobiotic metabolism *in vivo* than does CYP1A1 based on its much higher expression level and catalytic activity in liver (Shimada et al., 1996; Turesky et al., 2002). In addition, CYP1A2 is also an important enzyme in the bioactivation of heterocyclic aromatic amines (HCA), a chemical class to which AF belongs (Butler et al., 1989). In order to investigate the role of CYP1A2 in the *in vivo* metabolism of AF, three mouse lines including wild-type mice, *Cyp1a2*-null mice (*mCyp1a2*^{-/-}) and *CYP1A2*-humanized mice (*hCYP1A2*^{+/+}, *mCyp1a2*^{-/-}) were used for oral dosing of AF. Although all of the urine samples from wild-type, *Cyp1a2*-null and *CYP1A2*-humanized mice contained AF (I) and its nine urinary metabolites (II, VII-XIV), significant differences in the relative abundance of several metabolites among three mouse lines became evident after pooling all the peak areas of AF and its urinary metabolites and calculating the percentage of each metabolite in this metabolite cluster (Fig. 4 and Table 2). In wild-type mice (Fig. 4A), AF was mainly converted to the 3,*N*⁵-diOH-AF sulfate (IX) and *N*⁵-OH-AF-*N*⁵-glucuronide (XI), but in the *Cyp1a2*-null mice (Fig. 4B), the major metabolites were *N*⁵-OH-AF (II) and its glucuronide (XI). Comparing the metabolic profiles of wild-type and *Cyp1a2*-null mice, it was apparent that the removal of CYP1A2 from the metabolic system diminished the 3-hydroxylation reaction, which subsequently led to significantly decreased levels of the 3-hydroxy metabolites including 3-OH-AF sulfate (VIII), 3,*N*⁵-di-OH-AF sulfate (IX) and 3,*N*⁵-diOH-AF-*N*⁵-glucuronide (XIV), and to the accumulation of *N*⁵-hydroxy metabolites, such as *N*⁵-OH-AF (II), *N*⁵-OH-AF sulfate (VII) and *N*⁵-OH-AF-*N*⁵-glucuronide (XI). Therefore, 3-hydroxylation of AF was a selective CYP1A2-mediated reaction. This conclusion was further strengthened by the relative composition of urinary AF metabolites in *CYP1A2*-humanized mice. Introduction of the human *CYP1A2* gene into *Cyp1a2*-null mice resulted in recovery of the 3-hydroxylation reaction of AF and its downstream metabolites and led to comparable levels of metabolites II, VII, IX and XIV as found in wild-type mice (Fig. 4C). However, there still existed significant differences between wild-type and *CYP1A2*-humanized mice with regard to the metabolites VIII and XI. The higher level of 3-OH-AF sulfate (VIII) and the lower level of *N*⁵-OH-AF glucuronide (XI) in *CYP1A2*-humanized mice implied that 3-hydroxylation of AF was a more preferred reaction for human CYP1A2 than for mouse CYP1A2. Besides the major metabolites from 3-hydroxylation and *N*⁵-hydroxylation, an AF-*N*⁵-glucuronide (X) and two *N*^{4'}-hydroxylation products, *N*^{4'}-OH-AF-*N*^{4'}-glucuronide (XII) and *N*⁵,*N*^{4'}-diOH-AF-*N*-glucuronide (XIII), were also detected as minor metabolites found at comparable levels in the three mouse lines. It should also be noted that all three mouse lines including *Cyp1a2*-null mice retained similar levels of the AF parent compound (I) in their urine, which comprised about 10-15% of total urinary AF metabolites. This observation indicated that, even without the mouse CYP1A2 enzyme, AF was still extensively metabolized. Therefore, some other P450 enzymes were also involved in the metabolism of AF.

