Association of Human Cytochrome P450 1A1 (CYP1A1) and Sulfotransferase 1A1 (SULT1A1) Polymorphisms with Differential Metabolism and Cytotoxicity of Aminoflavone

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Abstract

Aminoflavone (AF), a clinically investigational novel anticancer agent, requires sequential metabolic activation by CYP1A1 and SULT1A1 to exert its antitumor activities. The purpose of this study was to determine the functional significance of common polymorphisms of human CYP1A1 and SULT1A1 on the metabolism and cytotoxicity of AF. To this end, Chinese Hamster V79 cells were genetically engineered to stably express human CYP1A1*1 (wild-type), CYP1A1*2C (I462V), or CYP1A1*4 (T461N) and coexpress human CYP1A1*1 with human SULT1A1*1 (wild-type), SULT1A1*2 (R213H), or SULT1A1*3 (M223V). The metabolism and cytotoxicity of AF were evaluated in these cellular models. All common variants of CYP1A1 and SULT1A1 were actively involved in the metabolic activation of AF, but with a varying degree of activity. Whereas CYP1A1 I462V variant exhibited a superior activity (mainly caused by a significantly higher Vmax) for hydroxylations of AF, expression of different CYP1A1 variants did not confer cell differential sensitivity to AF. The cells co-expressing CYP1A1*1 with SULT1A1*1, SULT1A1*2, or SULT1A1*3 displayed SULT1A1 allele-specific sensitivity to AF: SULT1A1*3 exhibited the highest sensitivity (IC50, 0.01 μmol/L), followed by SULT1A1*1 (IC50, 0.5 μmol/L), and SULT1A1*2 showed the lowest sensitivity (IC50, 4.4 μmol/L). These data suggest that the presence of low-activity SULT1A1*2 may predict poor response to AF, whereas the presence of high-activity CYP1A1/SULT1A1 alleles, especially combination of CYP1A1*2C and SULT1A1*3 or SULT1A1*1, may be beneficial to patients receiving AF. The present study provides a foundation for future clinical investigations of potential genetic biomarkers that may enable selection of patients for the greatest potential benefit from AF treatment. Mol Cancer Ther; 9(10); 2803–13. ©2010 AACR.

Introduction

Aminoflavone [AF; 5-amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one; NSC 686288] is a novel fluorinated diaminoflavone analogue that has shown antitumor activity in vitro and in vivo against a variety of human tumor types, including breast, renal, and ovarian cancers (1–4). AF is currently being evaluated in a lysine derivative prodrug form (AFP464), which is rapidly converted to AF in plasma by nonspecific plasma esterases, in phase I clinical trials at several institutions including the Karmanos Cancer Institute. It is entering phase II trials for the treatment of breast cancer. Mechanistic studies on the cytotoxicity of AF have revealed that AF induces DNA damage associated with DNA-protein cross-link, DNA single-strand breaks, and DNA replication–dependent DNA double-strand breaks (3, 5). AF also inhibits DNA synthesis and induces S-phase arrest (3). A substantial body of evidence suggests that AF requires metabolic activation by cytochrome P450 1A1 (CYP1A1) and sulfotransferase 1A1 (SULT1A1) to exert its antitumor activity (1, 2, 4, 6). Specifically, AF is metabolized by CYP1A1 to form hydroxylamines that are readily conjugated by SULT1A1, an unstable N-sulfooxy-AF metabolite that spontaneously decomposes, and highly reactive nitrene ions that react with cellular DNA and protein, leading to cell death and apoptosis (4). AF itself also induces its own metabolism by activation of transcription of CYP1A1 and SULT1A1 through the aryl hydrocarbon receptor pathway (1, 6).

The human genes for CYP1A1 and SULT1A1 are polymorphic. Besides the wild-type (CYP1A1*1), 10 alleles have been identified for human CYP1A1 (http://www.cypalleles.ki.se/). The most common single-nucleotide polymorphisms (SNP) are CYP1A1*2C (I462V) and CYP1A1*4 (T461N). Both result in amino acid substitutions in the heme binding region of the enzyme (7). Allele frequencies of CYP1A1*2C are 2.2% to 8.9% and 19.8% in Caucasians and Japanese, respectively (8–11). CYP1A1*4...
allele occurs in 2.0% to 5.7% of a Caucasian population (8–11). CYP1A1 polymorphisms have been associated with different enzyme activity (12–14) and inducibility (15), and altered susceptibility to cancer risk (16–18).

