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Arginine to lysine 108 substitution in recombinant CYP1A2 abolishes methoxyresorufin metabolism in lymphoblastoid cells

[1,2]Nicholas E. Hadjokas, [3]Renke Dai, [3]Fred K. Friedman, [2,4]Michael J. Spence, [5,6,7]Barry J. Cusack, [2,6,7]Robert E. Vestal & *[,2,4]Yongsheng Ma

[1]Pharmacologist, Research Service, Department of Veterans Affairs Medical Center, Boise, Idaho, ID 83702, U.S.A.; [2]Mountain States Medical Research Institute, Boise, Idaho, ID 83712, U.S.A.; [3]Laboratory of Metabolism, NCI, NIH, Bethesda, Maryland, MD 20892, U.S.A.; [4]Assistant Research Scientist, Research Service, Department of Veterans Affairs Medical Center, Boise, Idaho, ID 83702, U.S.A.; [5]Chief of Gerontology, Medical Service, Department of Veterans Affairs Medical Center, Boise, Idaho, ID 83702, U.S.A.; [6]Department of Medicine, University of Washington, Seattle, Washington, WA 98195, U.S.A. and [7]Department of Pharmacology, University of Washington, Seattle, Washington, WA 98195, U.S.A.

1 Cytochrome P4501A2 (CYP1A2) activates a large number of procarcinogens to carcinogens. Phytochemicals such as flavones can inhibit CYP1A2 activity competitively, and hydroxylated derivatives of flavone (galangin) may be potent, selective inhibitors of CYP1A2 activity relative to CYP1A1 activity. Molecular modelling of the CYP1A2 interaction with hydroxylated derivatives of flavone suggests that a number of hydrophobic residues of the substrate-binding domain engage in hydrogen bonding with such inhibitors.

2 We have tested this model using site-directed mutagenesis of these residues in expression plasmids transfected into the human B-lymphoblastoid cell line, AHH-1 TK+/−.

3 Consistent with the molecular model's predicted placement in the active site, amino acid substitutions at the predicted residues abolished CYP1A2 enzymatic activity.

4 Transfected cell lines contained equal amounts of immunoreactive CYP1A2.

5 Our results support the molecular model's prediction of the critical amino acid residues present in the hydrophobic active site, residues that can hydrogen bond with CYP1A2 inhibitors and modify substrate binding and/or turnover.

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Abbreviations: CYP, cytochrome P450; CYP1A1, cytochrome P4501A1; CYP1A2, cytochrome P4501A2; CYP1A2 108 R-K, cytochrome P4501A2 bearing amino acid substitution at residue 108 (arginine to lysine); CYP1A2 313 D-N, cytochrome P4501A2 bearing amino acid substitution at residue 313 (aspartate to asparagine); CYP1A2 321 T-V, cytochrome P4501A2 bearing amino acid substitution at residue 321 (threonine to valine); CYP1A2 385 T-V, cytochrome P4501A2 bearing amino acid substitution at residue 385 (threonine to valine); EROD, ethoxyresorufin-O-deethylase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; h1A1v2, transfectant human lymphoblastoid cell line that contains two CYP1A1 cDNA-containing expression cassettes per vector molecule; h1A2v2, transfectant human lymphoblastoid cell line that contains two CYP1A2 cDNA-containing expression cassettes per vector molecule; MROD, methoxyresorufin-O-deethylase; P450BM3, cytochrome P450 of Bacillus megaterium

