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An alternatively spliced cytochrome P4501A1 in human brain fails to bioactivate polycyclic aromatic hydrocarbons to DNA-reactive metabolites

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Abstract

CYP1A1, a cytochrome P450 enzyme, metabolizes polycyclic aromatic hydrocarbons to genotoxic metabolite(s) that bind to DNA and initiate carcinogenesis. RT-PCR amplification of the complete open reading frame of *CYP1A1* generated an amplicon of 1593 bp having deletion of 87 bp of exon-6 that translated into functional P450 enzyme. Unlike wild type CYP1A1, exon 6 del CYP1A1 did not metabolize polycyclic aromatic hydrocarbons such as, benzo(a)pyrene to genotoxic, ultimate carcinogens that form DNA adducts. Exon 6 del CYP1A1 metabolized ethoxyresorufin (the classical substrate for CYP1A1) less efficiently compared with wild type CYP1A1 while pentoxy and benzyloxyresorufin (classical substrates for CYP2B) were dealkylated more efficiently. In silico docking showed alteration of the substrate access channel in exon 6 del CYP1A1 such that benzo(a)pyrene does not bind in any orientation that would permit the formation of carcinogenic metabolites. Genotyping revealed that the splice variant was not generated due to differences in genomic DNA sequence and the variant was present only in brain but not in liver, kidney, lung, or heart from the same individual. We provide evidence that unique P450 enzymes, generated by alternate splicing in a histiospecific manner can modify genotoxic potential of carcinogens such as benzo(a)pyrene by altering their biotransformation pathway.

Keywords: benzo(a)pyrene, carcinogenesis, target organ toxicity, xenobiotic metabolism.
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Cytochrome P450 (E.C. 1.14.14.1; P450), a superfamily of heme proteins, is involved in the metabolism of a vast array of carcinogens, drugs, and endogenous compounds. Liver is the major organ involved in P450-mediated xenobiotic metabolism (Conney 2003). However, the importance of extrahepatic metabolism has been increasingly recognized especially with respect to their potential role in target organ toxicity. The preferential localization of xenobiotic metabolizing enzymes within specific cell types in these organs renders such cells a remarkable capacity to metabolize foreign compounds including carcinogens (McLemore *et al.* 1990; Hedlund *et al.* 2001). Studies from our laboratory and others have demonstrated the presence of a competent microsomal P450 system in rodent (Anandatheerthavarada *et al.* 1990) and human brain (Ravindranath *et al.* 1989) and its ability to metabolize a variety of xenobiotics (Ravindranath and Boyd 1995; Ravindranath *et al.* 1995).

Cytochrome P4501A1 (*CYP1A1*), a member of the P450 super family, is a major extrahepatic enzyme, and is induced upon administration of 3-methylcholanthrene, β-naphthoflavone or 2,3,7,8-tetrachlorodibenzo-p-dioxin (Jones *et al.* 1986). *CYP1A1* contributes notably to the toxicity of many carcinogens, especially polycyclic aromatic hydrocarbons (PAHs), as it is the principal enzyme that bio-activates inert

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The nucleotide sequence of human brain exon 6 del *CYP1A1* has been deposited in GenBank database (Accession No. AY871801).

Abbreviations used: BP, benzo(a)pyrene; *CYP,* cytochrome P450; Exon 6 del *CYP1A1,* exon 6 deleted *CYP1A1*; PAHs, polycyclic aromatic hydrocarbons; PCR, polymerase chain reaction.

hydrocarbons into DNA-binding reactive metabolites (Park *et al.* 1996). The presence of *CYP1A1* in brain could potentially play a role in the initiation of carcinogenesis *in situ,* as PAHs are lipophilic and can cross the blood brain barrier. Several epidemiological studies (Inskip *et al.* 1995) carried out on workers in petroleum industry (Burch *et al.* 1987; Lee *et al.* 1997) and on smokers (a major source of PAH exposure) have shown association with brain tumor incidence. GSTM3 allele is associated with brain tumor incidence (De Roos *et al.* 2006). Further, within the subgroup having GSTM3 allele, a stronger association was found between smoking and brain tumor incidence. Further induction of *CYP1A1* enzyme by PAHs increases the generation of reactive oxygen species, which may cause oxidative damage in brain (Strolin-Benedetti *et al.* 1999).

Significant differences are seen in the metabolism of psychoactive drugs mediated by brain and liver P450 enzymes. For example, drugs, such as, alprazolam are metabolized differently in liver and brain wherein relatively larger amount of the active metabolite is generated in brain compared with liver (Pai *et al.* 2002). These observations have indicated the possible existence of unique P450 enzymes in the brain that are different from the well-characterized hepatic P450s. RT-PCR amplification of exon 3 to 7 of *CYP1A1* RNA demonstrated the presence of a transcript having deletion of exon 6 in human brain but not in liver (Chinta *et al.* 2005). Sequence analysis indicated that the deletion of exon 6 in *CYP1A1* does not lead to premature termination or frame shift. Therefore, we attempted to clone full-length exon 6 del *CYP1A1* and characterize the function of the unique splice variant that is generated by alternate splicing in human brain, but not in liver or kidney.

Materials and methods

Materials

3-Hydroxy benzo[a]pyrene (3-OH-BP) was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repositories at Midwest Research Institute (Kansas City, MO, USA). Monoclonal antibody against rat P4501A1 was a gift from Dr Paul E. Thomas, Rutgers University, USA. Polyclonal antibody against β-actin was purchased (Clontech, Mountain View, CA, USA).

Human Tissue

Human brain and other tissues were obtained at autopsy from male and female traffic accident victims with no known neurological or psychiatric disorders through the Human Brain Tissue Repository, Department of Neuropathology, NIMHANS. The material was collected at postmortem in accordance to the ethical guidelines of the Government of India. The average age of the individuals was 38.2 years ± 18.9 (7–72 years) and postmortem delay between death and autopsy was 5.50 ± 2.45 h. Total RNA was isolated from human autopsy tissues and used for RT-PCR (Chomczynski 1993).

RT-PCR amplification of exons 3–7 of CYP1A1

RT-PCR was carried out using 25 autopsy human brain samples and human brain cortex, liver, lung, kidney, and heart from the same individual using forward and reverse primers, 5′ -TGGATGAGA ACGCCAATGTC-3′ and 3′-TGGGTTGACCCATAGCTTCT-5′, respectively.