With respect to SULT1A1, besides the wild-type (SULT1A1*1), the most common SNPs are SULT1A1*2 (R213H) and SULT1A1*3 (M223V). These allele frequencies vary in different ethnic populations: In Caucasians, SULT1A1*1 (frequency, 65.6%) is the most common allele by SULT1A1*2 (35.2%) and SULT1A1*3 (1.2%); in Chinese, SULT1A1*1 is the predominant allele (91.4%), whereas SULT1A1*2 and SULT1A1*3 alleles are rare (8% and 0.6%, respectively); in African American, both SULT1A1*2 and SULT1A1*3 alleles are common (29.4% and 22.9%, respectively; ref. 19). The allelic variants of SULT1A1 have been associated with altered enzyme activity and thermal stability of the enzyme, with the SULT1A1*2 allele being associated with low enzyme activity and thermal stability (20, 21).

AF can be considered as a “prodrug” that requires sequential metabolic activation by CYP1A1 and SULT1A1 to exert its antitumor activity. Besides the liver, CYP1A1 and SULT1A1 are expressed in extrahepatic tissues including normal (e.g., lung) and tumor cells. Thus, functional genetic polymorphisms in these enzymes may influence the metabolic activation and cytotoxicity of AF in both normal and tumor cells, thereby contributing to interindividual or interethnic variability in clinical outcomes of efficacy and safety of patients treated with AF. The purpose of this study was to determine the functional significance of common polymorphisms of human CYP1A1 and SULT1A1 genes on the metabolism and cytotoxicity of AF. To this end, Chinese Hamster lung fibroblast V79 cells were genetically engineered to stably express human CYP1A1*1 (wild-type), CYP1A1*2C (I462V), or CYP1A1*4 (T461N) and coexpress human wild-type CYP1A1 with human SULT1A1*1 (wild-type), SULT1A1*2 (R213H), or SULT1A1*3 (M223V). The metabolism and cytotoxicity of AF were evaluated in these cellular models.

Materials and Methods

Chemicals and reagents

AF (NSC 686288) was provided by the National Cancer Institute (Bethesda, MD). Resorufin, 7-ethoxyresorufin, dicumarol, and DMSO were purchased from Sigma. G418 (geneticin), hygromycin B, DMEM, Opti-MEM, penicillin, streptomycin, and pcDNA3.1(+) and pcDNA3.1/Hygro(+) expression vectors were obtained from Invitrogen. QuikChange Site-Directed Mutagenesis kit was obtained from Stratagene. The wild-type human CYP1A1 cDNA was kindly provided by Dr. F.J. Gonzalez (National Cancer Institute). The wild-type human SULT1A1 cDNA and Pierce bicinechonic acid (BCA) protein assay kit were purchased from Open Biosystems Thermo Scientific. Fetal bovine serum (FBS) was obtained from Atlanta Biologicals. Purified recombinant enzymes of CYP1A1 and SULT1A1 were obtained from BD Biosciences. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H- tetrazolium inner salt (MTS) assay kit was obtained from Promega.

Cell lines

Chinese Hamster V79 cell line was obtained from the American Type Culture Collection. The cells were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in the presence of 5% CO2. The identity of the V79 cell line was verified by the American Type Culture Collection using short tandem repeat profiling analysis. No authentication was done by us.

Generation of V79 cells stably expressing human CYP1A1*1, CYP1A1*2C, or CYP1A1*4

The wild-type human CYP1A1 cDNA was inserted into the expression vector pcDNA3.1(+) that contains G418 as selection antibiotic gene, and served as a template to generate the CYP1A1*2C and CYP1A1*4 recombinant expression vector by site-directed mutagenesis using QuikChange Site-Directed Mutagenesis kit. The entire coding region of CYP1A1, including the mutated sites, was verified by complete DNA sequence. The recombinant CYP1A1 expression vector was linearized with Scal (Invitrogen) and used for transfection of cells by electroporation. In brief, V79 cells (5 × 10⁵/400 μL Opti-MEM) were transfected with 20 μg of Scal-linearized pcDNA3.1/CYP1A1*1, pcDNA3.1/CYP1A1*2C, or pcDNA3.1/CYP1A1*4 plasmids in a 2-mm gene pulser cuvette using Gene Pulser Xcell electroporation system (200 V and 950 μF; Bio-Rad). Immediately after electroporation transfection, the cells were grown in fresh DMEM (containing 10% FBS) for 48 hours. The cells were then cultured in the presence of 1 mg/mL G418 (Geneticin) until resistant clones appeared. Multiple clones for each transfection were isolated and expanded. The levels of CYP1A1 mRNA and protein expression in each clone were determined by real-time reverse transcription-PCR (RT-PCR) and Western blot, respectively, as described below. Stable clones (denoted as V79-CYP1A1*1, V79-CYP1A1*2C, and V79-CYP1A1*4) expressing comparable CYP1A1 mRNA were selected and used for functional experiments. CYP1A1 activities in the selected clones were assessed by ethoxyresorufin-O-deethylase (EROD) assay.

