Characterization of Common CYP1B1 Variants with Different Capacity for Benzo[*a*]pyrene-7,8-Dihydrodiol Epoxide Formation from Benzo[*a*]pyrene

Eleni Aklillu,[1] Steinar Øvrebø,[3] Ingrid V. Botnen,[3] Charlotta Otter,[4] and Magnus Ingelman-Sundberg[2]

[1]Division of Clinical Pharmacology, Department of Laboratory Medicine, Karolinska University Hospital-Huddinge, [2]Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden; [3]Department of Toxicology, National Institute of Occupational Health, Oslo, Norway; and [4]AstraZeneca Mölndal R&D, Molecular Biology, Mölndal, Sweden

Abstract

Cytochrome P450 1B1 (CYP1B1), an extrahepatic enzyme inducible by smoking, is overexpressed in many tumors and catalyzes the metabolic activation of procarcinogens such as polycyclic aromatic hydrocarbons. In human, CYP1B1 is genetically polymorphic and five common missense mutations causing amino acid substitution have been identified. In this study, we have investigated CYP1B1 haplotypes present in a Spanish population and carried out functional analyses of the corresponding enzymes in yeast using benzo[*a*]pyrene as a substrate. *CYP1B1*1*, *CYP1B1*2*, *CYP1B1*3*, *CYP1B1*4*, *CYP1B1*6*, and *CYP1B1*7*, encoding combinations of the Arg48Gly, Ala119Ser, Leu432Val, Asn453Ser, and Ala443Gly amino acid substitutions, were present at frequencies of 14.3%, 25.5%, 38.8%, 18.1%, 0.4%, and 2.6%, respectively. The variant CYP1B1 forms were heterologously expressed with human reductase in *Saccharomyces cerevisiae* and kinetic analyses of benzo[*a*]pyrene metabolism were carried out. CYP1B1.7, having the amino acid substitutions Arg48Gly, Ala119Ser, Leu432Val, and Ala443Gly, exhibited a significantly decreased capacity ($P < 0.001$) for the formation of (±)-benzo[*a*]pyrene-*trans*-7,8-dihydrodiol from benzo[*a*]-pyrene as indicated by lower intrinsic clearance (V_{max}/K_m). A somewhat decreased clearance was observed for CYP1B1.4, whereas no significant differences in kinetic properties among the remaining variant enzymes were observed as compared with CYP1B1.1. Thus, genetic polymorphism in the *CYP1B1* gene, as defined by the haplotypes investigated, might cause interindividual differences in susceptibility (e.g., to lung cancer induced by smoking). The results indicate the necessity to make molecular epidemiologic investigations regarding the association of the specific *CYP1B1* haplotypes and cancer risk. (Cancer Res 2005; 65(12): 5105-11)

Introduction

Cigarette smoking is a major factor for the development of several different types of cancer including lung cancer (1). Many of the chemical carcinogens in cigarette smoke are members of the polycyclic aromatic hydrocarbon (PAH) family. Cytochrome P450 enzymes are central to the metabolic activation of PAHs to epoxide intermediates, which are converted with epoxide hydrolase to the ultimate carcinogens, diol-epoxides that interact with DNA or proteins to form adducts. Benzo[*a*]pyrene (B[*a*]P), a major carcinogenic constituent in tobacco smoke, is by far the best studied and most potent mutagens and carcinogens known (1). B[*a*]P is implicated in the cause of various cancer forms such as lung and colon cancer and people are exposed through smoking, occupational exposure, and intake of broiled or smoked food.

In human lung, B[*a*]P is first metabolically activated by cytochrome P450 1A1 (CYP1A1) and cytochrome P450 1B1 (CYP1B1) to form B[*a*]P-7,8-dihydroepoxide, which is further hydrolyzed by microsomal epoxide hydrolase to (±)-benzo[*a*]pyrene-*trans*-7,8-dihydrodiol (B[*a*]P-7,8-DHD). This compound is further metabolized by CYP1B1 to form (±)-benzo[*a*]pyrene-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide (2). B[*a*]P-diol-epoxide is implicated as to induce mutations in the *p53* gene in lung cancers caused by smoking (3, 4). CYP1A1 and CYP1B1 are expressed in human lung but their relative expression levels as well as their relative contribution in the bioactivation of B[*a*]P have not been clarified. Significantly increased expression levels of CYP1B1 as compared with CYP1A1 in nontumor and tumor human lung tissues has been reported (5, 6). In contrast, a recent study indicated that the CYP1B1 protein is expressed at levels 1/10 of those of CYP1A1 in lungs of nonsmokers and smokers (7). Some studies have indicated that CYP1B1 is more active than CYP1A1 in B[*a*]P activation (8, 9) whereas others have implicated that CYP1A1 is more active (2). However, enzyme induction caused by smoking results in 10-fold higher levels of both CYP1A1 and CYP1B1 proteins and about 10% of the population shows much greater enzyme induction (7, 10). Because CYP1A1 and CYP1B1 are known to bioactivate procarcinogens found in cigarette smoke, both enzymes might play important role in lung carcinogenesis.