RT-PCR amplification of complete ORF of CYP1A1

RT-PCR was performed using forward and reverse primers; 5′-GAACCTTCCCTGATCCTTGTG-3′ and 5′-GCCCAGATAGCAA-AACTGCA-3′ (27–1798), respectively. The RT-PCR product was used as template for nested PCR using the forward and reverse primers; 5′-TTGTGATCCCAGGCTCCAAG-3′ and 5′-GGTCTG-GCTGCCCAACCA-3′ (43–1720), respectively. RT-PCR amplification of the complete ORF of *CYP1A1* resulted in the generation of exon 6 del *CYP1A1* (GenBank Accession No. AY871801) in four samples, while wild type *CYP1A1* (GenBank Accession No. NM_000499) was amplified in one sample. The amplicon was ligated into pcDNA 3.1 vector for expression studies.

Amplification of genomic DNA by PCR

Genomic DNA was extracted from human brain cortex and PCR was performed using forward and reverse primers, 5′-GCTGCC-TGCCTAACTTCTTCT – 3′ and 5′-AGCAGGATAGCCAGGAA-GAGA-3′, respectively to amplify the genomic DNA from exons 3–7.

PCR amplification of exon 6 of CYP1A1

Polymerase chain reaction amplification was performed using primers from the flanking regions namely, exons 5 and 7 (forward and reverse primers, 5′-CCCTTCACCATCCCCCACA-3′, and 5′-ATGGGTTGACCCATAGCTTCT-3′). pCR 2.1 vector (TA Cloning Vector, Invitrogen Corporation, USA) containing the complete ORF of wild type *CYP1A1* was used as template. The expected size of the PCR product was 127 bp, which consisted of 87 bp of exon 6 and the flanking regions of exons 5 and 7.

Immunoblotting with membrane preparations from transfected cells

Membrane preparations containing both microsomal and mitochondrial protein (50 μg) isolated from Neuro2a cells (Pai *et al.* 2004) expressing wild type and exon 6 del CYP1A1 were analyzed by SDS-PAGE and transferred to nitrocellulose membrane. The blots were incubated sequentially with antiserum to rat P4501a1 (Anandatheerthavarada *et al.* 1990) and anti-rabbit IgG conjugated to alkaline phosphatase. Color was developed using chromogenic substrates for the alkaline phosphatase.

Assay of total P450 content and dealkylation of alkoxy resorufins

Total cytochrome P450 content in the membrane preparation from Neuro2a cells expressing CYP1A1 variants was measured from carbon monoxide reduced minus oxidized difference spectrum. *O*-Dealkylation of alkoxy resorufin was performed in microtitre plates as described (Kennedy and Jones 1994). Incubations performed in the absence of NADPH were incorporated as controls. Similar experiments were also performed following pre-incubation of the membranes with antiserum to P4501a1 for 30 min at 25°C

prior to the addition of substrate and NADPH. Formation of resorufin was measured fluorimetrically using 530 nm for excitation and 590 nm for emission.

Identification of DNA adducts by ^{32}P-post-labeling

COS-1 cells were transfected with cDNA to wild type and exon 6 del *CYP1A1* in both orientation, and were treated with benzo[a]pyrene (BP; 1 μmol/L), 3-methylcholanthrene (MC; 1 μmol/L), or vehicle for 16 h. DNA was isolated and nuclease-P1-enhanced version of the ^{32}P-post-labeling assay for DNA adducts was performed (Reddy and Randerath 1986). The labeled products were separated by two-dimensional polyethyleneimine-cellulose thin-layer chromatography (Moorthy and Randerath 1996) and exposed to autoradiography.

Aryl hydrocarbon hydroxylase (AHH) activity

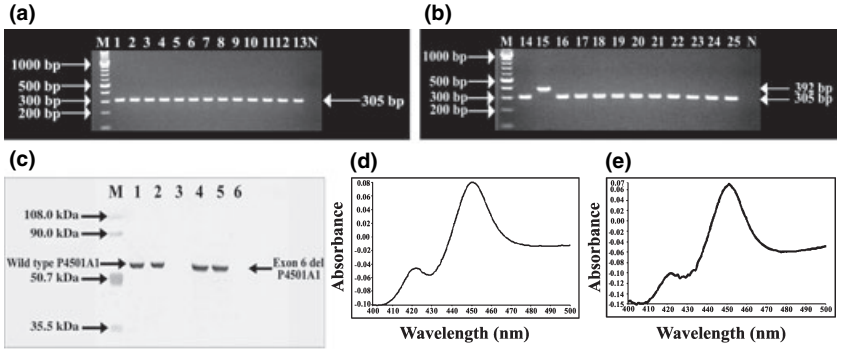
AHH activity was assayed in cell lysates expressing both wild type and exon 6 del *CYP1A1* (Nebert and Gelboin 1968). The expression level of *CYP1A1* was ascertained by western blotting using P4501A1-specific antibodies and normalized using β-actin.

Localization of exon 6 del CYP1A1 in cells and tissue by *in situ* hybridization

The 127 bp PCR product representing exon 6 was ligated into pCRII vector and digoxigenin labeled sense and antisense riboprobes were generated. Digoxigenin labeled riboprobes were also generated from the complete ORF of exon 6 del *CYP1A1* (GenBank No. AY871801). *In situ* hybridization was performed using paraffin embedded sections of brain, liver, and kidney from the same individual (Pai *et al.* 2004). Neuro2a cells were grown in chamber slides, fixed with buffered paraformaldehyde (4%, w/v) and processed for *in situ* hybridization.

In silico docking studies

AUTODOCK 3.0 was used to dock BP with wild type and exon 6 del CYP1A1 (Morris *et al.* 1998). All water molecules were



(h)

		Exon 5- Exon –7 cDNA sequence (5' ⟶ 3')
(1)	Exon 6 del CYP1A1	CTTCCTTCGTCCCCTTCACCATCCCCCAC AAG AAGCTATGGGTCAACCCATCTGAGTTCCT
(2)	Exon 6 del CYP1A1	CTTCCTTCGTCCCCTTCACCATCCCCCAC AGG AAGCTATGGGTCAACCCATCTGAGTTCCT

(i)

Genomic sequence representing exon-intron/intron-exon boundaries				
	E 5 – I 5	I 5 – E 6	E 6 – I 6	I 6 – E 7
Exon 6 del CYP1A1 (1)	CCCCAC**AGG**TAAGGCC	CTCCCT**AGC**ACAACAA	CATGAC**CAG**TAAGTTC	GGTTAC**AGGA**AGCTAT
Exon 6 del CYP1A1 (2)	CCCCAC**AGG**TAAGGCC	CTCCCT**AGC**ACAACAA	CATGAC**CAG**TAAGTTC	GGTTAC**AGGA**AGCTAT
Wild type CYP1A1	CCCCAC**AGG**TAAGGCC	CTCCCT**AGC**ACAACAA	CATGAC**CAG**TAAGTTC	GGTTAC**AGGA**AGCTAT