Generation of V79 cells stably coexpressing human CYP1A1*1 with SULT1A1*1, SULT1A1*2, or SULT1A1*3

The wild-type human SULT1A1 cDNA was inserted into the expression vector pcDNA3.1/Hygro(+) that contains hygromycin B as selection antibiotic gene, and served as a template to generate SULT1A1*2 and SULT1A1*3 recombinant expression vector by site-directed mutagenesis using QuikChange Site-Directed Mutagenesis kit. The entire coding region of SULT1A1,
including the mutated sites, was verified by complete DNA sequence. The recombinant SULT1A1 expression vectors were linearized with Sph1 (Invitrogen) and used for transfection of the V79-CYP1A1*1 cell line, as described above. The cells were cultured in the presence of 1 mg/mL G418 and 200 μg/mL hygromycin B 48 hours after transfection until resistant clones appeared. Multiple clones for each transfection were isolated and expanded. Protein expressions and mRNA levels of SULT1A1 and CYP1A1 in clones were determined by Western blot and real-time RT-PCR, as described below. Stable clones (denoted as V79-CYP1A1-SULT1A1*1, V79-CYP1A1-SULT1A1*2, and V79-CYP1A1-SULT1A1*3) expressing comparable SULT1A1 and CYP1A1 mRNA were selected and used for functional experiments.

Western blot detection of CYP1A1/SULT1A1

Cell lysates from the V79 cells that were transfected with the blank vector (negative control), CYP1A1 alone, or CYP1A1 plus SULT1A1 (~40 μg protein per lane) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. To detect protein expression of CYP1A1 and SULT1A1, membranes were probed with rabbit anti-CYP1A1 antibody (1:10,000; BD Biosciences) and mouse anti-SULT1A1 antibody (1:500; Sigma-Aldrich), respectively, and detected by the chemiluminescent system using horseradish peroxidase–conjugated goat anti-rabbit IgG (1:10,000; Santa Cruz Biotechnology) and donkey anti-mouse IgG (1:2,500; Santa Cruz Biotechnology) as the secondary antibody, respectively. The commercially available purified recombinant enzymes of CYP1A1 (5 μg per lane) and SULT1A1 (2 μg per lane; BD Biosciences) were used as the positive control. β-Actin was used as a loading control.

Real-time quantitative RT-PCR to determine mRNA levels of CYP1A1 and SULT1A1

Total RNA was extracted from cell pellets using RNeasy Plus Mini kit (Qiagen). The first-strand cDNA was synthesized using qScript cDNA SuperMix kit (Quanta Biosciences). Real-time PCR was done using FastStart SYBR Green Master (Roche Applied Science) on an iCycler iQs real-time PCR detection system (Bio-Rad). Thermal cycling conditions for CYP1A1 and SULT1A1 included an initial denaturation at 95°C for 8 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds and 72°C for 30 seconds. The reactions were normalized to GAPDH internal standard.

EROD assay

CYP1A1 enzyme activities in the CYP1A1-transfected stable clones were assessed by EROD activity, as described previously with modifications (22). Briefly, the cells (2 × 10^6 per well) were seeded in a 96-well plate and, 24 hours after seeding, washed once with PBS and incubated in culture medium containing varying concentrations of 7-ethoxyresorufin (0.5–16 μmol/L) at 37°C for 30 minutes. Dicumarol (10 μmol/L) was also added to the assay medium to prevent further metabolism of resorufin by the cytosolic enzyme diaphorase (23). At the end of incubation, a 75-μL aliquot of cell medium was collected from each well and transferred to another 96-well plate, to which 200 μL ethanol was added into each well. The formation of resorufin was determined fluorimetrically at 530-nm excitation and 590-nm emission wavelength using a Victor X3 2030 Multilabel Reader (Perkin-Elmer). A standard curve of resorufin (1–800 μmol/L) was prepared in culture medium. The protein concentration in each cell sample was determined by BCA protein assay according to the manufacturer’s protocol. The concentration of resorufin was normalized to cell protein concentration.

Metabolism of AF

The functional influence of CYP1A1 polymorphisms on the metabolism of AF was determined in the V79 cells stably expressing CYP1A1*1 (wild-type), CYP1A1*2C (I462V), or CYP1A1*4 (T461N). The cells were seeded in 10-cm Petri dishes (5 × 10^6 per dish). Twenty-four hours after seeding, the cells were incubated at 37°C in DMEM containing varying concentrations of AF (i.e., 0.5, 1, 2.5, 5, 10, and 25 μmol/L) for 6 hours; at the end of incubation, the cells were washed five times with ice-cold PBS, and cell pellet was collected and stored at −80°C until analysis. Two independent experiments were done, with duplicates in each experiment.