CYP1B1, commonly expressed in human lung (5), is inducible by cigarette smoking (11), B[*a*]P, and dioxin (12). The enzyme is involved in the metabolic activation of chemically diverse procarcinogens to reactive metabolites that cause DNA damage (13). CYP1B1 is overexpressed in a wide range of human cancers, including breast, colon, lung, esophagus, skin, lymph node, brain, and testis cancer. CYP1B1 has been suggested as a biomarker for tumor diagnosis (14, 15). The importance of CYP1B1 in chemical carcinogens is well illustrated using *Cyp1b1*-null mice. Embryonic fibroblast cells derived from *Cyp1b1*-null mice were found to be resistant to 7,12-dimethylbenz(*a*)anthracene (DMBA)–mediated tumorigenesis (16) and the *Cyp1b1*-null mice were protected from DMBA-induced malignant lymphomas (16), bone marrow

Requests for reprints: Eleni Aklillu, Division of Clinical Pharmacology, Karolinska University Hospital-Huddinge, C-168, Karolinska Institutet, SE-141 86 Stockholm, Sweden. Phone: 46-8-5858-1056; Fax: 46-8-5858-1070; E-mail: eleni.aklillu@labmed.ki.se.

cytotoxicity, and preleukemia (17), but the incidences of adenoma and adenocarcinoma of the lung were increased as compared with wild-type mice.

The human *CYP1B1* gene is located on chromosome 2 and has three exons within the coding region starting at exon 2. The mRNA is 5.2 kb and encodes a protein of 543 amino acids (18). In human, nine common single nucleotide polymorphisms (SNP) of which five cause amino acid substitutions and seven *CYP1B1* variant alleles have been identified thus far (ref. 19; see http://www.imm.ki.se/CYPalleles/cyp1b1.htm). Several studies investigated any association of SNPs in the human *CYP1B1* gene with the incidence of various cancer forms. Association of CYP1B1 polymorphism with increased risk for ovarian cancer (20), endometrial cancer, renal cancer (21, 22), smoking-related head and neck cancer (23, 24), lung cancer (25) as well as prostate cancer in Caucasians (26, 27) and in Japanese population (28) is reported. An association of CYP1B1 polymorphism with increased risk of breast cancer in Japanese (25) and Chinese populations (29) has also been stated (25, 29), but no association has been found in Caucasian women (30, 31). In addition, evidence has been presented for an association between the frequency of tobacco-induced *p53* mutations and *CYP1B1* polymorphisms in patients with head and neck squamous cell cancer (24). It has been anticipated that these polymorphisms might cause an altered function of the enzyme thereby determining interindividual differences in susceptibility for carcinogenesis.

CYP1B1 exhibits variable constitutive expression and the magnitude of induction varies within the population (12). Tobacco-induced expression of CYP1B1 in human lung extensively varies between individuals both at mRNA and protein levels as assessed in endobronchial mucosal biopsies taken from active cigarette smokers (7, 32). Interindividual variation in the metabolic activation of B[*a*]P by human tissues as well as in levels of B[*a*]P-DNA or protein adducts in humans has been noted (33). This might give rise to variations in B[*a*]P-mediated carcinogenesis and susceptibility to various cancer forms.

In light of the occurrence of seven different variant CYP1B1 forms, we were interested to investigate whether there were differences among these enzymes in the capacity for metabolism of B[*a*]P possibly contributing to the explanation regarding differences in individual susceptibility (e.g., to lung cancer). We found that two of the forms had a decreased intrinsic clearance for B[*a*]P when expressed heterologously.