Comparison of *in vitro* metabolism of AF by MLM from wild-type, *Cyp1a2*-null mice and *CYP1A2*-humanized mice and by HLM

Urinary metabolite profiling revealed a significant influence of CYP1A2 in the metabolism of AF through a 3-hydroxylation reaction and the potential interspecies difference with regard to the catalytic activity of CYP1A2 (Fig. 4 and Table 2). To confirm these observations, 20 μ M of AF was incubated with MLM from wild-type, *Cyp1a2*-null and *CYP1A2*-humanized mice and pooled HLM, respectively. As shown in Fig. 5A and 5B, two monohydroxylamines, *N*⁵-OH-AF (II) and *N*^{4'}-OH-AF (III), were generated by all three MLM from wild-type, *Cyp1a2*-null and *CYP1A2*-humanized mice, as well as by pooled HLM, and both metabolites

could be further converted to $N^5,N^{4'}$ -diOH-AF (**V**) in all microsomal incubations. Consistent with the *in vivo* data (Fig. 4 and Table 2), both 3-OH-AF (**IV**) and 3, N^5 -diOH-AF (**VI**) were absent after incubation of AF with the MLM from *Cyp1a2*-null mice because of the lack of the CYP1A2-mediated 3-hydroxylation reaction. Furthermore, the interspecies difference between mouse and human CYP1A2 on the 3-hydroxylation activity was observed since the incubation of AF with MLM from CYP1A2-humanized mice and HLM resulted in much higher yields of 3-OH-AF (**IV**) than from MLM of wild-type mice.

Comparison of *in vitro* metabolism of AF by recombinant P450s

Extensive metabolism of AF in *Cyp1a2*-null mice implied the potential involvement of other P450s in the oxidation of AF (Table 2). Therefore, 20 μ M of AF was incubated with a panel of recombinant human P450s, including CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. Chromatograms generated by three most reactive P450s (CYP1A1, CYP1A2 and CYP2C19) are presented in Fig. 6A, and the relative activities of the whole P450 panel on the generation of three monohydroxylated metabolites (**II-IV**) and two dihydroxylated metabolites (**V-VI**) are illustrated in Fig. 6B-F. As shown in Fig. 6, CYP1A2 was more selective in the conversion of AF to 3-OH-AF (**IV**), while CYP1A1 was more active than CYP1A2 in the production of N^5 -OH-AF (**II**) and $N^{4'}$ -OH-AF (**III**). The catalytic activities of CYP1A1 and CYP1A2 for the formation of $N^5,N^{4'}$ -diOH-AF (**V**) were comparable, but CYP1A2 was far more active than CYP1A1 for the formation of 3, N^5 -diOH-AF (**VI**), since it required CYP1A2-mediated 3-hydroxylation. Besides CYP1A P450s, two CYP2C P450s, CYP2C9 and CYP2C19, were also active in the *N*-hydroxylation of AF to generate metabolites **II** and **III**. In addition, both CYP3A4 and CYP2D6 also had low but measurable levels of AF metabolizing activity (Fig. 6B-D).

Relative contribution of CYP1A2, CYP2C and CYP3A enzymes in the metabolism of AF

Since both CYP2C and CYP3A P450s are two of the most abundant P450s in liver (Guengerich, 2005), their relative contribution to the formation of the three major monohydroxylated metabolites (**II**, **III** and **IV**) were evaluated by an enzyme kinetic experiment. As shown in Fig. 7A-B, it is clear that CYP2C19 may be an important enzyme for the *N*-hydroxylation of AF in humans, especially at high substrate concentrations, though the contribution from CYP2C9 and CYP3A is likely to be negligible. Nevertheless, comparing to CYP1A2 (K_m values for **II** and **III** are 4.0 μ M and 5.7 μ M, respectively), AF-metabolizing activity of CYP2C19 (K_m values for **II** and **III** are 14.9 μ M and 17.5 μ M, respectively) was still much lower. By calculating the ratio between relative V_{max} and K_m (V_{max}/K_m), enzyme efficiencies of CYP1A2 for the formation of metabolites **II** and **III** were more than six- and five-fold greater than CYP2C19, respectively. Also, consistent with other *in vivo* and *in vitro* data (Table 2, Fig. 4-6), enzyme kinetic analysis further confirmed that 3-hydroxylation is a CYP1A2-dominant reaction, in which the K_m value of CYP1A2 is 3.3 μ M (Fig. 7C). In addition, results from the pre-incubations of HLM with widely-used chemical inhibitors showed that α -naphthoflavone (CYP1A2 inhibitor) can significantly inhibit the formation of all three monohydroxylated AF metabolites, especially to 3-OH-AF (**IV**), though sulfaphenazole (CYP2C9 inhibitor) and mephenytoin (CYP2C19 inhibitor) failed to affect AF metabolism (Fig. 7D).