AF was also incubated with CYP1A1 Supersomes (BD Biosciences) as the positive control. The reaction mixtures (total volume, 0.2 mL) containing 50 μmol/L AF, 1.3 mmol/L NADPH, 3.3 mmol/L glucose-6-phosphate, 0.4 units/mL glucose-6-phosphate dehydrogenase, 3.3 mmol/L magnesium chloride, and 100 pmol/mL CYP1A1 enzyme in 100 mmol/L potassium phosphate buffer (pH 7.4) were incubated at 37°C for 30 minutes and terminated by adding 100 μL acetone and centrifugation at 14,000 rpm at 4°C for 10 minutes. The supernatant was collected and stored at −80°C until analysis. An incubation with control Supersomes was done simultaneously with the negative control.

The functional influence of SULT1A1 polymorphisms on the metabolism of AF was determined in the V79-CYP1A1-SULT1A1*1, V79-CYP1A1-SULT1A1*2, and V79-CYP1A1-SULT1A1*3 cells. The cells were seeded in 10-cm Petri dishes (5 × 10^6 per dish). Twenty-four hours...
after seeding, the cells were incubated at 37°C in DMEM containing 1 and 10 μmol/L AF for 6 hours; at the end of incubation, the cells were washed five times with ice-cold PBS, and cell pellet was collected and stored at −80°C until analysis. Three independent experiments were done, with duplicates in each experiment.

Bioanalytic assay

The intracellular concentrations of AF and its metabolites were determined using a validated high-performance liquid chromatography (HPLC) method. The cell pellet was resuspended in 300 μL PBS. Into an aliquot of 250 μL cell suspension, 1 mL of acetyl acetate was added, vortex mixed for 1 minute, and centrifuged at 14,000 × g and 4°C for 10 minutes. The supernatant was evaporated to dryness under nitrogen gas, and the residual was reconstituted in 100 μL of mobile phase (acetonitrile/0.5% formic acid in water, 42:58, v/v), and an aliquot of 50 μL was injected to Shimadzu LC-10ADvp HPLC system equipped with a photodiode detector (Shimadzu Corp.). AF and its metabolites were separated on an X Terra RP18 column (5 μm, 150 × 3.9 mm inside diameter; Waters) with a mobile phase consisting of acetonitrile−0.5% formic acid in water (42:58, v/v) at a flow rate of 1 mL/min. The detection wavelength was set at 350 nm, and the autosampler temperature was set at 4°C. The calibration curve for AF was constructed over the concentration range of 0.02 to 200 μmol/L. The within- and between-day precision and accuracy for the calibrators and quality control plasma samples were <15%. Because the reference standards of AF metabolites were not available, the concentrations of all metabolites were calculated from the calibration curve of AF. The protein content in each cell pellet sample was determined by BCA protein assay using human serum albumin as standards. The intracellular concentrations of AF and its metabolites were normalized to cell protein content.

The monohydroxy and dihydroxy metabolites of AF were identified using a Waters Quattro Micro triple quadrupole mass spectrometer (MS; Waters). HPLC separation was done on a Waters X Terra MS column (50 mm × 2.1 mm inside diameter) with a gradient mobile phase consisting of acetonitrile−0.5% formic acid in water at a flow rate of 0.3 mL/min. The eluent was monitored at the m/z of 321.1 (for AF), 337.1 (for monohydroxy-AF), and 353.1 (for dihydroxy-AF) under the following MS conditions: source temperature of 120°C, desolvation temperature of 350°C, cone voltage of 40 V, and capillary voltage of 300 V.

Cytotoxicity of AF

The cytotoxicity of AF in the V79 cell lines stably expressing CYP1A1 alone or coexpressing CYP1A1 and SULT1A1 was evaluated by MTS assay. The subconfluent cells were trypsinized and seeded in 96-well plates at the seeding number of 1 × 10^4 per well. Twenty-four hours after seeding, the cells were incubated in culture
medium containing AF at the concentrations of 0 (control), 0.01, 0.1, 0.5, 1, 5, and 10 μmol/L for 72 hours. At the end of treatment, drug-containing medium from all wells was removed, and cells were fed with 100 μL of drug-free medium followed by addition of 20 μL of the combined MTS/phenazine methosulfate solution. The plates were incubated at 37°C for 4 hours, after which the amount of soluble formazan produced by cellular reduction of MTS was measured spectrophotometrically at 490 nm using a Victor X3 2030 Multilabel Reader (Perkin-Elmer). The MTS assay for each cell lines was done in quadruplicate in three independent experiments.