Fig. 1 Amplification and expression of *CYP1A1* variants from human brain. (a & b): Exons 3 to 7 of *CYP1A1* cDNA was amplified by RT-PCR from 25 samples of human brain cortex obtained at autopsy. An amplicon of 305 bp indicating deletion of exon 6 was observed in all samples except one (Sample I), which showed the presence of exon-6 (392 bp; lane 15 in b). (c) Mitochondria and microsomes (50 μg) from cells transfected with sense (lanes 1 & 2) and antisense (lane 3) wild type *CYP1A1* cDNA and sense (lanes 4 & 5) and antisense (lane 6) exon 6 del CYP1A1 were immunostained with antiserum to P4501a1. Dithionite reduced carbon monoxide binding spectrum of membrane preparations from Neuro2a cells expressing exon 6 del (d) and wild type P4501A1 (e) protein. The P450 specific content was 0.984 and 1.15 nmoles of P450/mg protein for exon 6 del and wild type P4501A1. The complete ORF of exon 6 del *CYP1A1* was amplified from 4 human brain samples (f) and wild type *CYP1A1* was amplified from one sample (g). 'M' indicates molecular weight markers. 'N' indicates negative control. (h) Exons 3–7 of *CYP1A1* cDNA was PCR amplified in two samples of human brain cortex (1 and 2) and an amplicon of 305 bp was detected indicating the deletion of 87 bp of exon 6. Sequencing revealed the G → A substitution in the 3′ end of exon 5. (i) Genomic DNA sequence of the region exon 5–7 was similar in the samples 1 and 2. The genomic DNA sequence was also identical in the individual who expressed wild type *CYP1A1*.

removed except two that bind to backbone atoms of residues in the I helix since AUTODOCK uses implicit solvation parameters. The four backbone residues binding waters are G293, A294, E297, and T298 in the crystal structure of the CYP2C5 template, and these residues are understood to be directly involved in proton transport during catalysis (Wester *et al.* 2003). The corresponding residues in the wild type and exon 6 del CYP1A1 homology models are conserved. All non-polar hydrogens were merged to the connected carbon atom and Kollman united-atom charges were added. The heme charges were dealt with separately being generously provided by Dr Mark Paulsen (Northern Michigan University) in which atom assignments were made with quantum mechanical derivations providing an electrostatic potential fit. ChemDraw Ultra and Chem 3D Pro programs within ChemOffice were used to prepare the BP coordinates and minimize the structure. BP is a rigid molecule, therefore; there were no rotatable bonds to be considered. Grid maps were generated with AUTOGRID. Affinity grid dimensions were 86 Å × 84 Å × 86 Å with a spacing of 0.425. The three-dimensional grid was mainly located on the distal side of the heme where one edge was slightly protruding below the plane of the heme. This allowed the entire distal portion of both proteins to be sampled through conformational space for potential binding sites. The Lamarckian genetic algorithm was used to test docking conformations and the beginning BP state was located outside of the protein near F–G loop. A total of fifty dockings were performed for both proteins with a population size of 50 individuals, a maximum of 250 000 energy evaluations, 27 000 maximum number of generations, a mutation rate of 0.02, a crossover rate of 0.8 and only one individual was allowed to survive to the next generation.

Results

Cloning and expression of CYP1A1 from human brain

The region spanning exons 3 to 7 of *CYP1A1* cDNA was amplified by RT-PCR using total RNA from 25 samples of human brain cortex obtained at autopsy. An amplicon of 305 bp was observed in all samples indicating absence of exon 6, except in one sample, wherein a 392 bp amplicon was observed, indicating that the individual (lane 15 of Figs 1a and b) expressed the wild type *CYP1A1*. We were unable to detect 2 bands in any samples, which would have indicated the presence of both wild type and exon 6 del *CYP1A1*. We then amplified the complete ORF of *CYP1A1* from five human brain samples including the brain sample that showed the presence of the wild type *CYP1A1*. PCR product of 1593 bp was seen in four samples (Fig. 1f) while 1680 bp product was seen in the brain sample mentioned above (Fig. 1g). Sequencing revealed that 1593 bp amplicon was devoid of 87 bp of exon-6 (GenBank No. AY871801) and 1680 bp amplicon was similar to wild type *CYP1A1* (GenBank No. NM_000499). When Neuro2a cells were transfected with cDNAs encoding exon 6 del *CYP1A1,* the expressed protein was a functional P450 similar to that seen in cells transfected with wild type CYP1A1 as ascertained from the reduced carbon monoxide difference spectra (Figs 1d and e). The expressed protein could also be detected by immunoblotting using antiserum to P4501a1 (Fig. 1c).