Discussion

Structure-activity relationship (SAR) protocols guided the development of AF as a chemotherapeutic agent. Based on the fact that natural or synthetic flavonoids can elicit anti-estrogenic and/or estrogenic activities, AF was derived from the structure of flavone (Havsteen, 2002). By adding two amino groups as electron donors and three fluoride atoms plus a methyl group for blocking metabolism, AF possesses much higher cytotoxicity against the estrogen receptor (ER)-positive cell line MCF-7 than its parent compounds (Akama et al., 1996; Akama

et al., 1997). Recent results from the NCI 60 cell line screenings indicated that the bioactivity of AF may surpass the original expectation of developing an anti-estrogen for breast cancer therapy (http://www.dtp.nci.nih.gov/docs/dtp_search.html). Interestingly, although one of the original medicinal chemistry strategies for developing this compound was to decrease the potential metabolism in the flavone moiety (Akama et al., 1997), which was thought to have a deactivating effect on its bioactivity, the results from this study clearly demonstrated that AF was still extensively metabolized, since multiple metabolites were detected after *in vitro* incubation and oral administration, and the parent compound only accounted for about 10-15% of all detected urinary metabolites in the three mouse lines used in this study.

To identify and profile of phase I and phase II metabolites of AF, the ultra-performance liquid chromatograph (UPLC) system coupled with a time-of-flight mass spectrometer (TOFMS) were adopted to achieve high-resolution chromatographic separation and accurate mass measurements (in the 5-10 ppm range of real mass). By combining the information on the retention time and the mass of each ion, computer-assisted analysis can automatically interrogate the mass chromatograms and spectra for identifying the potential AF metabolites. Compared to a manual search, this strategy was more efficient and exhaustive. It should be noted that in-source fragmentation occurred for several sulfate and glucuronide metabolites, presumably because of weak chemical bonds between the parent compound and the conjugated moiety. With careful examination and adjustment of conditions on the mass spectrometer, the identities of those fragments can be unambiguously resolved. Structures of major AF metabolites were elucidated through analyzing the fragmentation pattern recorded by tandem mass spectrometry. Like typical flavone compounds, the retro Diels-Alder (rDA) reaction contributes to the formation of major daughter ions of AF and its metabolites by breaking the C-ring (Mullen et al., 2002). Examining the product ions from the rDA reaction and the breaking of C-C bond between B-ring and C-ring, can pinpoint whether the phase I reaction happens on the A-ring, B-ring or C-ring. As for phase II metabolites, the conjugation sites of sulfation and glucuronidation were putatively assigned based on the common rules of drug metabolism and previous knowledge of the *N*-glucuronidation of hydroxylamine (Kadlubar et al., 1977; Kaderlik et al., 1994).

As mentioned above, previous studies on the AF bioactivation and metabolism have focused on the CYP1A P450s, CYP1A1 and CYP1A2 (Kuffel et al., 2002; Loaiza-Perez et al., 2004). Results from urinary metabolite profiling (Table 2), *in vitro* incubation (Fig. 5-6) and chemical inhibition by α -NF (Fig. 7D) in this study also support the view that CYP1A2 plays an important role in AF metabolism, especially for the 3-hydroxylation reaction. However, as shown in Table 2, mice without CYP1A2 can still extensively metabolize AF, which suggest that CYP1A2 was not the sole enzyme responsible for AF metabolism *in vivo*. Indeed, incubation of AF with a panel of recombinant human CYPs revealed that CYP2C9 and CYP2C19 have significant *N*-hydroxylation activities (Fig. 6), and enzyme kinetic analysis further indicated that CYP2C19 has a pharmacologically relevant K_m value for the formation of hydroxylamines (**II** and **III**) (Fig. 7). Although mephenytoin, a CYP2C19 substrate and inhibitor, did not inhibit the HLM-mediated metabolism, which is possibly attributed to the ineffective competitive inhibition mechanism, a preliminary inhibition experiment showed that anti-rat CYP2C11 antibody can significantly inhibit the *N*-hydroxylation reactions catalyzed by MLM (data not shown). It is known that there is some degree of overlap in the substrate specificity of CYP1A and CYP2C enzymes (Venkatakrishnan et al., 1999; Otake and Walle, 2002). Because of their prominence in hepatic xenobiotic metabolism system, CYP2C enzymes should could contribute to the *N*-hydroxylation of AF, though the 3-hydroxylation reaction is highly CYP1A2-selective. Further *in vivo* and *in vitro* studies are required to understand the role of CYP2C P450s in AF metabolism and the potential interspecies difference.