Introduction

Cytochromes P450 (CYP) are a superfamily of haemoprotein enzymes responsible for the metabolism, detoxification and elimination of many endogenous and exogenous chemicals (Black & Coon, 1987; Porter & Coon, 1991). The P4501A gene family has two members, CYP1A1 and CYP1A2. CYP1A1 is a highly conserved xenobiotic-metabolizing enzyme with common induction properties and catalytic activities evident among mammals, chicken and fish (Gonzalez, 1992). CYP1A1 is induced in lung tissue by polycyclic aromatic hydrocarbons and is involved in the phase I metabolism of these compounds, which may result in lung carcinogenesis. CYP1A2 is constitutively expressed in human liver where it is capable of metabolizing and activating numerous promutagens and procarcinogens, including 2-acetylaminofluorene, acetanilide, aflatoxin B_1, and 4-aminobiphenyl and other arylamines (Guengerich, 1988; Guengerich & Shimada, 1991). Environmental or genetic factors that specifically modulate the activities of CYP1A1 or CYP1A2 may significantly modify risk for development of cancers associated with these P450s without affecting metabolism by other P450 superfamilies.

Previous studies, including those from our laboratory, have demonstrated that many factors contained in plant-derived foods, including some flavonoids, are potent, selective inhibitors of CYP1A isozymes and therefore could be potential agents for cancer chemoprevention (Digiovanni, 1990; Hertog *et al*., 1993; Zhai *et al*., 1998a,b). Analysis of the comparative inhibition of CYP1A1 and CYP1A2 by a series of flavonoids with different hydroxyl substitutions revealed that 3,5,7 trihydroxyflavone (galangin) was the most

*Author for correspondence at: VA Medical Center, Research Service (151), 500 West Fort Street, Boise, Idaho, ID 83702-4598, U.S.A. E-mail: yongsheng.ma@med.va.gov

potent CYP1A2 inhibitor (K_i of 8 nM) (Zhai *et al*., 1998a). Among the hydroxylated flavones investigated, galangin also exhibited the greatest isozyme selectivity. Galangin exhibited 5 fold greater inhibition of CYP1A2 than of CYP1A1 based upon analysis of reciprocal IC_{50} values to facilitate comparison of their potencies.

The substrate-specificities of mammalian P450s have been well characterized. However, the structural basis of substrate recognition is unclear since, except for a modified rabbit 2C5 (Williams *et al*., 2000), three-dimensional structures have not been determined experimentally as has been done for bacterial P450s (Dai *et al*., 2000). To identify potent, selective inhibitors of these isozymes, it is crucial to locate the sites of interaction between P450 and substrates/inhibitors (Halpert, 1995; Poulos, 1988). Hence, computational, molecular modelling approaches in drug metabolism, particularly for the study of cytochromes P450, are being developed (Szklarz & Halpert, 1997; Lewis, 1999; Dai *et al*., 2000). We have developed a computer model for the structure of CYP1A2 to explain its interaction and differential inhibition by derivatives of flavone (Dai *et al*., 1998). The model predicts that the potent, selective CYP1A2 inhibitor galangin interacts with the substrate binding domain of CYP1A2 *via* three hydrogen-bonding interactions in the hydrophobic active site. The 3-hydroxyl oxygen atom of galangin is hydrogen-bonded with the side-chain NH group of Arg-108. The 3-hydroxyl and 4-carbonyl groups of galangin are both capable of hydrogen bonding to the side chain of Thr-385. The 7-hydroxyl group of galangin is hydrogen-bonded to the side chain of Gln-141. In contrast, flavone, a less potent or selective inhibitor, engages in hydrogen-bonding only with Thr-321.

To validate the computer model, we have made conservative amino acid replacements at these sites using site-directed mutagenesis of expression plasmids that were subsequently transfected into a human lymphoblastoid cell line (AHH-1 TK+/−). The model suggests that substitution of lysine for Arg-108 should decrease the hydrogen-bonding of substrates while preserving positive-charge interactions with the nearby Asp-110 residue. Thr-321, located in the I helix, corresponds to Thr-252 of bacterial P450cam and Thr-319 of rat P4501A2, binds molecular oxygen during catalysis (Poulos *et al*., 1987; Tuck *et al*., 1993).