**Data analysis**

The enzyme kinetic constants of maximum rate of reaction ($V_{max}$) and substrate affinity ($K_m$) for formations of hydroxylated AF metabolites were estimated by fitting the pooled rate measurement data from two independent experiments (with duplicates for each) to Michaelis-Menten equation (Eq. A). The drug concentration producing 50% inhibition of cell growth compared with the control ($EC_{50}$) was determined by fitting the pooled cell survival data from three independent experiments to an inhibitory effect Sigmoid $E_{max}$ model (Eq. B). All fittings were done using nonlinear regression with the software WinNonlin 5.0 (Pharmsight Corp.). The estimated values

![Figure 2](https://example.com/figure2.jpg)
of enzyme kinetic constants ($V_{\text{max}}$ and $K_m$) and EC$_{50}$ were presented as mean ± SE as determined by nonlinear regression.

\[ v = \frac{V_{\text{max}} \times S}{K_m + S} \]  

where $S$ is substrate concentration, $v$ is the rate of metabolite formation, $V_{\text{max}}$ is maximum metabolite formation velocity, and $K_m$ is the substrate concentration at which 50% of $V_{\text{max}}$ is obtained.

\[ E = E_{\text{max}} \times \left(1 - \frac{C \gamma}{EC_{50} + C \gamma}\right) \]  

where $E$ represents percent cell survival under treatment relative to the control (no drug treatment), $E_{\text{max}}$ is percent cell growth of the control (100%), $C$ is the drug concentration, EC$_{50}$ is the drug concentration producing 50% inhibition of cell growth compared with control, and $\gamma$ is the Hill coefficient.

**Results**

**Stable expression of CYP1A1/SULT1A1 in V79 cells**

Chinese Hamster lung fibroblast V79 cells, genetically engineered to express metabolizing enzymes, are widely used for determining the contributions of specific enzymes to the metabolic profile of a given xenobiotic or for examining the role of metabolism in the drug cytotoxicity (24, 25). To determine the functional influence of CYP1A1 polymorphisms on the metabolism and cytotoxicity of AF, the V79 cells were stably transfected with human CYP1A1$^{*1}$ (wild-type), CYP1A1$^{*4}$ (T461N), or CYP1A1$^{*2C}$ (I462V). To further determine the functional influence of SULT1A1 polymorphisms on the metabolic activation of AF, the V79 cells were cotransfected with human CYP1A1$^{*1}$ and SULT1A1$^{*1}$ (wild-type), SULT1A1$^{*2}$ (R213H), or SULT1A1$^{*3}$ (M223V). Real-time RT-PCR and Western blot indicated a comparable mRNA/protein expression of CYP1A1 or SULT1A1 among the selected stable clones expressing different variants of CYP1A1 or SULT1A1 (Fig. 1A and B).

EROD assay suggested that the V79-CYP1A1$^{*2C}$ cells exhibited a significant higher catalytic rates in terms of $V_{\text{max}}$ (3,111 ± 185 pmol/min/mg protein) compared with the V79-CYP1A1$^{*4}$ ($V_{\text{max}}$, 1,269 ± 918 pmol/min/mg protein) and V79-CYP1A1$^{*1}$ cells ($V_{\text{max}}$, 918 ± 28 pmol/min/mg protein; Fig. 1C). Our results are in good agreement with earlier investigations in which a higher EROD activity of the CYP1A1 I462V variant was observed for the purified recombinant enzyme from *Escherichia coli* or baculovirus/insect expression systems (26, 27) as well as for microsomes prepared from sf9 cells coexpressing human CYP1A1 variants and cytochrome P450 reductase (28).

### Table 1. Enzyme kinetic constants for formations of hydroxylated AF metabolites in the V79 cells with stable expression of CYP1A1$^{*1}$ (wild-type), CYP1A1$^{*4}$ (T461N), or CYP1A1$^{*2C}$ (I462V)