Another interesting observation on the relative composition of urinary metabolites was the much lower levels of N^4' -hydroxylation products in urine than N^5 - and 3-hydroxylation products (Table 2), although the relative abundance of N^4' -OH-AF (**III**) after microsomal incubations was comparable to that of N^5 -OH-AF (**II**) and 3-OH-AF (**IV**) (Fig. 5). During the course of this study, it was noted that when stored at room temperature or 4°C, the half-life of N^4' -OH-AF (**III**) in the microsomal incubation extracts was much shorter than other monohydroxylated AF metabolites (**II** and **IV**) (data not shown). In addition, the abundance of the parent ion of N^4' -OH-AF (**III**) in the MS² fragmentation experiment was also much lower than N^5 -OH-AF (**II**) and 3-OH-AF (**IV**) with the same collision energy (Fig. 2), and it disappeared completely with the increased collision energy. Therefore, this hydroxylamine metabolite is likely to be unstable and chemically reactive. Since reactive hydroxylamine compounds are destined for conversion into genotoxic electrophiles through acetylation and sulfation (Turesky, 2002), it is possible that N^4' -OH-AF (**III**) can be further transformed to more reactive species that can covalently bind to DNA and protein, as shown in a recent study on the bioactivation of AF by sulfotransferase (Meng et al., 2006). It would be interesting to investigate whether N^4' -OH-AF (**III**) is the major reactive and cytotoxic metabolite of AF in future studies. Furthermore, N^4' -acetyl-AF, an AF metabolite found in rat plasma (Phillips et al., 2000), has not been detected in urine although its presence in plasma was also observed in this study. A possible explanation is that acylases, which exist in high abundance in kidney, may deacetylate N^4' -acetyl-AF back to AF (Yamauchi et al., 2002).

One of the drawbacks of using mouse urine samples for metabolism studies, especially for the purpose of quantitation, is the high degree of variance in urine volume and quality caused by dosing, water intake, evaporation and contamination from diet and feces. By calculating the relative percentage of each metabolite in the whole metabolite cluster, the aforementioned variability problem among the samples can be significantly reduced since the concentrations of all urinary components change proportionally with the alteration in experimental conditions. As shown in Table 2, the results from this study demonstrated that urinary metabolite profiling based on the relative composition was able to establish a data set with small variance, and therefore it can function as an efficient tool to investigate the influence of genetic modification on drug metabolism. This strategy may also be applicable to drug-drug interaction studies.

The use of genetically modified animal models, including gene knockout and transgenic mice, provides a powerful tool for studying xenobiotics metabolism. *Cyp1a2*-null mice have been used to demonstrate the important role of CYP1A2 on the pharmacokinetics and metabolism of caffeine (Buters et al., 1996), and *CYP1A2*-humanized mice to the interspecies difference in metabolism of the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (Cheung et al., 2005). This study further proved the value of both animal models for studying the metabolism of CYP1A2 substrates as well as interspecies differences between human and mouse. Besides their application to drug metabolism, these animal models can also be used to determine the role of drug metabolizing enzymes in the metabolism of endogenous chemicals and the modulation of normal homeostasis, and for understanding the role of a candidate enzyme in the carcinogenicity of known carcinogens (Gonzalez, 2003).

In summary, by combining the accurate mass measurement and the software-assisted global metabolite profiling strategy, thirteen AF metabolites, from CYP-mediated oxidation and phase II glucuronidation and sulfation, were identified, and a comprehensive *in vivo* metabolic pathway of AF was constructed (Fig. 8). Besides one known metabolite, twelve novel metabolites were structurally elucidated by LC-MS/MS analysis. Significant differences in the relative composition of urinary metabolites among three mouse lines demonstrated that, i) CYP1A2 plays an important role in the AF metabolism, although it is not the sole enzyme capable of oxidizing AF, ii) 3-hydroxylation is a CYP1A2-selective reaction, and iii) there is a clear interspecies difference between mouse and human CYP1A2 in the metabolism of AF.

In vitro incubations of AF with microsomes and recombinant P450s as well as enzyme kinetics confirmed these observations, and further indicated that CYP2C enzymes might also contribute to AF metabolism.