In this work we validate the model using site-directed mutagenesis and cellular expression of wild-type and mutant CYP1A2 cDNA combined with *in vitro* enzyme substrate metabolism studies. Our results satisfactorily validate the molecular model by supporting the model's identification of amino acid residues that are in the CYP1A2 active site. Perturbation of the residues hypothesized to be in the active site modified enzyme activity. We conclude that Arg-108, Thr-321, and Thr-385 are likely to be critical sites of such CYP1A2 interaction with both inhibitors and substrates; be it by hydrogen bonding, steric hindrance, and/or hydrophobic interaction.

Methods

Chemicals

RPMI medium 1640 (with L-histidinol, without L-glutamine, without L-histidine) was purchased from GENTEST (Woburn, MA, U.S.A.) while conventional RPMI medium 1640 was obtained from Sigma (St. Louis, MO, U.S.A.). Horse serum, L-glutamine, hygromycin B, methoxyresorufin, ethoxyresorufin, and other chemicals utilized in these studies were purchased from Sigma (St. Louis, MO, U.S.A.). The oligonucleotides were synthesized by GIBCO-BRL, Life Technologies (Palo Alto, CA, U.S.A.).

Construction of expression plasmids

The eukaryotic expression vector HyHo-pL4 was a gift from Dr Bruce W. Penman (GENTEST Co., Woburn, MA, U.S.A.). The HyHo-pL4 vector confers resistance to hygromycin B and L-histidinol during subsequent selection of transfected cells. CYP1A2 cDNA (−8 EcoRI to DraI +1556, see ref. Jaiswal *et al*., 1986) was a gift from Dr Frank J. Gonzalez (NCI, NIH, Bethesda, MD, U.S.A.). This CYP1A2 cDNA was first cloned into pBlueKS vector at *Eco*RI and *Eco*RV sites to get proper subcloning sites. The insert was then released with *Xba*I and *Xho*I. It was then cloned into *Xba*I and *Sal*I (compatible with *Xho*I) sites of the expression vector HyHo-pL4 and designated p1A2W/T.

Mutagenesis

Mutant oligonucleotides were synthesized and gel-purified by GIBCO-BRL, Life Technologies (Palo Alto, CA, U.S.A.). Complementary mutant oligos, size and nucleotide alterations are shown in Figure 1. The mutant plasmids were constructed with QuikChange™ Site-Directed Mutagenesis kit using p1A2W/T as a template according to the manufacturer's manual (Stratagene, La Jolla, CA, U.S.A.). Mutations were confirmed by sequencing analysis (Biotechnology Center, Utah State University, Logan, UT, U.S.A.).

Cell culture and stable transfection

The parental AHH-1 TK+/− is a human B-lymphoblastoid cell line and was a gift from Dr Bruce W. Penman (GENTEST Co., Woburn, MA, U.S.A.). Cells were grown in RPMI 1640 supplemented to 9% v v^{-1} horse serum and incubated in a 37°C incubator with a 5% CO_2 atmosphere. Cells were split every other day and the density was maintained between 2×10^5 to 1.2×10^6 cells ml^{-1}. Cells were transfected by electroporation. Ten μg of plasmid DNA in 20 μl water were transfected into the 1×10^6 cells in 0.8 ml in a 0.4 cm gap cuvette by

Figure 1 CYP1A2-cDNA mutations investigated in this study and the oligonucleotides utilized to create them. Underlined sequences represent complementary oligonucleotides utilized to construct mutant cDNA. The dotted, underlined sequences represent codons that were modified to create amino acid substitutions. The codons generated in mutant cDNA and the amino acid modification made are listed immediately beneath each substitution.

electroporation at 2.5 KV/resistance mode, 360 Ω resistance and 700 V charging voltage using a BTX600 electroporation unit (BTX Inc., San Diego, CA, U.S.A.). Following electroporation, selection for cells expressing CYP1A2 was achieved by growth of transfectant cells in RPMI 1640 media in the presence of 400 μg ml^{-1} hygromycin B for two days followed by three days of growth in the same media containing 200 μg ml^{-1} hygromycin B. Cells fully resistant to hygromycin B were then grown in RPMI 1640 media containing 2 mM histidinol without histidine and maintained at a concentration of 2×10^5 to 1.2×10^6 cells ml^{-1}. Cells fully resistant to histidinol were obtained in 2–3 weeks.