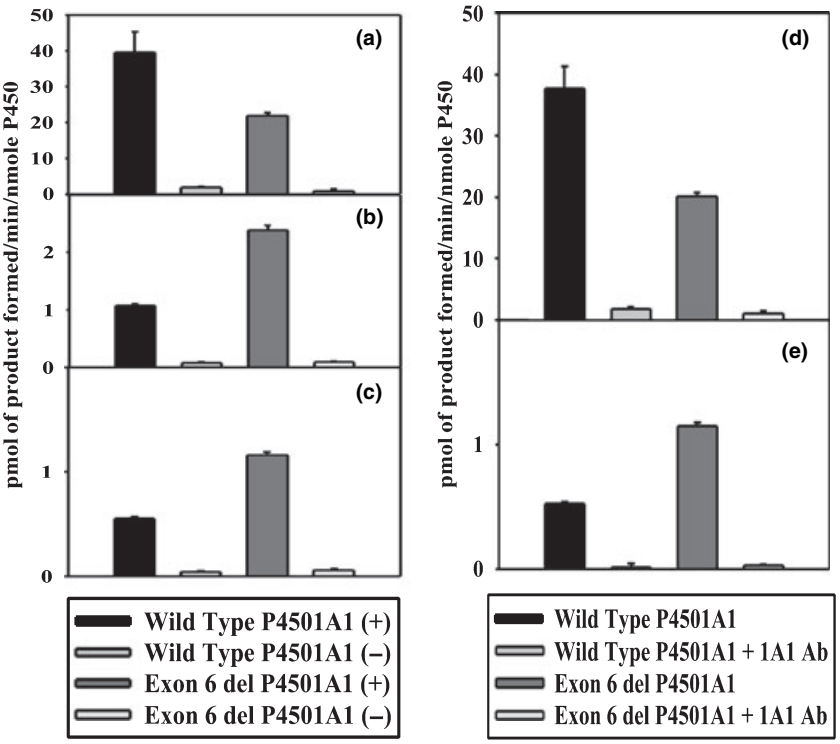


Fig. 2 Dealkylation of alkoxy resorufins by *CYP1A1* variants from human brain. Membrane preparations containing both mitochondria and microsomes from Neuro 2A cells expressing wild type and exon 6 del P4501A1 were incubated with ethoxy (a), pentoxy (b) and benzyloxy (c) resorufin and the formation of resorufin by O-dealkylation was measured. Preincubation with antiserum to P4501a1 inhibits dealkylation of ethoxy (d) and benzyloxy (e) resorufin. Values are depicted as NADPH dependent rates and are mean ± SD (n = 3 individual experiments). Cells transfected with cDNA in reverse orientation were incorporated as controls.

Cells transfected with cDNAs in the reverse orientation did not have any detectable P450 as examined by immunoblot and P450 assay (data not shown).

RT-PCR amplification of exons 3–7 of *CYP1A1* from two human brain samples (1 and 2 of Fig. 1h) yielded a 305 bp product indicating the presence of exon 6 del *CYP1A1*. In sample 1, the 3′ end of exon 5 showed a G → A substitution as compared with sample 2. In order to determine if this substitution was the result of changes in genomic DNA sequence, we amplified the region spanning exons 3–7 using genomic DNA from human brain. The genomic DNA sequences of the sample from the individual who expressed wild type *CYP1A1* (Fig. 1i; Fig. S1) and not the exon 6 del *CYP1A1* were identical to samples 1 and 2 which expressed only exon 6 del *CYP1A1*.

Dealkylation of alkoxy resorufin by exon 6 del P4501A1

Membrane preparations containing both mitochondria and microsomes were prepared from Neuro2a cells transfected with cDNAs to *CYP1A1* variants. Exon 6 del P4501A1 deethylated ethoxyresorufin, the classical substrate of P4501A1 much less efficiently compared with wild type P4501A1 (Fig. 2a), whereas pentoxy and benzyloxy resorufin, classical substrates of P4502B were dealkylated more efficiently by exon 6 del P4501A1 compared with wild type P4501A1 (Fig. 2b and c). Dealkylation of ethoxy and benzyloxy resorufin could be inhibited by antiserum to P4501a1 indicating that this biotransformation was indeed mediated by P4501A1 (Fig. 2d and e).