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Abbreviations

AF, Aminoflavone; CYP, cytochrome P450; MLM, mouse liver microsomes; HLM, human liver microsomes; MS², tandem mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; UPLC, ultra-performance liquid chromatography; TOFMS, time-of-flight mass spectrometry; rDA, retro Diels-Alder reaction; α -NF, α -naphthoflavone.

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Fig. 1.
Structure of Aminoflavone (AF). AF structure is annotated following standard nomenclature of the flavonoids.

Fig. 2.

LC-MS/MS structural elucidation of phase I AF metabolites generated by microsomal incubation. Conditions for LC-MS/MS analysis and microsomal incubation were described in the *Materials and Methods*. MS² fragmentation was conducted with collision energy ramping from 10 to 40 eV, except a fixed collision energy of 35 eV for inlaid spectrum in Fig. 2D. **A.** MS² fragmentation of AF (**I**). **B.** MS² fragmentation of *N*⁵-hydroxy-AF (**II**). **C.** MS² fragmentation of *N*^{4'}-hydroxy-AF (**III**). **D.** MS² fragmentation of 3-hydroxy-AF (**IV**). **E.** MS² fragmentation of *N*⁵,*N*^{4'}-dihydroxy-AF (**V**). **F.** MS² fragmentation of 3,*N*⁵-dihydroxy-AF (**VI**). Major daughter ions from fragmentation were interpreted in the inlaid structural diagrams.

Fig. 3.

LC-MS/MS structural elucidation of major phase II metabolites of AF in urine. Urine samples from wild-type mice were collected for 24 hours after oral dosing with 50 mg/kg AF. Chromatographic and spectroscopic conditions for LC-MS/MS were described in the *Materials and Methods*. MS² fragmentation was conducted with collision energy ramping from 10 to 40 eV. **A.** MS² fragmentation of 3-hydroxy-AF sulfate (**VIII**). **B.** MS² fragmentation of 3,*N*⁵-dihydroxy-AF sulfate (**IX**). **C.** MS² fragmentation of AF-*N*⁵-glucuronide (**X**). **D.** MS² fragmentation of *N*⁵-hydroxy-AF-*N*⁵-glucuronide (**XI**) with inlaid spectrum of deconjugated *N*⁵-hydroxy-AF ion. **E.** MS² fragmentation of *N*^{4'}-hydroxy-AF-*N*^{4'}-glucuronide (**XII**). **F.** MS² fragmentation of 3,*N*⁵-dihydroxy-AF-*N*⁵-glucuronide (**XIV**). Major daughter ions from fragmentation were interpreted in the inlaid structural diagrams.

Fig. 4.

Representative chromatograms of major AF metabolites in urine samples of wild-type (*mCyp1a2*^{+/+}), *Cyp1a2*-null (*mCyp1a2*^{-/-}) and *CYP1A2*-humanized (*hCYP1A2*^{+/+}, *mCyp1a2*^{-/-}) mice. Chromatographic and spectroscopic conditions for LC-MS analysis were described in the *Materials and Methods*. Ions within the 20 ppm range of theoretical accurate mass ($[M+H]^+$) of AF and its metabolites (321.0851; 337.0800; 417.0368; 433.0317; 497.1172; 513.1121 and 529.1070) were extracted from each 10-min mass scan on the urine samples from three mouse lines. The identities of metabolites (**I**, **II**, **VII-XIV**) were presented in Table 2.

Fig. 5.

In vitro oxidation of AF by MLM from wild-type, *Cyp1a2*-null and *CYP1A2*-humanized mice and by pooled HLM. **A.** Representative chromatograms of monohydroxylated AF (337⁺) and dihydroxylated AF (353⁺) ions (**II-VI**). Ions within the 20 ppm range of theoretical accurate masses (337.0800 and 353.0749) were extracted from each 10-min mass scan on the samples generated by incubating 20 μ M AF with 0.5 mg/ml MLMs from three mouse lines and pooled HLM. **B.** Relative activity of MLMs and HLM for the generation of phase I AF metabolites (**II-VI**). Enzymatic activity of the microsome with the highest yield of targeted metabolite was set arbitrarily as 1. Relative activity was presented as mean $\pm$ SD (n=3).

Fig. 6.