Additionally, as a control for functional studies, the h1A2v2 and h1A1v2 cell lines were obtained from GENTEST (Woburn, MA, U.S.A.). These cell lines constitutively expresses CYP1A2 or CYP1A1 at high levels due to transfection with vectors containing two CYP1A-cDNA expression cassettes per vector molecule.

Preparation of microsomes

Microsomes were prepared as described by Penman *et al.* (1993). Briefly, 5×10^8 cells were treated with 20 μg ml^{-1} 5-aminolevulinic acid to induce P450 expression one day prior to harvesting cells. Cells were then collected in 250 ml centrifuge bottles at 4°C, 1000 r.p.m. for 7 min and the pellet were washed in 10 ml ice cold saline and centrifuged at 1500 r.p.m. for 5 min at 4°C. The pellet was resuspended in ice cold H_2O (4 ml gm^{-1} of cells), transferred to a large glass/glass dounce and disrupted with 10 strokes of pestle A followed by a brief pulse with a cell sonicator. The disrupted cell suspension was diluted with 0.5 M potassium phosphate to yield a final concentration of 0.1 M potassium phosphate and centrifuged at $9000\times g$ for 10 min. The supernatant was centrifuged at $100{,}000\times g$ for 6 min (at speed). The high-speed pellet was quickly resuspended in 0.1 M potassium phosphate buffer and frozen as aliquots at −80°C. Microsomal protein concentrations were determined by BCA protein assay (Pierce Chemical Company, Rockford, IL, U.S.A.) using bovine serum albumin as the standard.

Preparation of cellular protein extracts

Cellular proteins were harvested with Mammalian Protein Extraction Reagent (M-PER®, Pierce Chemical Co., Rockford, IL, U.S.A.) according to the manufacturer's protocol. Cells (10^7 cells) were harvested and mixed gently with 0.5 ml M-PER® for 10 min. Cellular debris was pelleted by centrifugation at $27{,}000\times g$ for 15 min, supernatant transferred to a clean tube and stored at −80°C until further analysis (Western analysis).

CYP1A functional activity (methoxy-resorufin-O-deethylase [MROD] or ethoxy-resorufin-O-deethylase [EROD] activity)

MROD activity was measured in whole cells according to Crespi *et al.* (1985). Briefly, cell cultures were electronically counted in a Coulter EPICS XL flow cytometer subsequent to generation of a unicellular suspension by 6-passes through an 18-g needle. Approximately 11.1×10^6 cells were harvested and resuspended in 2.0 ml media (no phenol red). Cells (180 or 90 μl per well) were dispensed into wells of a 96-well plate and all wells were adjusted to 180 μl volume. Substrates or vehicle (20 μl of methoxyresorufin, ethoxyresorufin, or DMSO vehicle) were added to wells to yield final concentrations of 2.5 μM and 0.1% v v^{-1} DMSO. The apparent Km's of wild-type CYP1A2 for methoxyresorufin and ethoxyresorufin were reported to be 0.77 and 0.39 μM, respectively (Zhai *et al.*, 1998a), but another study suggests a higher Km for ethoxyresorufin (1.7 μM; Parikh *et al.*, 1999). In preliminary studies we observed Km's of 0.25 and 0.50 μM for methoxyresorufin and ethoxyresorufin, respectively. Cell culture plates were incubated at 37°C for up to 4 h and the fluorescence of the resorufin metabolite using either substrate was measured in Labsystems Fluoroskan Ascent FL fluorometer at an excitation wavelength of 544 nm and an emission wavelength of 612 nm. Standard curves of resorufin were prepared for each experiment by dispensing 5, 10, 20, 50, 100, 150, or 200 pmoles of resorufin per well (200 μl well^{-1}). Fluorescence was linearly related to picomoles of resorufin (fluorescence = 0.0215 pmole^{-1}, $R^2 = 0.99$). An extinction coefficient of 23,000 cm^{-1} M^{-1} at 462 nm (in methanol) was used for methoxy- and ethoxyresorufin, while an extinction coefficient of 53,500 cm^{-1} M^{-1} at 572 nm (in potassium phosphate buffer, 50 mM, pH > 9) was used for resorufin.