<table>
<thead>
<tr>
<th>Formation</th>
<th>$V_{\text{max}}$ (pmol/h/mg protein)</th>
<th>$K_m$ (μmol/L)</th>
<th>$V_{\text{max}}/K_m$ (μL/h/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formation of 4'-N-OH-AF (RT, 10.0 min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1A1$^{*1}$ (wild-type)</td>
<td>34.8 ± 2.8</td>
<td>3.2 ± 0.8</td>
<td>10.9</td>
</tr>
<tr>
<td>CYP1A1$^{*4}$ (T461N)</td>
<td>44.5 ± 4.0</td>
<td>4.6 ± 1.2</td>
<td>9.7</td>
</tr>
<tr>
<td>CYP1A1$^{*2C}$ (I462V)</td>
<td>112 ± 11.2</td>
<td>7.2 ± 1.7</td>
<td>15.6</td>
</tr>
<tr>
<td>Formation of 5'-N-OH-AF (RT, 5.9 min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1A1$^{*1}$ (wild-type)</td>
<td>8.5 ± 3.1</td>
<td>24.7 ± 15.1</td>
<td>0.3</td>
</tr>
<tr>
<td>CYP1A1$^{*4}$ (T461N)</td>
<td>7.3 ± 2.4</td>
<td>17.5 ± 10.7</td>
<td>0.4</td>
</tr>
<tr>
<td>CYP1A1$^{*2C}$ (I462V)</td>
<td>14.3 ± 2.7</td>
<td>4.2 ± 2.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Formation of 3-OH-AF (RT, 10.8 min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1A1$^{*1}$ (wild-type)</td>
<td>3 ± 0.2</td>
<td>1.2 ± 0.3</td>
<td>2.5</td>
</tr>
<tr>
<td>CYP1A1$^{*4}$ (T461N)</td>
<td>2.4 ± 0.2</td>
<td>1.5 ± 0.6</td>
<td>1.7</td>
</tr>
<tr>
<td>CYP1A1$^{*2C}$ (I462V)</td>
<td>8.3 ± 1.0</td>
<td>2.2 ± 0.9</td>
<td>3.9</td>
</tr>
<tr>
<td>Formation of 4',5'-N-OH-AF (RT, 3.5 min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1A1$^{*1}$ (wild-type)</td>
<td>5.2 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td>5.5</td>
</tr>
<tr>
<td>CYP1A1$^{*4}$ (T461N)</td>
<td>5.6 ± 0.3</td>
<td>1.8 ± 0.4</td>
<td>3.1</td>
</tr>
<tr>
<td>CYP1A1$^{*2C}$ (I462V)</td>
<td>14.7 ± 1.5</td>
<td>3.5 ± 1.1</td>
<td>4.2</td>
</tr>
</tbody>
</table>

NOTE: Enzyme kinetic constants of $V_{\text{max}}$ and $K_m$ were estimated by fitting the pooled rate measurement data from two independent experiments (with duplicates in each experiment) to Michaelis-Menten equation. Data represent mean ± SE as determined by nonlinear regression.
CYP1A1 allele-specific metabolism of AF

When incubated with the V79-CYP1A1*1, V79-CYP1A1*2C, or V79-CYP1A1*4 cells, AF produced a similar metabolite profile as that from incubation with CYP1A1 Supersomes (Fig. 2), suggesting that the metabolism of AF in these cell lines is predominantly mediated by CYP1A1. Consistent with the findings from earlier studies (29), HPLC and liquid chromatography-tandem MS (LC-MS/MS) analyses suggested that the major metabolites of AF formed in the CYP1A1 Supersomes reaction as well as in CYP1A1-expressing cells were 4N-OH-AF [retention time (RT), 10.0 minutes], 5N-OH-AF (RT, 5.9 minutes), 3-OH-AF (RT, 10.8 minutes), 4N,5N-diOH-AF (RT, 3.4 minutes), and 3,5N-diOH-AF (RT, 4.4 minutes; Fig. 2). Metabolic kinetic studies revealed that all CYP1A1 variants catalyzed hydroxylations of AF, yet with varying catalytic activity (Table 1; Fig. 3). Whereas there was no apparent difference in the enzyme kinetic constants of V_{max} and K_m between the cells expressing CYP1A1 wild-type and T461N variant, the cells expressing I462V variant exhibited significantly higher catalytic capacity in terms of the V_{max} for the formations of both monohydroxylated and dihydroxylated AF metabolites. The V_{max} for I462V for the formations of 4N-OH-AF, 5N-OH-AF, 3-OH-AF, and 4N,5N-OH-AF was estimated as 3.2-, 1.7-, 2.8-, and 2.8-fold, respectively, of those for the wild-type enzyme (Table 1; Fig. 3). With regard to the K_m, the I462V had a higher affinity for the formation of 5N-OH-AF (K_m, 4.2 μmol/L for I462V versus 24.7 μmol/L for wild-type) while exhibiting a higher K_m value for the formations of 4N-OH-AF, 3-OH-AF, and 4N,5N-OH-AF than the wild-type enzyme (Table 1). Taken together, I462V exhibited a 1.4-, 11.3-, and 1.6-fold higher intrinsic enzyme activity or catalytic efficiency (V_{max}/K_m) for the formations of 4N-OH-AF, 5N-OH-AF, and 3-OH-AF, respectively, than the wild-type (Table 1).