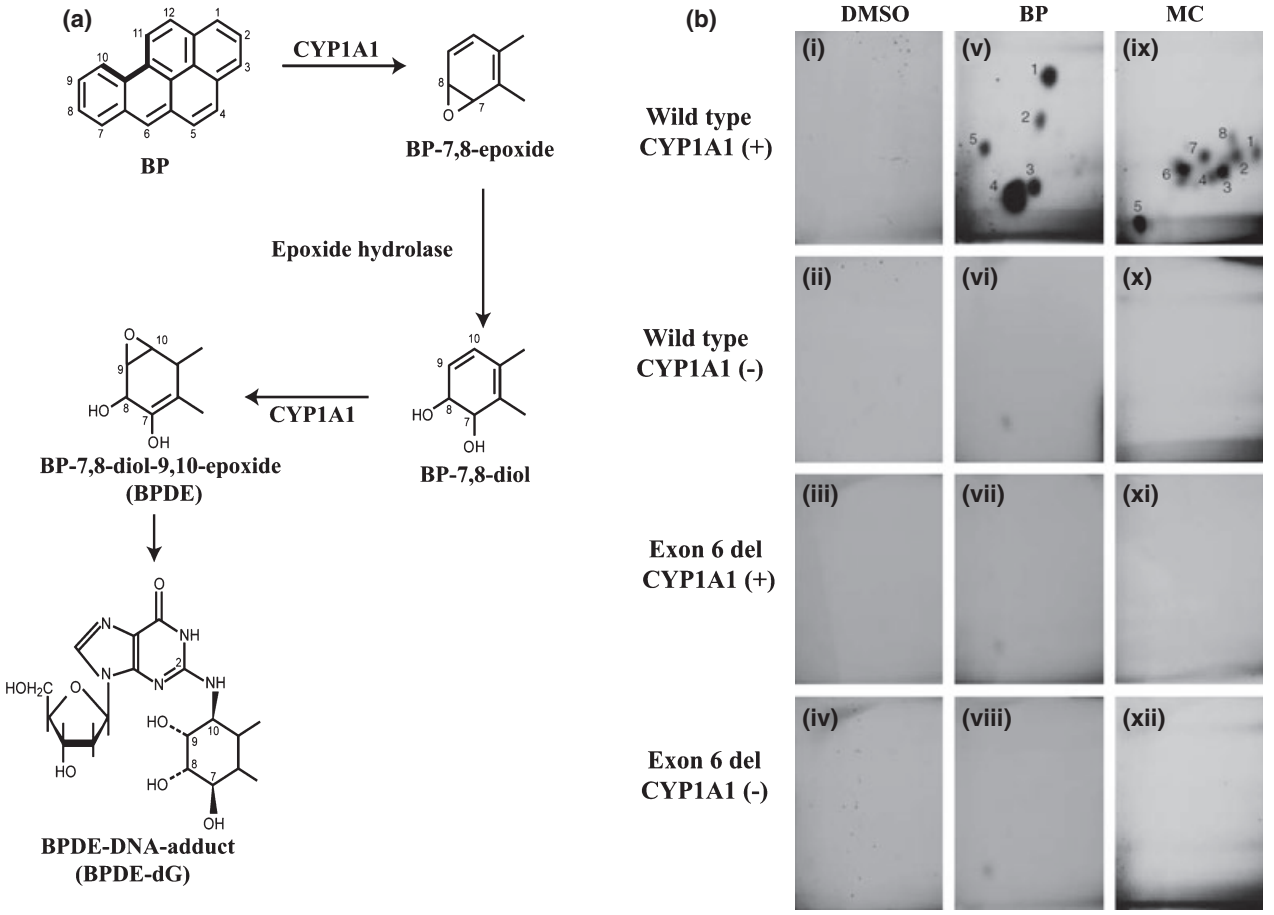


Fig. 3 Bioactivation of PAH to DNA binding adducts by *CYP1A1* variants. (a) Biotransformation pathway of BP bioactivation to DNA adduct formation. The major metabolic activation pathway of BP entails the CYP1A1-dependent metabolic activation at the 7, 8 bond to yield an epoxide, which then undergoes epoxide hydrolase-mediated hydration to BP-7, 8-diol. BP-7, 8-diol is in turn transformed to BPDE by *CYP1A1*-mediated epoxidation at the 9,10 position. The major BPDE-DNA adduct results from nucleophilic attack of the exocyclic N^2 position of guanine on the C-10 position of (+)-*anti*-BPDE. (b) COS-1 cells were transfected with cDNA to wild type or exon 6 del *CYP1A1* for 24 h, followed by treatment with DMSO (i–iv, vehicle), BP (1 μmol/L) (v–viii), or MC (1 μmol/L) (ix–xii), as described under Materials and Methods. DNA adducts were analyzed by ^{32}P-post-labeling. (+) and (−) denote plasmids containing cDNAs that were cloned in the sense and antisense orientation, respectively.

Formation of DNA adducts by metabolites of polycyclic aromatic hydrocarbons

The mechanism by which BP interacts with DNA and initiates carcinogenic processes involves the formation of a biologically active carcinogen, BP-7,8-diol-9,10-epoxide (BPDE; Fig. 3a). BP or MC, but not vehicle, induced the formation of multiple adducts in cells transfected with wild type *CYP1A1* cDNA (Fig. 3b). BP and MC gave rise to 5 (nos. 1–5) and 8 (nos. 1–8) adduct spots, respectively. Similar DNA adducts were not detectable in BP or MC-exposed cells that were transfected with the exon 6 del CYP1A1. Metabolism of BP to 3-OH-BP by exon 6 del P4501A1.

Metabolism of BP to 3-OH-BP by exon 6 del P4501A1

Cells transfected with wild type *CYP1A1* efficiently hydroxylated BP to 3-hydroxy benzo[a]pyrene. However, cells expressing exon 6 del CYP1A1 failed to hydroxylate BP and no activity was detectable (Fig. 4a). In fact, the response was similar to the cells that were transfected with *CYP1A1* cDNA (reverse orientation). In order to ensure that the transfection efficiency was similar amongst the experiment groups, we performed immunoblot experiments using antibody to rat P4501a1, which cross-reacts with human P4501A1. The transfection efficiency did not vary significantly between the different groups (Fig. 4b).

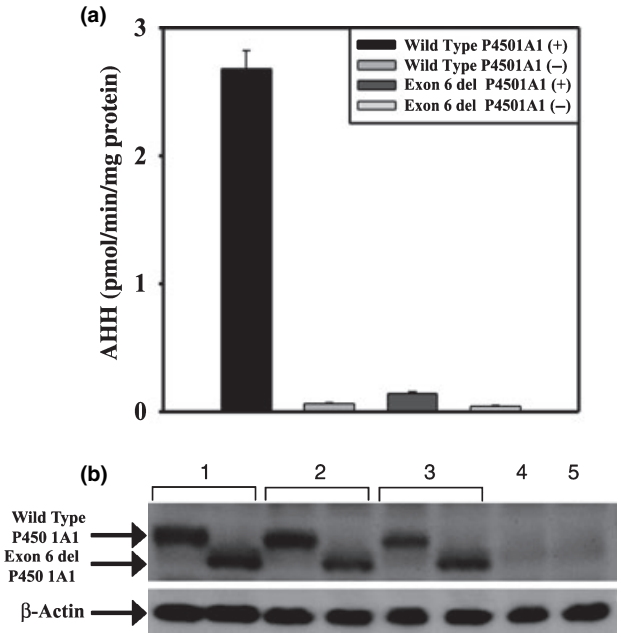


Fig. 4 Aryl hydrocarbon hydroxylase activity (a) and immunoblot (b) analyses of protein pellets from cells transfected with cDNA to exon 6 del and wild type *CYP1A1*. (a) AHH activity is expressed as pmol of 3-hydroxybenzo[a]pyrene formed per min per mg protein in cells transfected with wild type and exon 6 del *CYP1A1* cDNA in forward (+) and reverse (–) orientation. Data is represented as mean ± sd of 3 independent transfection experiments performed in triplicate. (b) Immunoblot of transfected cells using monoclonal antibody to rat P4501a1. Lanes 1, 2, and 3 represent protein samples (100 μg) from three independent transfection experiments for the wild type *CYP1A1* or exon 6 del *CYP1A1* constructs. Lanes 4 and 5 represent proteins from cells transfected with the constructs, cloned in reverse orientation. The blots were normalized with β-actin antibody. The ratios of pixel densities of wild type *CYP1A1* and the variant protein normalized to those of β-actin were 0.42 ± 0.04 and 0.49 ± 0.07, respectively.

Localization of exon 6 del and wild type CYP1A1 mRNA by fluorescence in-situ hybridization

Neuro2a cells transiently transfected with wild type or exon 6 del *CYP1A1* showed intense staining when hybridized with full-length cDNA to exon 6 del *CYP1A1*. However, cells transfected with exon 6 del *CYP1A1* showed no staining (Fig. 5b) when hybridized with the probe representing exon 6 alone. Neuro2a cells transfected with wild type or exon 6 del *CYP1A1* in reverse orientation did not exhibit any fluorescence. The corresponding nuclear staining of the cells with DAPI is shown in Fig. 5b.

CYP1A1 variants in human brain, lung, liver, and kidney

In situ hybridization was performed using 127 bp amplicon representing exon 6 of *CYP1A1* using human brain, liver and kidney sections from the same individual. Expression of exon 6 of *CYP1A1* was detected in liver and kidney but not in the brain from the same individual (Fig. 6a) indicating that the *CYP1A1* transcript in brain did not contain exon 6. However, when *in situ* hybridization was carried out using full-length exon 6 del *CYP1A1*, the expression of the same could be discerned in the cortical neurons of the human brain. The region spanning exons 3–7 of *CYP1A1* cDNA was amplified by PCR using cDNA synthesized from total RNA of human brain, liver, kidney, lung and heart tissues from the same individual obtained at autopsy. An amplicon of 305 bp (Fig. 6b; lane 1) was observed in brain but not in the other tissues wherein an amplicon of 392 bp was observed indicating the expression of wild type *CYP1A1*.