Western analysis

Microsomal proteins (10 μg) were subjected to SDS–PAGE on discontinuous gels (4–10%). Proteins were transferred onto a Hybond-P: PVDF membrane (Amersham, Arlington Heights, IL, U.S.A.) in 25 mM Tris, 192 mM glycine, and 10% methanol at 30 V overnight at 4°C. Nonspecific binding sites were blocked by incubating the membrane for 1 h with shaking in Tris-buffered saline (100 mM Tris, 150 mM NaCl), 0.1% Tween-20, and 1% fish gelatin (TBS-T & 1% FG). 'Blocked' membrane was incubated with primary antibody (GENTEST goat anti-CYP1A1/1A2, 500 fold dilution) in TBS-T and 0.1% FG for 2 h with shaking at room temp. After being washed four times with TBS-T, no FG, the membrane was incubated for 1 h in TBS-T and 0.1% FG with 100,000× diluted peroxidase-conjugated affini-Pure rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, U.S.A.). Finally, the membrane was washed four times with TBS-T, no FG, and developed with enhanced chemiluminescence substrate (Amersham, Arlington Heights, IL, U.S.A..). To establish the electrophoretic mobility of CYP1A2 in this system, microsomes containing human lymphoblast-expressed CYP1A2 (catalog number M103c, GENTEST, Woburn, MA, U.S.A.) were utilized as a standard. The predominant band of immunoreactive CYP1A2 protein migrated as an approximately 50–55 kilodalton band relative to a series of molecular weight standards. Film exposures were analysed with a video image analyser (Alpha Imager 2000, version 3.24, Alpha Innotech Corp., San Leandro, CA, U.S.A.).

Results

Briefly, the codons encoding amino acid residues 108, 313, 321, and 385 were modified in the DNA of expression plasmids using a QuikChange™ Site-Directed mutagenesis kit.

The mutagenized plasmid DNA (or wild type CYP1A2 cDNA) was subsequently transfected into a lymphoblastoid cell line (AHH-1 TK+/−). Cells were then selected for successful transfection by growth in media containing hygromycin B and L-histidinol. Figure 1 depicts the relevant coding sequence of the expression plasmid from nucleotide 305 through nucleotide 1195. The mutant oligonucleotides utilized to develop the mutant expression plasmids, the modified codons in the mutant oligonucleotides, and the amino acid substitutions are depicted in Figure 1.

The functional activity of the P450 constructs was evaluated by monitoring the metabolism of the CYP1A2 substrate methoxyresorufin to resorufin (Burke *et al*., 1985). The kinetics of methoxyresorufin metabolism by the h1A2v2 cell line (which contains two CYP1A2-cDNA containing expression cassettes per vector molecule) is shown in Figure 2. Enzyme activity was proportional to cell numbers (0.621×10^6 or 0.311×10^6 cells in 0.2 ml) and was linear for about 1 h, with a subsequent decline in reaction rate. Consequently, cell lines were compared for resorufin production after 1 h incubation at 37°C.