SULT1A1 allele-specific metabolism of AF

Because the sulfate metabolites of AF were unstable and not detectable under the present HPLC condition, the reductions in the intracellular concentrations of AF and hydroxylated metabolites may indicate the extent of SULT1A1-mediated sulfation in the cells coexpressing CYP1A1 and SULT1A1. As shown in Fig. 4A, after the cells were incubated with 1 μmol/L AF for 6 hours, the intracellular concentration of AF in the cells expressing

![Figure 3](https://example.com)
CYP1A1 alone and coexpressing CYP1A1 with SULT1A1*1, SULT1A1*2, and SULT1A1*3 was 42%, 19%, 44%, and 14%, respectively, of that in the V79-vector cells (control); similarly, after the cells were incubated with 10 μmol/L AF for 6 hours, the intracellular AF concentrations in the respective cells were 64%, 27%, 64%, and 26%, respectively, of that in the control. These results suggested that the cells coexpressing CYP1A1 with SULT1A1*1 or SULT1A1*3, but not SULT1A1*2, produced significantly more overall metabolism of AF than those expressing CYP1A1 alone. The SULT1A1 allele-specific metabolism was consistently observed for the disappearance of a detectable AF hydroxylated metabolite (N,N'-N-OH-AF; Fig. 4B). Taken together, these data indicated that SULT1A1.2 exhibited a lower enzyme activity in the phase II metabolism of AF, whereas SULT1A1.3 showed an equivalent activity compared with the wild-type enzyme.

**SULT1A1 allele-specific sensitivity to AF**

The dose-response curves and IC_{50} values of AF in the V79-vector cells (control) and in the cells stably expressing CYP1A1 alone or coexpressing CYP1A1 and SULT1A1 are shown in Fig. 5 and Table 2. The V79-vector cell line was resistant to AF. Expression of different allelic variant of CYP1A1 all conferred cell sensitivity to AF without apparent allelic-specific difference in the IC_{50} values. Co-expressions of SULT1A1 with CYP1A1 rendered cells more sensitive to AF, yet with SULT1A1 allele-specific sensitivity: The cells expressing SULT1A1*3 exhibited the highest sensitivity, followed by SULT1A1*1, whereas SULT1A1*2 exhibited the lowest sensitivity (Fig. 5; Table 2).

**Discussion**

AF, a clinically investigational novel anticancer agent, requires metabolic activation by CYP1A1 and SULT1A1 to exert its antitumor activities (2, 4). In the present in vitro

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**Table 2.** IC_{50} values of AF

<table>
<thead>
<tr>
<th>No.</th>
<th>Cell line</th>
<th>IC_{50} (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>V79</td>
<td>&gt;10</td>
</tr>
<tr>
<td>2</td>
<td>V79-CYP1A1*1</td>
<td>8.1 ± 0.6</td>
</tr>
<tr>
<td>3</td>
<td>V79-CYP1A1*4</td>
<td>7.6 ± 0.7</td>
</tr>
<tr>
<td>4</td>
<td>V79-CYP1A1*2C</td>
<td>8.4 ± 0.6</td>
</tr>
<tr>
<td>5</td>
<td>V79-CYP1A1-SULT1A1*1</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>6</td>
<td>V79-CYP1A1-SULT1A1*2</td>
<td>4.4 ± 1.8</td>
</tr>
<tr>
<td>7</td>
<td>V79-CYP1A1-SULT1A1*3</td>
<td>0.01 ± 0.01</td>
</tr>
</tbody>
</table>

NOTE: IC_{50} values were estimated by fitting the pooled survival data from three independent experiments to an inhibitory effect Sigmoid E_{max} model. Data represent mean ± SE as determined by nonlinear regression.
study, we showed the functional significance of common polymorphisms within human CYP1A1 and SULT1A1 genes on the metabolism and cytotoxicity of AF. Our results showed that all common variants of CYP1A1 and SULT1A1 were actively involved in the metabolic activation of AF, but with a varying degree of activity. Most remarkably, the cells expressing CYP1A1*2C (I462V) exhibited a superior catalytic activity (mainly caused by a significantly higher V_max) in the phase I metabolism (i.e., hydroxylations) of AF, whereas cells expressing SULT1A1*2 (R213H) possessed a diminished capacity to sulfate and activate hydroxylated AF metabolites.