Relative mono-hydroxylation and di-hydroxylation activities by recombinant human P450s. **A.** Representative chromatograms of monohydroxylated AF (337⁺) and dihydroxylated AF (353⁺) ions (**II-VI**) after incubation of 20 μM AF with recombinant human CYP1A1, CYP1A2 and CYP2C19 enzymes. Ions within the 20 ppm range of theoretical accurate masses (337.0800 and 353.0749) were extracted from each 10-min mass scan on the samples generated by Supersome incubation. **B.** Relative activity of P450s for the generation of N⁵-OH-AF (**II**). **C.** Relative activity of P450s for the generation of N^{4'}-OH-AF (**III**). **D.** Relative activity of P450s for the generation of 3-OH-AF (**IV**). **E.** Relative activity of P450s for the generation of N⁵,N^{4'}-diOH-AF (**V**). **F.** Relative activity of P450s for the generation of 3,N⁵-diOH-AF (**VI**). Enzymatic activity of the P450s with the highest yield of targeted metabolite was set arbitrarily as 1. Relative activity was presented as mean ± SD (n=3).

Fig. 7.

Relative contribution of CYP1A2, CYP2C and CYP3A P450s to AF metabolism. AC. Enzyme kinetics of recombinant human CYP1A2 (■), CYP2C9 (○), CYP2C19 (▼) and CYP3A4 (◇) isozymes on the formation of N^5 -OH-AF (II), $N^{4'}$ -OH-AF (III) and 3-OH-AF (IV) were evaluated after the incubations with AF ranging from 200 nM to 80 μ M. Relative activity was calculated as the percentage of the highest activity detected in all kinetic reactions. **D.** Effect of specific CYP inhibitors on HLM-mediated phase I metabolism. 10 μ M of α -naphthoflavone (α -NF), 100 μ M of sulfaphenazole (SUL) or 100 μ M of mephenytoin (MEPH) was pre-incubated with HLM before adding 20 μ M of AF. Catalytic activity of HLM for the formation of each phase I metabolite (II, III and IV) was set arbitrarily as 1. Relative activity was presented as mean $\pm$ SD (n=3).

Fig. 8.

Major *in vivo* AF metabolism pathways. Both phase I and phase II metabolizing enzymes can convert AF (**I**), collectively or separately, into more hydrophilic metabolites. P450-mediated oxidation reactions at C³, N⁵ and N^{4'} positions transform AF to three monohydroxylated metabolites (**II-IV**), which can be further metabolized to two dihydroxylated metabolites (**V** and **VI**). Among these reactions, 3-hydroxylation is CYP1A2-selective. Besides direct *N*-conjugation of AF (**I**) to metabolite **X**, UDP-glucuronosyltransferases (UGT) can also convert phase I metabolites (**II**, **III**, **V**, and **VII**) into glucuronides (**XI**, **XII**, **XIII**, and **XIV**), respectively. Furthermore, cytosolic sulfotransferases (SULT) can metabolize phase I metabolites (**II**, **IV**, and **VI**) to sulfates (**VII**, **VIII**, and **IX**), respectively. Solid line represents phase I oxidation reaction, and dashed line for phase II conjugation reaction. Thicker line symbolizes the pathway to major urinary metabolites in one or all mouse lines in this study, and thinner line for the pathway to minor urinary metabolites.

Table 1

Phase I AF metabolites from microsomal incubations. Three monohydroxylated (**II-IV**) and two dihydroxylated AF metabolites (**V** and **VI**) after microsomal incubations were separated by UPLC and identified by accurate mass measurement and MS² fragmentation in Q-TOF mass spectrometer. Mass accuracy was calculated as the error of real mass from the theoretical accurate mass ($[M+H]^+$) in part per million (ppm).