MROD metabolism by the transfected cell lines is depicted in Figure 3 with data transformed to pmoles resorufin per 10^6 cells h^{-1}. The wild-type 1A2 transfectant produced 48.4 pmoles resorufin 10^6 cells h^{-1}, while the h1A2v2 cell line from GENTEST produced 46.9 pmoles resorufin 10^6 cells h^{-1}. We also observed that the h1A1v2 cell line which expresses CYP1A1 metabolized methoxyresorufin (43.6 pmoles 10^6 cells h^{-1}). As expected, this is significantly greater metabolism than that of the parental AHH-1 TK+/− parental cell line that metabolized 1.4 pmoles 10^6 cells h^{-1}. Methoxyresorufin metabolism was not evident in cells that were transfected with a CYP1A2-cDNA encoding an arginine to lysine modification at amino acid residue 108 (designated CYP1A2 108 R-K). Nor was MROD activity evident in cells transfected with a CYP1A2-cDNA encoding a threonine to valine modification at amino acid residue 321 (designated CYP1A2 321 T-V). Finally, cells transfected with a CYP1A2-cDNA encoding a threonine to valine modification at amino acid residue 385 (designated CYP1A2 385 T-V) were also devoid of MROD activity.

We confirmed the lack of enzyme activity in the mutant constructs by checking their metabolism of the CYP1A1 substrate ethoxyresorufin as shown in Figure 4. The h1A1v2 cell line rapidly metabolized this substrate to resorufin (101 pmoles 10^6 cells h^{-1}) while the h1A2v2 cell line was less active (26.1 pmoles 10^6 cells h^{-1}). The wild-type CYP1A2 transfectant was also less active with this substrate than with methoxyresorufin (29.4 pmoles 10^6 cells h^{-1}). None of the cell lines bearing mutant cDNA transfections (108 R-K, 321 T-V, or 385 T-V) exhibited

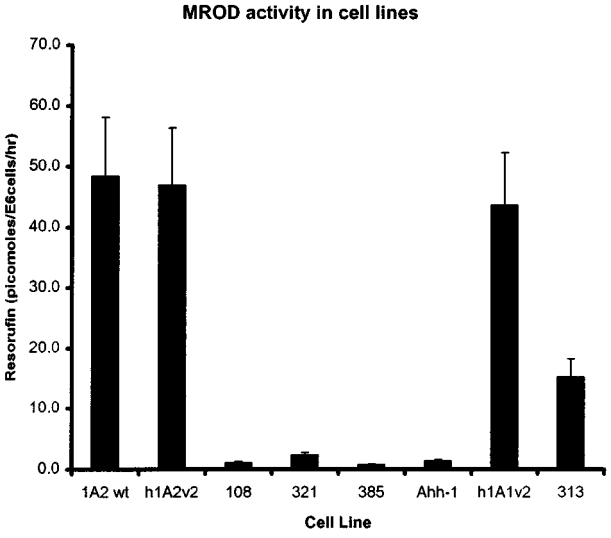

Figure 3 Methoxyresorufin metabolism by transfected cell lines expressing wild-type or mutant CYP1A2-cDNA constructs. Cells were incubated with methoxyresorufin (2.5 μM) for 1 h at 37°C and metabolism monitored by analysis of fluorescent product as described in Methods. Picomoles of resorufin produced by 1×10^6 cells after 60 min at 37°C is depicted. Results represent means and standard deviations from six separate experiments.

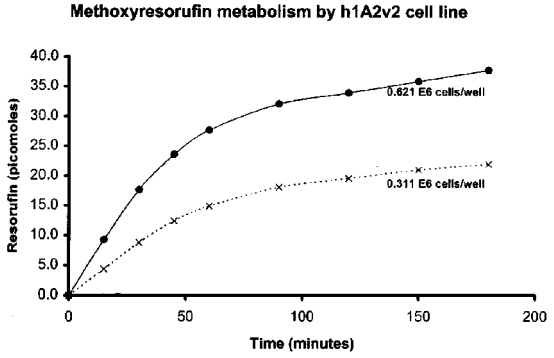

Figure 2 Kinetics of methoxyresorufin metabolism by h1A2v2 cells. A 0.2 ml suspension of 0.621×10^6 (solid line) or 0.311×10^6 (dashed line) cells were incubated with methoxyresorufin (2.5 μM) for 3 h at 37°C and metabolism monitored by analysis of fluorescent product as described in Methods. Results are expressed as picomoles of resorufin and represent the means from four replicate-wells in a representative experiment.