Docking studies

Our docking experiments predict that BP may have two major binding orientations in the CYP1A1 active site. The first cluster presents the 7, 8, 9, 10 aromatic ring near the heme iron with a docking energy of −10.00 kcal/mol (Fig. 7a) and the second cluster presents position 3 close to the heme iron with a docking energy of −9.86 kcal/mol (Fig. 7b). There was a total of six clusters found using a 0.5A root mean square cut off acceptance value. Cluster 1 represents 36/50 (72%) and cluster 2 represents 7/50 (14%) of the docking conformations. The remaining clusters were not included since they had fewer than 3 representative dockings. BP also has two main docking clusters out of 11 found for exon 6 del CYP1A1 and both are situated in a manner where position 3 is in close proximity to the iron (Figs 7c and d). Cluster 1 represents 16/50 (32%) dockings with –9.81 kcal/mol docking energy and cluster 2 represents 10/50 (20%) with –9.74 kcal/mol docking energy.

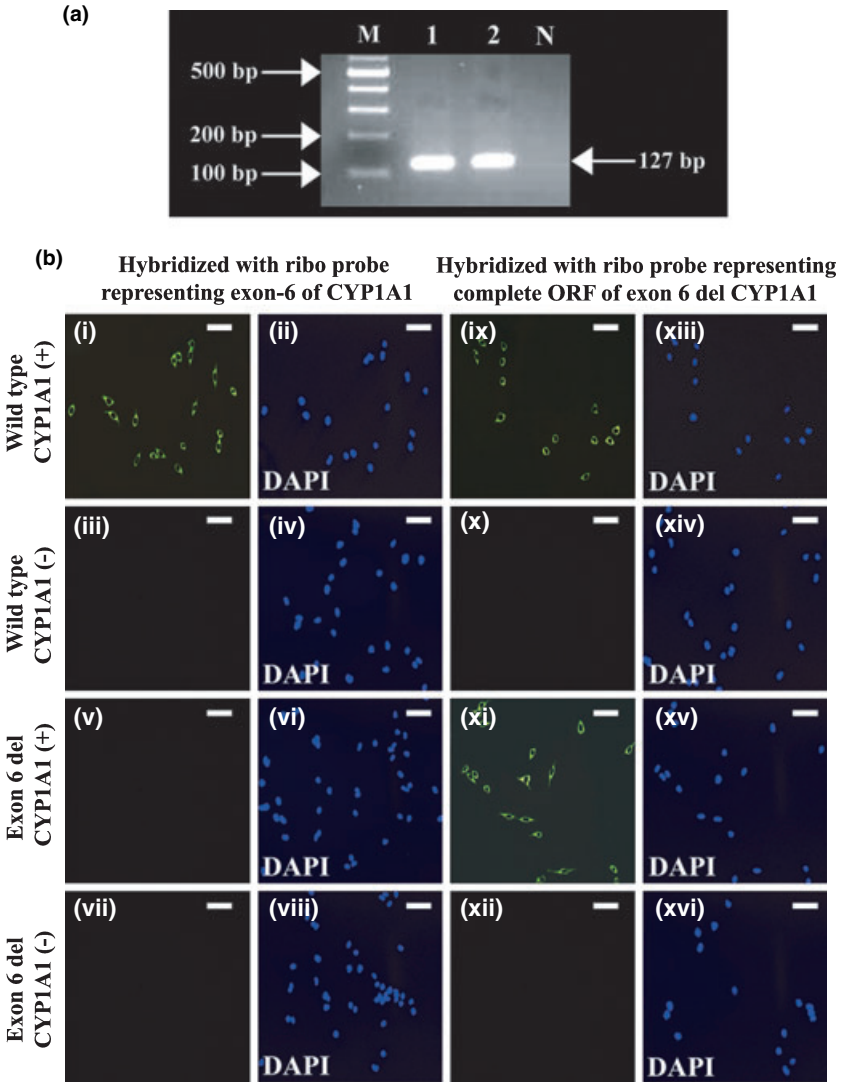


Fig. 5 Detection of exon 6 del *CYP1A1* by in situ hybridization. (a) cDNA to complete open reading frame of wild type *CYP1A1* was used as template to amplify a 127 bp product representing exon 6 (87 bp) along with flanking regions of exons 5 and 7. 'M' indicates molecular weight markers. 'N' indicates negative control. (b) Neuro 2A cells were transfected with wild type (i, ii, xi, x) and exon 6 del (v, vi, xiii, xiv) *CYP1A1* and hybridized with 127 bp amplicon representing exon-6 of *CYP1A1* (i–viii) or full-length exon 6 del *CYP1A1* (ix–xvii). The region representing exon 6 was detected only in cells transfected with wild type *CYP1A1* (i), while hybridization with the full-length exon 6 del *CYP1A1* resulted in fluorescent staining in both sets of transfected cells. The corresponding DAPI stained images are also depicted. Bar = 50 μm.

Discussion

The presence of unique, tissue-specific P450 enzymes generated through alternate splicing (Pai *et al.* 2004; Chinta *et al.* 2005) provides a mechanism by which active metabolites can be potentially formed at site of action of xenobiotics within the target organ, the brain. The constitutive expression of *CYP1A1* in rat and human brain has been demonstrated by RT-PCR amplification of selected regions of *CYP1A1* gene (Schilter and Omiecinski 1993; McFayden *et al.* 1998). We now present the expression and functional characterization of the exon 6 del *CYP1A1* variant from human brain. Genomic DNA sequence did not reveal any significant difference between the two samples expressing wild type and exon 6 del *CYP1A1* indicating that the alternate splicing events governing the deletion of exon 6 were not related to genetic polymorphism of genomic DNA. Another point of interest was the single base substitution (G → A) seen in the cDNA sequence representing 3′ end of exon 5 in two samples both of whom expressed exon 6 del *CYP1A1*, notwithstanding their identical genomic DNA sequence (Figs 1h and i). Further, the exon 6 del *CYP1A1* was detected only in brain but not in lung, liver, or kidney from the same individual, implying brain-specific splicing events. Nervous system has a propensity for generating alternate spliced forms and splicing defects may not be related to differences in genomic sequence but may be regulated by mechanisms involving spliceosomal complex and RNA binding proteins, which are poorly understood (Grabowski and Black 2001).