Materials and Methods

Chemicals for high-performance liquid chromatography analyses. (±)-Benzo[*a*]pyrene-*trans*-7,8-dihydrodiol (B[*a*]P-7,8-DHD), (±)-benzo[*a*]pyrene-*trans*-9,10-dihydrodiol (B[*a*]P-9,10-DHD), and (±)-3-hydroxybenzo[*a*]pyrene (B[*a*]P-3OH) were purchased from the National Cancer Institute, Chemical carcinogen Repository (Midwest Research Institute, Kansas City, MO). Methanol was from Lab-Scan (Stillorgan, Ireland). B[*a*]P was purchased from Sigma (St. Louis, MO).

Genotyping of the CYP1B1 missense mutations. The genomic DNA used was from 116 healthy unrelated Spaniards who participated in a previous study concerning genetic polymorphism in the *CYP2A6* gene (34). The ethics committee at Karolinska Institutet approved the use of these samples for the current investigation.

Genotyping for CYP1B1, missense mutations in exon 2 (142C > G, 355G > T) and in exon 3 (4326C > G, 4390A > G, 4360C > G), was done using a two-step allele-specific PCR method. For primer sequence and PCR conditions, see ref. 19. In brief, a region covering the polymorphic sites in each exon was amplified in the first PCR (PCR I) from about 50 ng genomic DNA followed by SNP-specific PCR II. Analysis of 142C > G and 355G > T was done by first amplifying part of exon 2 and analysis of 4326C > G, 4390A > G, and 4360C > G was carried out by amplifying part of exon 3, which spans the three polymorphic sites in a PCR I reaction. PCR I product was subsequently used as a template in allele-specific PCR II.

CYP1B1 haplotype analysis. All subjects heterozygous for SNPs within each exon were further analyzed for linkage disequilibrium using molecular haplotyping as previously described (19). In brief, part of each exon covering the polymorphic sites amplified in PCR I was used as a template in PCR II after a 10-fold dilution in water. In PCR II, the haplotypes were determined using two allele-specific primers in both forward and reverse directions in all four possible combinations. All subjects heterozygous for SNPs in both exon 2 and exon 3 were further reanalyzed for linkage disequilibrium in the following way: The two alleles were first separated by allele-specific long PCR with respect to the presence or absence of the 142C > G SNP (Arg48Gly), followed by genotyping of each individual allele for the SNPs in exon 3. The long PCR products were diluted 10-fold in water and 1 μL was used as a template in a PCR II reaction, where the corresponding SNP in exon 3 in which the subject was heterozygous was analyzed.

Construction of pYeDP60-CYP1B1 expression plasmids. The wild-type human CYP1B1 cDNA (*CYP1B1*1*) was amplified selectively and cloned into the pYeDP60 expression plasmid (35). The various CYP1B1 cDNAs were generated by site-directed mutagenesis using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the instructions of the manufacturer with appropriate primer pairs as described in ref. 19. Additional rounds of site-directed mutagenesis generated constructs containing more than one SNP sequentially. In brief, the *CYP1B1*1* plasmid was used as a template to generate the *CYP1B1*2* (142C > G causing Arg48Gly; 355G > T causing Ala119Ser), *CYP1B1*3* (4326C > G causing Leu432Val), and *CYP1B1*4* (4390A > G causing Asn453Ser). The *CYP1B1*6* (142C > G causing Arg48Gly; 355G > T causing Ala119Ser; 4326C > G causing Leu432Val) variant was made using the *Not*I-*Sac*I fragment from the *CYP1B1*3* plasmid to replace the corresponding part in the *CYP1B1*2* plasmid. The variant *CYP1B1*7* was generated by introducing the 4360C > G causing Ala443Gly in the *CYP1B1*6* plasmid. All plasmids were sequenced using the ABI Prism Big Dye terminator cycle sequencing kit and analyzed with ABI Prism 377 DNA sequencer to ensure the correct constructs and to exclude any potential PCR artifacts.

Expression of human CYP1B1 variants in yeast. The different human CYP1B1 enzyme variants were coexpressed with human reductase in *Saccharomyces cerevisiae*. The yeast strain INVSc1-HR *MAT αhis3Δ1 leu2 trp1-289 ura3-52* (*pFL-35 human reductase*), expressing human cytochrome P450 reductase, was a gift from the LINK project (a program of the University of Dundee/Biotechnology and Biology Research Council/Department of Trade and Industry/Pharmaceutical Industry). Yeast cells were transfected with the pYeDP60-CYP1B1 expression plasmids and microsomal preparations were made from expanded single colonies that were inoculated into selective medium and grown as described in ref. 36.