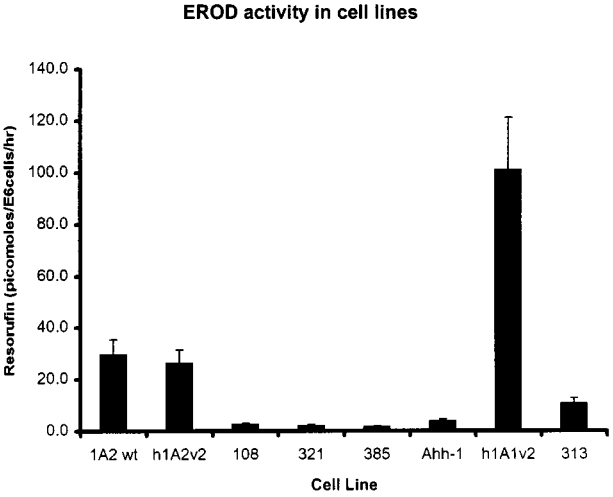

Figure 4 Ethoxyresorufin metabolism by transfected cell lines expressing wild-type or mutant CYP1A2-cDNA constructs. Cells were incubated with ethoxyresorufin (2.5 μM) for 1 h at 37°C and metabolism monitored by analysis of fluorescent product as described in Methods. Picomoles of resorufin produced by 1×10^6 cells after 60 min at 37°C is depicted. Results represent means and standard deviations from six separate experiments.

greater EROD activity than the parental AHH-1 TK+/− cell line. Hence, the mutations did not convert MROD activity into EROD activity.

In addition to our initial identification of active site residues 108, 321 and 385, we subsequently predicted that Asp-313 is also in this region and engages in a charge interaction with mexilitine, a much less potent competitive inhibitor of CYP1A2 than galangin (Wei *et al*., 1999). We thus modified residue 313 from aspartate to asparagine (313 D-N) and functionally assessed the resulting mutant. MROD and EROD activities were diminished but evident in cells transfected with 313 D-N cDNA. The activities by cells bearing the 313 D-N construct were about 30% of wild-type (15.2 and 10.6 pmoles 10^6 cells h^{-1}, respectively for MROD and EROD). Hence, site-directed mutagenesis of four residues that interact with inhibitors and were predicted to appear in the active site, were found to either abolish or significantly reduce CYP1A2 activities.

To verify that absence of methoxyresorufin metabolism by CYP1A2 108 R-K was not the result of gross differences in CYP1A2 expression, cells transfected with the mutant constructs were compared with wild type transfectants with respect to levels of immunoreactive CYP1A2 in cellular protein extracts prepared from each cell line. As shown in Figure 5, immunoreactive CYP1A2 was not evident in protein extracts from the Ahh-1 TK+/− parental cell line, but was evident in all other cell lines examined in this study. The mutant cell lines did not display significant differences in immunoreactive CYP1A2 relative to wild-type transfectants, but expression was clearly less than that in the h1A2v2 cell line that has two expression cassettes per vector molecule. Analysis of film exposures from five experiments with a video image analyser revealed that the CYP1A2 band from h1A2v2 cell line was about twice as dark as the band from the wild type cell line (average pixel density of h1A2v2 = 89 ± 8 (mean ± s.e.m.), wild-type = 51 ± 8; 255 = black). Hence, the absence of methoxyresorufin metabolism by CYP1A2 108 R-K, or the other mutant cell lines, was not due to the absence of CYP1A2 protein.