With regard to the functional significance of the I462V and T461V variants of CYP1A1, our findings are in striking agreement with an earlier investigation using purified recombinant CYP1A1 enzymes, in which CYP1A1.2 harboring I462V showed a significantly higher catalytic efficiency (V_max/K_m), mainly caused by a significant higher V_max toward hydroxylations of 17β-estradiol and estrone, whereas CYP1A1.4 harboring T461N exhibited activity comparable with the wild-type enzyme (13). A superior activity of the I462V variant was also observed toward a CYP1A1 probe substrate ethoxyresorufin in the present study (Fig. 2) as well as in earlier studies (27). Different inducibility has been reported among the allelic variants of CYP1A1 (30, 31). For example, CYP1A1*2A and CYP1A1*2B harboring a 3801T>C transition, resulting in a new MspI restriction endonuclease site, are associated with a highly inducible phenotype of the enzyme (32). However, the observed superior activity of the cells expressing CYP1A1*2C (I462V) for hydroxylations of AF could not be explained by differential enzyme expression or inducibility because (a) real-time RT-PCR and Western blot did not detect any apparent differences in mRNA and protein expression levels of CYP1A1 among the selected stable clones expressing CYP1A1*1, CYP1A1*2C, and CYP1A1*4 (Fig. 1) and (b) treatment of the CYP1A1-expressing V79 cells with AF did not induce CYP1A1 mRNA expression (probably due to the lack of aryl hydrocarbon receptor in V79 cells; data not shown). A more rational underlying mechanism could be variant-specific interactions of the enzyme with P450 reductase involving differential electron transfer. This interpretation is supported by a homology model of human CYP1A1 based on the CYP2C5 crystallographic template, which locates the mutated residue Val1084 on the proximal face of the heme group (adjacent to the thiolate ligand; ref. 7), a region where the interaction with P450 reductase is generally assumed to take place (33). It is plausible that the I462V amino acid substitution alters the electronic state of the heme group (adjacent to the thiolate ligand) (34), leading to an electronic state which affects the activity and stability of the complex.

It is noteworthy that tamoxifen, a widely used drug in the world for the treatment and prevention of estrogen receptor–positive breast cancer, undergoes a similar sequential metabolic activation by CYP2D6 and SULT1A1. Specifically, tamoxifen is metabolized predominantly by CYP2D6 to form two active metabolites, 4-hydroxytamoxifen (4-OH-TAM) and 4-hydroxy-N-desmethyltamoxifen (endoxifen), which show much
greater affinity for the estrogen receptor than tamoxifen (36, 37); furthermore, 4-OH-TAM and endoxifen are metabolized by SULT1A1 to form highly reactive products, leading to DNA adducts (38, 39). There is substantial evidence that functional polymorphisms (including SNPs and gene copy number variations) of CYP2D6 and SULT1A1 are associated with tamoxifen pharmacokinetics and clinical outcomes of efficacy and adverse effects (40–44). Of note, a recent study involving 1,325 breast cancer patients receiving tamoxifen suggests that the presence of two functional CYP2D6 alleles is associated with better clinical outcomes, and the presence of nonfunctional or reduced-function alleles with worse outcomes (44). In addition, the low-activity SULT1A1*2 allele has been associated with significantly poorer overall survival in breast cancer patients receiving tamoxifen (49).

Likewise, given the crucial roles of CYP1A1 and SULT1A1 in AF bioactivation, it is likely that functional polymorphisms in human CYP1A1 and SULT1A1 genes may influence clinical outcomes of patients treated with AF. The present study clearly indicated that the CYP1A1*2C (I462V) variant exhibited superior activity in the phase I metabolism (hydroxylations) of AF, whereas the SULT1A1*2 variant conferred the lowest cellular sensitivity to AF. It would be expected that the presence of low-activity SULT1A1*2 allele may predict poor response to AF, whereas the presence of high-activity CYP1A1/SULT1A1 alleles, especially the combination of CYP1A1*2C and SULT1A1*3 or SULT1A1*1, may be beneficial to patients receiving AF. The present study provided a foundation for future clinical investigations of potential genetic biomarkers that may enable selection of patients for the greatest benefit from AF treatment. Obviously, additional polymorphisms within regions of the CYP1A1 and SULT1A1 genes other than the open reading frame, such as with the promoter or introns as well as variation in the regulation of transcription or gene copy numbers, could also have functional or clinical significance on the metabolic activation of AF. The haplotypes of the polymorphisms in CYP1A1 and SULT1A1 could be highly significant in terms of the pharmacokinetics, efficacy, and safety of AF. All of those possibilities need to be explored in the future in vitro and clinical studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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