Analysis of benzo[*a*]pyrene metabolism. Rates of CYP1B1-dependent metabolism of B[*a*]P in yeast microsomes were analyzed as previously described (2) with slight modifications. In brief, incubation mixtures contained microsomes corresponding to 5 pmol of the CYP1B1 enzyme and varying concentrations (0, 4, 8, 16, 32, 64, and 80 μmol/L) of B[*a*]P dissolved in DMSO were buffered to pH 7.4 with 100 mmol/L sodium phosphate in a final volume of 0.25 mL. Recombinant human microsomal epoxide hydrolase (Gentest, BD Biosciences, San Jose, CA) was added to a final concentration of 0.25 μg/μL. Final concentration of DMSO in reaction mixtures was kept at <1%. Each variant allele was assayed twice in triplicate using two separate microsomal preparations for each variant enzyme. In all incubations, equivalent protein amounts (0.7 mg/incubation) were used by addition of microsomes from yeast transfected with the empty pYeDP60 plasmid. After 2 minutes of preincubation at 37°C, the reaction was initiated by adding NADPH to a final concentration of 1 mmol/L. Reactions were terminated after 5 minutes by addition of 1 volume of ice-cold acetone and kept on ice for 10 minutes. B[*a*]P and its metabolites were extracted twice with 1 mL of ethyl acetate. The organic-phase extracts were collected

after centrifugation for 10 minutes at 3,500 rpm and dried by evaporation under a nitrogen stream.

High-performance liquid chromatography analysis of (±)-benzo[*a*]pyrene-*trans*-7,8-dihydrodiol extract. The concentrations of the B[*a*]P standard metabolites were determined by UV absorbance of the dried residues dissolved in ethanol and employing extinction coefficient from the National Cancer Institute, Chemical carcinogen Repository (Midwest Research Institute, Kansas City, MO) as previously described (37). The dried residues were dissolved in 100 μL ethanol and 40 μL were injected into the high-performance liquid chromatography (HPLC) column. For B[*a*]P standard samples, pure B[*a*]P was weighed and dissolved in acetone. The separation was done on a Nova-pak C_{18} 3.9 × 150 mm column (Waters, Milford, MA) with a flow of 1.0 mL/min with a Waters 625 LC System (Waters), equipped with a LC 240 (Perkin-Elmer, Ltd., Beaconsfield, United Kingdom) fluorescence detector. Two different excitation/emission wavelength settings were used. For determination of B[*a*]P-tetrols, we used an excitation wavelength of 341 nm and measured emission at 381 nm; for the B[*a*]P-dihydrodiols, excitation was at 253 nm and emission at 410 nm; and for B[*a*]P-3OH and B[*a*]P, we used an excitation wavelength of 380 nm and emission at 431 nm. The excitation and emission wavelength pair was changed during the determination, 0 minute 380/431 nm, 0.5 minute 341/381, 253/410, and 380/431 nm. The B[*a*]P metabolites were separated by a linear gradient of methanol and water, 40 minutes at 30% methanol to 100% methanol, followed by 10 minutes at 100% methanol, and then 19 minutes at 30% methanol before the next injection. For HPLC analysis, using a signal-to-noise ratio of 3, the lower detection limit for B[*a*]P-7,8-DHD was 0.006 pmol/pmol/min. The lower limit of quantification, using a signal-to-noise ratio of 9, was 0.019 pmol/pmol/min (equivalent to 0.475 pmol in each 40 μL injection into the HPLC column). The lowest detectable HPLC peak area in our analysis for B[*a*]P-7,8-DHD was 44.9, which corresponds to 0.137 pmol/pmol/min.

Enzyme kinetics of (±)-benzo[*a*]pyrene-*trans*-7,8-dihydrodiol formation by CYP1B1 variants. For each CYP1B1 variant enzyme, kinetic variables (K_m and V_{max}) for the formation of B[*a*]P-7,8-DHD were determined by nonlinear regression in which data from HPLC were fitted to the following Michaelis-Menten equation: $v = V_{max} S / (K_m + S)$, where v is the rate of reaction, V_{max} is the maximum velocity of the reaction, S is the substrate concentration, and K_m is the Michaelis constant (apparent K_m). Intrinsic metabolic clearance (CL_{int}) was calculated as the ratios of V_{max} to K_m. Calculations were done using Sigma Plot enzyme kinetics module1.1 software (SPSS, Inc., Chicago, IL). In each instance, the simple one enzyme-one substrate Michaelis-Menten model was ascertained to best describe the data based on the appearance of both Michaelis-Menten and Eadie-Hofstee data plots.