CYP1A1 enzyme is primarily involved in metabolic activation of PAHs (Fig. 3a) and other environmental chemicals to reactive intermediates that can form DNA adducts, critical molecular events in the initiation of carcinogenesis (Thakker *et al.* 1976). Exon 6 del *CYP1A1* in human brain is unable to bioactivate PAHs to metabolites

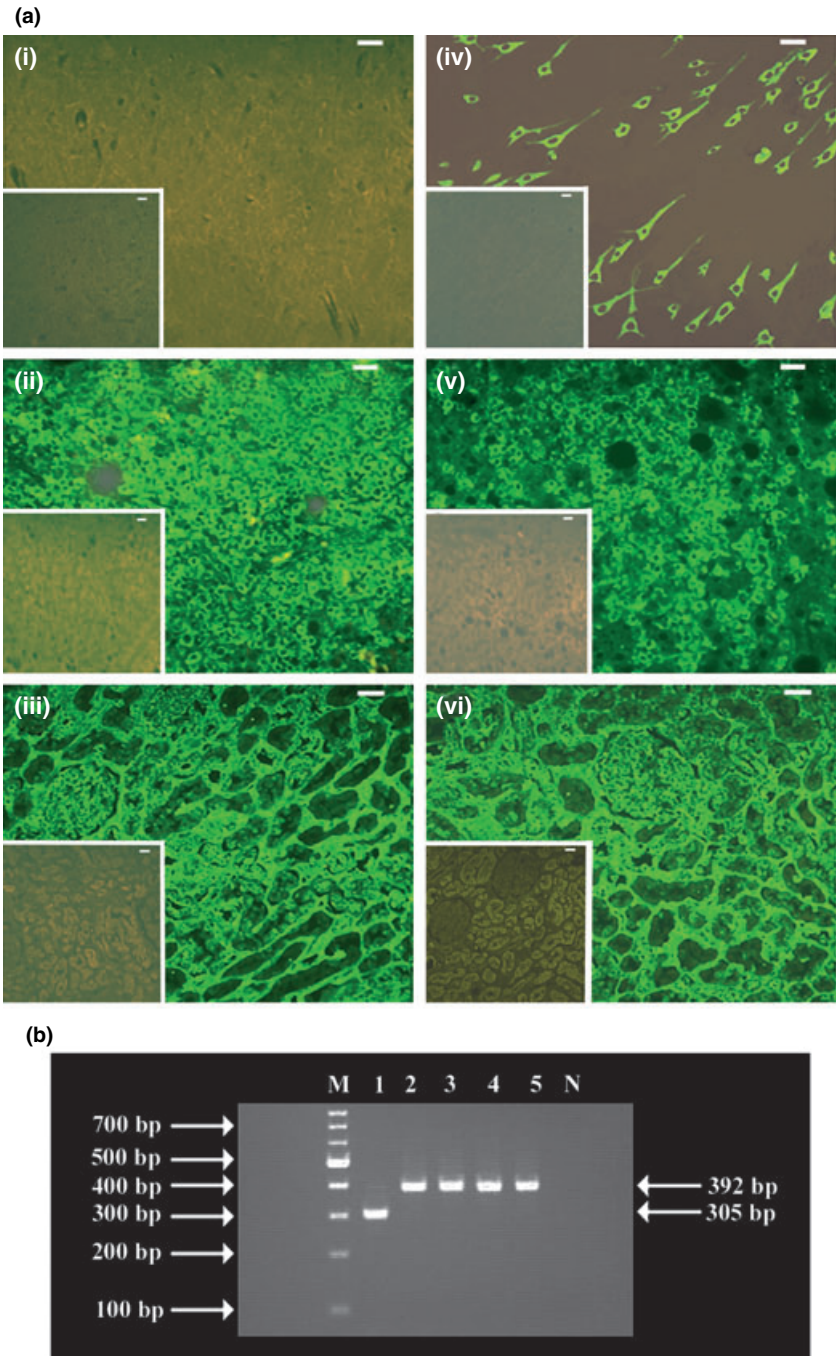


Fig. 6 Detection of mRNA of *CYP1A1* variants in human tissues. (a) Exon 6 of CYP1A1 transcript was localized in liver and kidney but not in cortical neurons of human brain from the same individual (ii, iii and i, respectively). The transcript representing the full-length *CYP1A1* gene was detectable in brain, liver and kidney (iv, v and vi, respectively). Insets depict corresponding control sections hybridized with sense riboprobes. Bar = 50 μm except in the liver, where bar = 25 μm. (b) Exon 3–7 of the *CYP1A1* gene was amplified by RT-PCR from of human brain, liver, kidney, lung and heart (lanes 1–5, respectively) from the same individual obtained at autopsy. An amplicon of 305 bp was observed in brain while an amplicon of 392 bp representing inclusion of exon 6 was seen in other tissues. 'M' indicates molecular weight markers. 'N' indicates negative control.

that could covalently bind to DNA while multiple DNA adducts were formed in PAH-exposed cells that were transfected with wild type *CYP1A1*. Further, RT-PCR experiments clearly demonstrate that human brain samples which express exon 6 del *CYP1A1* do not express the wild type *CYP1A1* indicating that in these brain tissues, the PAH would not be bioactivated to DNA binding metabolites.

Following exposure to BP, the major DNA adducts formed in cells transfected with wild type *CYP1A1* cDNA were most likely derived from 7(R),8(S)-dihydroxy-9 (S), 10 (R) epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDEI; Fig. 3a). The BP adducts seen in Fig. 3b, based on their chromatographic behavior, appear to have been derived from binding of (+) *anti*-BPDE1 to N^2 of deoxyguanosine. The major adduct (no. 4) results from nucleophilic attack of the exocyclic N^2 position of guanine on the C-10 position of (+)-*anti*-BPDE (Jerina *et al.* 1991). Because of the possibility of both *cis* and *trans* addition of the nucleophile, anti-BPDE forms three