Discussion

Our results validate predictions of the CYP1A2 model regarding active site amino acid residues. Arg-108 was predicted to engage in hydrogen bonding with galangin. Our rationale for substitution of lysine into this site was that hydrogen-bonding would be decreased while a positive charge would be maintained and charge interaction with CYP1A2

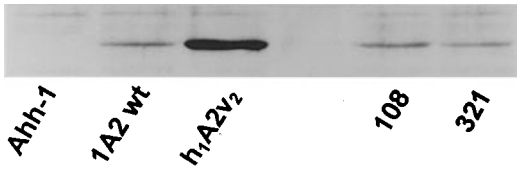

Figure 5 Western analysis of immunoreactive CYP1A2 present in transfected cell lines. Cellular proteins were extracted from transfectant cells and 10 μg were separated by SDS–PAGE and analysed as described in Methods. Lane assignments, left to right are: Ahh-1 TK+/−, CYP1A2 wild-type, h1A2v2, empty lane (molecular weight standards), CYP1A2 108 R-K, and CYP1A2 321 T-V.

Asp-110 preserved. On the other hand, a glutamine substitution at Arg-108 would be expected to preserve the capacity for hydrogen-bonding while eliminating the positive charge at the putative active site. This latter substitution would determine whether Arg-108 plays a role in stabilizing negatively charged substrates, such as carboxylated quinolones, through a charge interaction network. Carboxylated quinolones are well known inhibitors of CYP1A2 activity (Fuhr *et al*., 1992). The absence of CYP1A2 enzyme activity upon substitution of lysine at Arg-108 supports the model's identification of this residue in the active site.

Similarly, substitution of valine for Thr-385 also resulted in the loss of CYP1A2 enzymatic activity. According to our model, Thr-385 of CYP1A2 hydrogen bonds with the 3-hydroxyl and 4-carbonyl groups of galangin. If Thr-385 is in the CYP1A2 active site, its modification may be expected to affect the binding of substrates as well as inhibitors. The absence of enzyme activity in this mutant most probably derives from diminished substrate binding, but may also result from decreased enzyme turnover with bound substrate.

Our model predicts that flavone is a less potent CYP1A2 inhibitor than 3,5,7-trihydroxyflavone (galangin) because flavone interacts with the CYP1A2 active site *via* only one hydrogen bond while galangin utilizes multiple hydrogen bonds. Flavone's 4-carbonyl group is predicted to hydrogen bond with Thr-321 of CYP1A2, a residue that may be involved in binding molecular oxygen during the catalytic cycle. Hence, it was not surprising that substitution of valine for Thr-321 also resulted in the loss of enzyme activity, a loss that could result from not only reduced substrate binding but also inhibition of oxygen transfer. In agreement with our finding, mutation of this residue to proline also reduces EROD activity (Parikh *et al*., 1999). In contrast, substitution with serine, which preserves hydrogen bonding, did not change this activity.

Comparison of the transfectant cell lines by Western analysis revealed that the differences in MROD activity were not the result of gross differences in expression of CYP1A2 protein. Mutant transfectants had qualitatively as much immunologically detectable CYP1A2 protein as wild-type transfectants, although significantly less than that from h1A2v2 cells which contain two expression cassettes per vector. Spectral quantitation of CYP1A2 was not possible because the level of expression is beneath the detection limit of the spectrophotometer. Only microsomes prepared from the h1A2v2 or h1A1v2 cell lines have been reported to exhibit such a carbon monoxide-induced absorbance peak at 450 nm, presumably due to approximately 2 fold greater CYP expression by these cell lines (Penman *et al*., 1993).

While site-directed mutagenesis studies can identify critical amino acid residues required for substrate-specific catalysis and validate models of P450 structure, an amino acid substitution may also alter substrate binding by long range, conformational perturbation of the enzyme active site and/or by modifying electron transfer from NADPH cytochrome P450 reductase. Hence, caution needs to be exercised in interpreting results from such studies alone. Another method to probe these enzyme-substrate interactions and validate a molecular model involves the analysis of interactions between different substrates with the same residues in the P450 active site which avoids possible perturbations of cytochrome P450 structure by site-directed mutagenesis. This latter method of

substrate-, rather than amino acid-substitution, has the advantage of ensuring the retention of native protein sequence and structure. With this caveat in mind, the results of our site-directed mutagenesis studies suggest that Arg-108, Asp-313, Thr-321, and Thr-385 are important components of the CYP1A2 active site, as suggested by our molecular model.

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