Results

Genotyping and CYP1B1 single nucleotide polymorphism frequencies. One hundred sixteen healthy unrelated Spaniards were genotyped for the five missense mutations present in *CYP1B1*, namely, 142C > G, 355G > T, 4326C > G, 4390A > G, and 4360C > G, which cause Arg48Gly, Ala119Ser, Leu432Val, Asn453Ser, and Ala443Gly amino acid substitutions, respectively. The frequencies of the SNPs in the Spanish population investigated were 28.4%, 28.4%, 41.8%, 18.1%, and 2.6%, respectively.

Single nucleotide polymorphism mapping and determination of the CYP1B1 haplotypes. Haplotype reconstruction in every individual who was heterozygous for two or more SNPs was achieved by separately amplifying the two chromosomes using SNP-specific PCR, followed by regenotyping of the separated alleles for the remaining SNP. We employed molecular haplotyping analyses to examine linkage disequilibrium between the different SNPs. The efficiency of the haplotyping method has been previously evaluated by cloning the assigned haplotypes in a pBluescript plasmid followed by sequencing (19). With five missense mutations in the *CYP1B1* gene, theoretically $2^5 = 32$ possible different *CYP1B1* haplotypes could exist. In the population, however, only six were found, namely, *CYP1B1*1*, *CYP1B1*2*, *CYP1B1*3*, *CYP1B1*4*, *CYP1B1*6*, and *CYP1B1*7*, at allele frequencies of 14.7%, 25.4%, 38.8%, 18.1%, 0.4%, and 2.6 %, respectively (Table 1). The 4390A > G causing Asn453Ser (*CYP1B1*4*) exists alone independently of the other SNPs.

Estimation of haplotype frequencies and prediction of haplotype phase from routine genotyping data play an important role in linkage disequilibrium between SNPs, and can be achieved by various methods. Besides performing molecular haplotyping, we also used the Optimal Step Length EM Algorithm (38) for the identification and estimation of haplotype frequency using the online available web site (http://genome3.cpmc.columbia.edu/cgi-bin/GENOME/oslem/doHaplo.cgi), assessed May 30, 2004. The observed haplotypes and their frequencies identified by our molecular haplotyping method did not differ significantly [χ^2 test, degrees of freedom (*df*) = 5, P = 1.0] from the predicted haplotype and their frequencies calculated with computer algorithm (Table 1).

Fourteen different combinations of these haplotypes were present among the individuals examined, of which the *CYP1B1*2/*3* combination was found to be the most frequent one (Table 2). Seventeen individuals were heterozygous for 4326C > G and 4390A > G and molecular haplotyping for these SNPs indicated that two SNPs were not linked in these individuals and they were thus assigned as *CYP1B1*3/*4* genotype. Only one subject was found to be heterozygous for *CYP1B1*6,* and six individuals were heterozygous for *CYP1B1*7.* In all the subjects carrying 142C > G, 355G > T, a complete linkage was seen and it can be concluded accordingly that the rare *CYP1B1*5* was not present in the Spanish population investigated. Only two individuals were homozygous for the *CYP1B1*1* allele. The distribution of the observed genotype did not

Table 1. *CYP1B1* alleles found in a Spanish population (n = 116), their observed haplotype frequencies determined by allele-specific PCR-RFLP and predicted haplotype frequency by using Optimal Step Length EM Algorithm

Allele	Protein	Nucleotide changes	Amino acid substitution(s)	Observed allele frequency	Predicted allele frequency (%)
*CYP1B1*1*	CYP1B1.1	None		14.7	14.3
*CYP1B1*2*	CYP1B1.2	142C > G, 355G > T	Arg48Gly, Ala119Ser	25.4	25.8
*CYP1B1*3*	CYP1B1.3	4326C > G	Leu432Val	38.8	38.7
*CYP1B1*4*	CYP1B1.4	4390A > G	Asn453Ser	18.1	18.1
*CYP1B1*6*	CYP1B1.6	142C > G, 355G > T, 4326C > G	Arg48Gly, Ala119Ser, Leu432Val	0.4	0.5
*CYP1B1*7*	CYP1B1.7	142C > G, 355G > T, 4326C > G; 4360C > G	Arg48Gly, Ala119Ser, Leu432Val, Ala443Gly	2.6	2.6