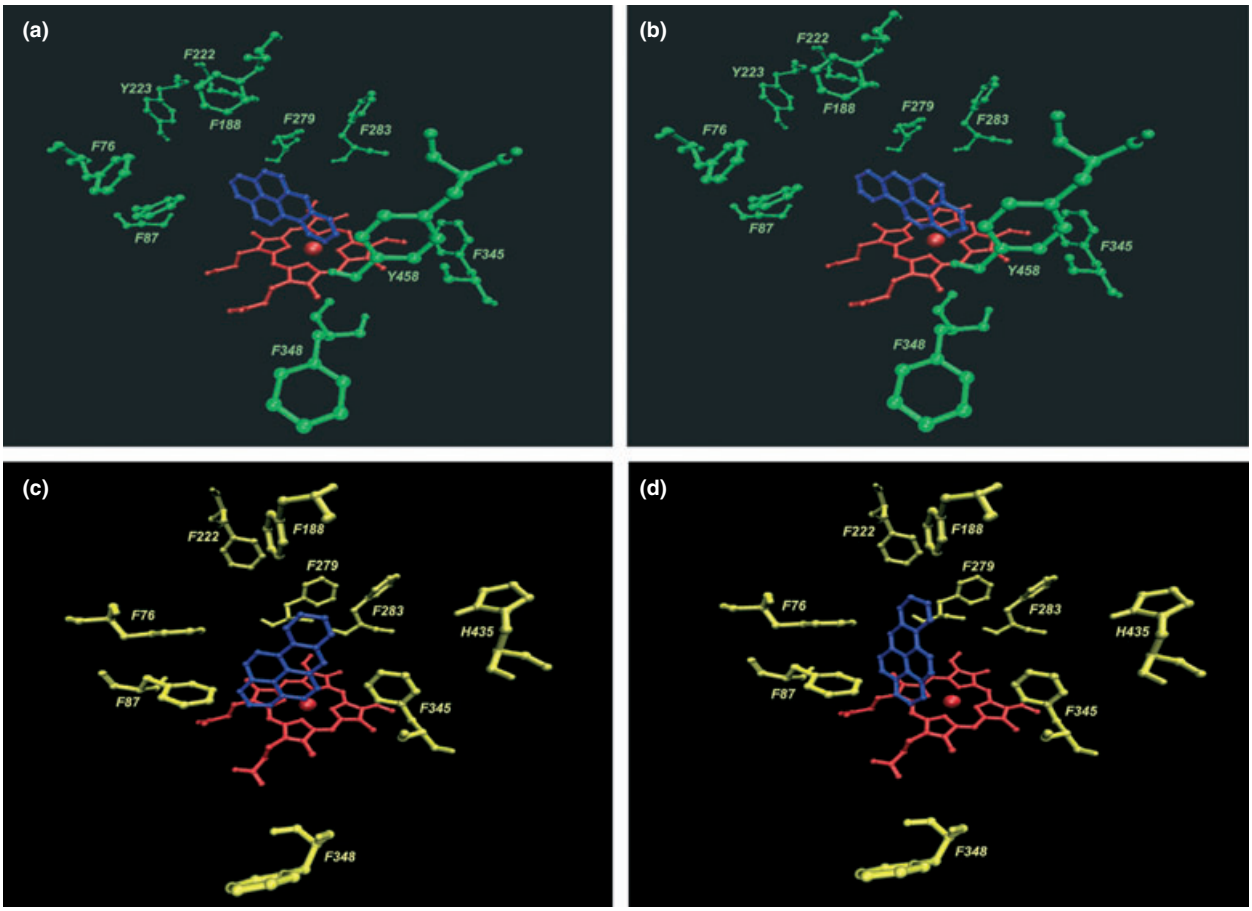


Fig. 7 Docking of benzo(a)pyrene into the active site of CYP1A1 variants. BP is shown in blue and heme shown in red. Aromatic residues within 10 Å of BP capable of π-ring stacking are shown in green. (a) 72% of the dockings fit within cluster 1, which positions the 7, 8, 9 10 ring closest to Fe-heme. (b) Cluster 2 accounts for 14% of the dockings, where position 3 is situated near Fe-heme. BP is shown in blue and heme shown in red. Aromatic residues within 10 Å of BP capable of π-ring stacking are shown in yellow. (c) Cluster 1 comprises 32% of the dockings and position 3 lies close to Fe-heme (d) Cluster 2 makes up 20% and also present position 3 close to Fe-heme.

other covalent adducts (nos. 1–3; Hall and Grover 1990). The identity of adduct number 5 is not known, but is probably formed by the reaction of *anti*-BPDE with deoxyadenosine. MC-DNA adducts also appear to have derived from dihydrodiol epoxides.

In silico binding of BP in the active site of CYP1A1 suggests two main binding orientations, one in which the substrate sits in a manner to allow the formation of the reactive ultimate diol epoxide carcinogen (cluster 1) and the other presents position 3 nearest to the heme iron (cluster 2), which would likely produce the 3-OH BP non-carcinogenic metabolite. BP does not appear to bind exon 6 del CYP1A1 in any orientation permissible of generating the carcinogenic diol epoxide. All sites considered reactive in the docking were within 5.5 Å from the heme iron, which offers a reasonable distance for oxidation (de Graaf *et al.* 2005).

The difference in binding modes between these two enzymes may lie partly in the repositioning of F345 in exon 6 del CYP1A1, which is part of substrate recognition site 5 (SRS5). This residue is adjacent to that portion of the protein deleted by the alternative splicing of parent CYP1A1 and the homology model suggests that it may compensate by sliding closer to the heme in the distal pocket. Therefore, it may sterically hinder BP from situating where the 7, 8, 9, 10 ring positions may be oxidized as seen with CYP1A1. This is confirmed by the *in vitro* ^{32}P post-labeling data wherein wild type *CYP1A1* made more hydroxylated products than exon 6 del *CYP1A1* suggesting that wild type *CYP1A1* is more catalytically efficient at total metabolism of BP than the exon 6 del *CYP1A1*.

The presence of the *CYP1A1* variant in the human brain and its inability to bioactivate PAHs would render neuroprotection in individuals exposed to carcinogenic PAHs. This would be of importance in view of the observed association between brain tumor incidence and exposure to PAHs (Inskip *et al.* 1995). The presence of the splice variant not only has

implications for metabolism of carcinogens, but also the metabolism of anti-neoplastic agents (Rooseboom *et al.* 2004). We now present evidence for the existence of novel site-specific biotransformation pathways that can potentially mediate metabolism of xenobiotics at the site of action by mechanisms that are very different from known pathways in liver. Identification of novel histio-specific P450 enzymes generated by alternate splicing of known genes or as yet unidentified genes may help understand metabolism at site of action and potentially predict the vulnerability of specific cell types within tissues to neoplastic transformation mediated through bioactivation of carcinogens.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1 Electropherogram depicting the partial sequence of CYP1A1 generated by amplification of cDNA (A) and genomic DNA (B) from human brain cortex samples. (A) Electropherogram of the cDNA sequence (exon 5-7) of CYP1A1 brain variant depicting absence of exon 6. A single nucleotide difference (G → A) is seen between samples 1 and 2 at the 3′ end of exon 5 (1166 bp relative to the ATG). (B) Amplification of the same region using the genomic DNA from the above two individuals revealed no difference between samples 1 and 2. The genomic DNA sequence of the similar amplicon from the individual, who expressed wild type CYP1A1 but not the brain variant, did not show any difference as compared to the samples 1 and 2.

This material is available as part of the online article from http://www.blackwell-synergy.com

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