ID	Formula	Accurate mass $[M+H]^+$	Name	Mass error (ppm)	RT (min)
II	C ₁₆ H ₁₁ F ₃ N ₂ O ₃	337.0800	N ⁵ -OH-AF	1.2	5.15
III	C ₁₆ H ₁₁ F ₃ N ₂ O ₃	337.0800	N ^{4'} -OH-AF	3.6	5.89
IV	C ₁₆ H ₁₁ F ₃ N ₂ O ₃	337.0800	3-OH-AF	0.3	5.94
V	C ₁₆ H ₁₁ F ₃ N ₂ O ₄	353.0749	N ⁵ ,N ^{4'} -diOH-AF	0.8	4.91
VI	C ₁₆ H ₁₁ F ₃ N ₂ O ₄	353.0749	3,N ⁵ -diOH-AF	0.3	5.02

Urinary profiles of AF metabolites in wild-type (*mIa2*^{+/+}) and *CypIa2*-null (*mIa2*^{-/-}) and *CYP1A2*-humanized (*hIa2*^{+/+}, *mIa2*^{-/-}) mice. Twenty-four h urine samples (n=4 for each mouse line) after oral dosing with 50 mg/kg AF were collected and analyzed as described in the *Materials and Methods*. Screening and identification of major metabolites were performed by using Metabol_lynx™ software based on accurate mass measurement. After structural elucidation of major metabolites by MS² fragmentation (Fig. 3), the abundance of each metabolite was represented as a relative peak area (area % ± SD) by calculating its percentage in the total peak area of AF and its urinary metabolites.

Table 2

ID	Formula	Accurate mass [M+1] ⁺	Reaction	RT (min)	<i>mIa2</i> ^{+/+} (Area %)	<i>mIa2</i> ^{-/-} (Area %)	<i>hIa2</i> ^{+/+} <i>mIa2</i> ^{-/-} (Area %)	MS ² fragments
I	C ₁₆ H ₁₁ F ₃ N ₂ O ₂	321.0851	parent	6.17	12.8±2.16	13.7±3.48	9.20±4.75	321.084 186.038 136.057
II	C ₁₆ H ₁₁ F ₃ N ₂ O ₃	337.0800	N ⁵ -OH	5.15	4.21±1.33 [*]	13.2±4.56 [*]	5.62±1.83 [◆]	337.079 320.080 202.035 136.059
VII	C ₁₆ H ₁₁ F ₃ N ₂ O ₆ S	417.0378	N ⁵ -OH + sulfation	4.25	0.31±0.62 [*]	4.19±0.70 [*]	0.35±0.70 [◆]	337.082 202.038 136.059
VIII	C ₁₆ H ₁₁ F ₃ N ₂ O ₆ S	417.0368	3-OH + sulfation	5.70	3.33±0.57 ^{##}	1.71±0.36 [◆]	24.3±5.67 ^{##}	337.082 186.038 152.053
IX	C ₁₆ H ₁₁ F ₃ N ₂ O ₇ S	433.0317	3-OH + N ⁵ -OH + sulfation	4.80	43.9±4.94 [*]	3.82±1.13 [*]	35.3±5.37 [◆]	353.076 336.076 202.036 152.054 497.117
X	C ₂₂ H ₁₉ F ₃ N ₂ O ₈	497.1172	N ⁵ -gluc	5.21	6.26±0.26	7.04±1.25	5.66±0.29	393.112 363.093 321.085 186.038 136.054
XI	C ₂₂ H ₁₉ F ₃ N ₂ O ₉	513.1121	N ⁵ -OH + N-gluc	4.74	14.0±4.96 ^{##}	44.3±3.26 [*]	2.04±0.53 ^{##}	513.115 337.079 319.071 291.075 202.033 136.057
XII	C ₂₂ H ₁₉ F ₃ N ₂ O ₉	513.1121	N ⁵ -OH + N-gluc	5.30	6.46±1.34	7.31±3.27	9.11±2.19	513.115 337.079 320.078 293.075 186.038
XIII	C ₂₂ H ₁₉ F ₃ N ₂ O ₁₀	529.1070	N ⁵ -OH + N ⁵ -OH + N-gluc	3.88	1.75±0.32	1.48±0.17	2.31±0.39	529.101 395.079 353.070 336.070 309.059
XIV	C ₂₂ H ₁₉ F ₃ N ₂ O ₁₀	529.1070	N ⁵ -OH + 3-OH + N-gluc	4.45	7.02±1.42 [*]	3.29±0.61 [*]	6.14±0.94 [◆]	202.039 529.110 353.076 336.066 202.035 152.051

* The symbol denotes significant difference between wild-type and *CypIa2*-null mice (P<0.01)
for significant difference between wild-type and *CYP1A2*-humanized mice (P<0.01)

◆ for significant difference between *Cyp1a2*-null and *CYP1A2*-humanized mice ($P<0.01$).