Table 2. Frequency of *CYP1B1* genotypes Spaniards listed in decreasing order of frequency

Genotype	No. subjects	
	Observed	Predicted (Hardy-Weinberg)
*CYP1B1*2/*3*	24 (20.7 %)	23
*CYP1B1*3/*4*	17 (14.7 %)	16.4
*CYP1B1*3/*3*	16 (13.8 %)	17.5
*CYP1B1*1/*3*	14 (12.1 %)	13.5
*CYP1B1*2/*4*	10 (8.6 %)	10.7
*CYP1B1*1/*2*	8 (6.9 %)	8.7
*CYP1B1*2/*2*	7 (6.0 %)	7.5
*CYP1B1*1/*4*	7 (6.0 %)	6.5
*CYP1B1*4/*4*	4 (3.4 %)	3.8
*CYP1B1*3/*7*	3 (2.6 %)	2.4
*CYP1B1*1/*1*	2 (1.7 %)	2.5
*CYP1B1*2/*7*	2 (1.7 %)	1.6
*CYP1B1*1/*7*	1 (0.9 %)	1.0
*CYP1B1*2/*6*	1 (0.9 %)	0.5

NOTE: Fourteen different combinations of *CYP1B1* alleles were found among 116 Spaniards. The observed genotype distribution pattern was consistent with the expected frequencies according to Hardy-Weinberg equilibrium using χ^2 test ($P > 0.05$, $df = 1$).

significantly deviate from the one expected by the Hardy-Weinberg law as revealed from χ^2 test.

Catalytic properties of CYP1B1 variant enzymes on the formation of (±)-benzo[*a*]pyrene-*trans*-7,8-dihydrodiol. The different human CYP1B1 cDNA variants, generated by site-directed mutagenesis, were cloned into the pYeDP60 expression vector and expressed in *S. cerevisiae*, strain INVSc1-HR, which is genetically modified to overexpress the human P450 reductase. All CYP1B1 variants were well expressed in yeast and properly folded to give a typical P450 spectrum in the CO-reduced form with a peak at 450 nm. The expression levels were similar, although not identical for all forms (Table 3). The kinetic properties of the six enzyme variants found in Spaniards, CYP1B1.1, CYP1B1.2, CYP1B1.3, CYP1B1.4, CYP1B1.6, and CYP1B1.7, were analyzed using B[*a*]P as a substrate. Formation of B[*a*]P-7,8-DHD was analyzed by incubating enzymes with varying concentrations of B[*a*]P in the presence of microsomal epoxide hydrolase. Representative HPLC chromatograms from incubation mixtures with CYP1B1.1 and CYP1B1.7 are given in Fig. 1.

The K_m, V_{max}, and intrinsic clearance (V_{max}/K_m) are given in Table 3. Michaelis-Menten plot for the formation of B[*a*]P-7,8-DHD by the different CYP1B1 variant enzymes expressed in yeast is illustrated in Fig. 2. Incubations with B[*a*]P revealed that CYP1B1.2, CYP1B1.3, and CYP1B1.6 exhibited similar kinetic properties as CYP1B1.1. Although CYP1B1.4 showed the lowest K_m value, the intrinsic clearance was significantly lower compared with CYP1B1.1 using one-way ANOVA with post hoc test (Bonferroni's multiple comparison test, $P < 0.01$). Interestingly, CYP1B1.7 had a significantly reduced V_{max} and increased apparent K_m for the formation of B[*a*]P-7,8-diol and the intrinsic clearance was only 20% of that of CYP1B1.1 ($P < 0.001$, independent *t* test, ANOVA with Bonferroni post hoc test). Thus, CYP1B1.7 has a dramatically decreased ability for the formation of B[*a*]P-7,8-DHD from B[*a*]P.

Discussion

The data obtained show that the kinetics for CYP1B1.7-dependent metabolism of B[*a*]P to B[*a*]P-7,8-diol is indeed much different from CYP1B1.1, whereas no or only small differences were

Table 3. Comparison of the pharmacokinetics of conversion of B[*a*]P to B[*a*]P-7,8-DHD by different human CYP1B1 variant enzymes, expressed in *S. cerevisiae*

CYP1B1 variant	Amino acid substitutions	Expression level (pmol/mg)	Formation of B[*a*]P-7,8-DHD			4-Hydroxylation of 17β-estradiol*		
			K_m (μmol/L)	V_{max} (pmol/pmol P450/min)	V_{max}/K_m	K_m (μmol/L)	V_{max} (pmol/pmol P450/min)	V_{max}/K_m
CYP1B1.1	None	14.6	21.1 ± 3.8	1.82 ± 0.12	0.086	5.3 ± 0.3	3.8 ± 0.1	0.71
CYP1B1.2	Arg48Gly, Ala119Ser	3.6	23.1 ± 5.8	0.91 ± 0.7	0.040	6.4 ± 1.9	4.7 ± 0.2	0.73
CYP1B1.3	Leu432Val;	7.1	23.3 ± 3.13	1.48 ± 0.07	0.064	6.4 ± 0.2	3.3 ± 0.2	0.51
CYP1B1.4	Asn453Ser	5.2	16.4 ± 4.1†	0.34 ± 0.03‡	0.021†	7.0 ± 1.0	3.6 ± 0.6	0.51
CYP1B1.6	Arg48Gly, Ala119Ser, Leu432Val	16.1	22.2 ± 6.7	1.22 ± 0.13	0.055	12.1 ± 1.0‡	2.1 ± 0.1	0.17§
CYP1B1.7	Arg48Gly, Ala119Ser, Leu432Val, Ala443Gly	7.4	47.4 ± 6.8§	0.76 ± 0.11†	0.016§	13.4 ± 1.3‡	1.7 ± 0.2‡	0.12

NOTE: For comparison, the kinetics of formation of 4-hydroxy estradiol from 17β estradiol as previously published (18) is given.
Kinetic variables are presented as means ± SD from three independent analyses in triplicate using two separate microsomal preparations for each enzyme variant.
*Data from ref. 19.
†Significant difference at $P < 0.05$ compared with CYP1B1.1 using independent *t* test and ANOVA with Bonferroni post hoc test.
‡Significant difference at $P < 0.01$ compared with CYP1B1.1 using independent *t* test and ANOVA with Bonferroni post hoc test.
§Significant difference at $P < 0.001$ compared with CYP1B1.1 using independent *t* test and ANOVA with Bonferroni post hoc test.

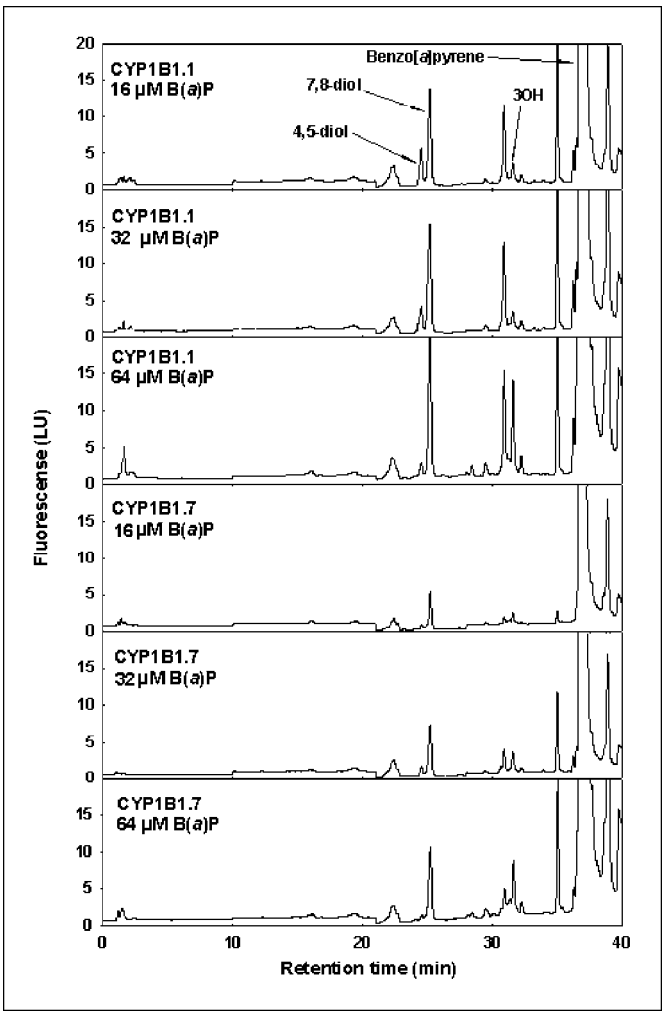

Figure 1. HPLC analysis of B[*a*]P metabolism by heterologously expressed CYP1B1.1 and CYP1B1.7 at three different substrate concentrations (16, 32, and 64 μmol/L). The metabolites identified are B[*a*]P-3OH (*3OH*), B[*a*]P-7, 8-DHD (*7,8-diol*), and B[*a*]P-4,5-DHD (*4,5-diol*).

seen using the other variant CYP1B1 forms. No significant influence of CYP1B1.6 on the formation of B[*a*]P-7,8-DHD from B[*a*]P was seen, which differs from previous findings regarding the metabolism of estradiol, where a lower clearance was seen (18). This indicates a substrate-dependent alteration in the function of CYP1B1.6. By contrast, CYP1B1.7, having in addition a Ala443Gly substitution, showed significantly reduced activity for the metabolism of both estradiol and B[*a*]P (Table 3). The Ala443 amino acid is conserved in the mouse, rat, and human CYP1B1 sequences. As glycine residues are critical for protein bending, it is reasonable to suggest that this amino acid substitution affects folding and function of the enzyme.

In humans CYP1B1 oxidizes all-*trans*-retinol to all-*trans*-retinal, the rate-limiting step for retinoic acid biosynthesis (39). Most cases of primary congenital glaucoma are caused by deleterious mutations in the *CYP1B1* gene (40), and *Cyp1b1* knockout mice show ocular drainage structure abnormalities consistent with those seen in humans with primary congenital glaucoma and mutations (41). Recently, *CYP1B1* alleles carrying the Ala443Gly substitution have been reported in German and French primary congenital glaucoma patients, and an association with the disease phenotype was suggested (42, 43). Interestingly, we have found that the Ala443Gly substitution is relatively common in an African population (18). Its frequency was 7% as compared with 2.6% in the Spaniards here investigated and, furthermore, primary congenital glaucoma is more frequent (5-10%) and occurs earlier in Black than in White subjects.

Because of the reduced capacity of the *CYP1B1*7* variant to form carcinogenic metabolites such as 7,8-diol and 4-OH estradiol from B[*a*]P and estradiol, respectively, *CYP1B1*7* may be advantageous in conferring an individual's protection from B[*a*]P- and estrogen-induced carcinogenesis. On the other hand, carriers of this variant gene might have reduced capability for metabolism of endogenous compounds, possibly affecting the risk for eye abnormalities and increased risk of primary congenital glaucoma.

We found no significant differences in the catalytic properties between CYP1B1.1, CYP1B1.2, CYP1B1.3, and CYP1B1.6 in the metabolism of B[*a*]P. Accordingly, Shimada et al. (44) reported similar activities of these variant enzymes, expressed together with human NADPH-P450 reductase in bacteria, in the metabolism of B[*a*]P and DMBA. Also, no significant alteration in the conversion of B[*a*]P-7,8-DHD into tetrahydrotetrols by the presence for these variant enzymes expressed in yeast was reported by Mammen et al. (45).

Our results indicate that although CYP1B1.4 had the lowest K_m value, the V_{max} and V_{max}/K_m values were significantly lower compared with CYP1B1.1. Thus, CYP1B1.4 might be of a high-affinity but low-capacity variant enzyme. Similarly, a 2-fold decrease in both K_m and V_{max} for CYP1B1.4 enzyme, compared with CYP1B1.1 enzyme expressed in yeast for the conversion of B[*a*]P-7,8-DHD to (±)-benzo[*a*]pyrene-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxides, has been reported before (45). Recently, expression of CYP1B1 variant enzymes in COS-1 cells revealed a selective posttranslational degradation of CYP1B1.4, causing a 2-fold reduction in the cellular level of the CYP1B1.4, significantly reduced enzyme half-life, and ethoxyresorufin *O*-deethylase activity for CYP1B1.4 as compared with other CYP1B1 variant enzymes (46). The authors indicated polymorphism-dependent posttranslational regulation of CYP1B1

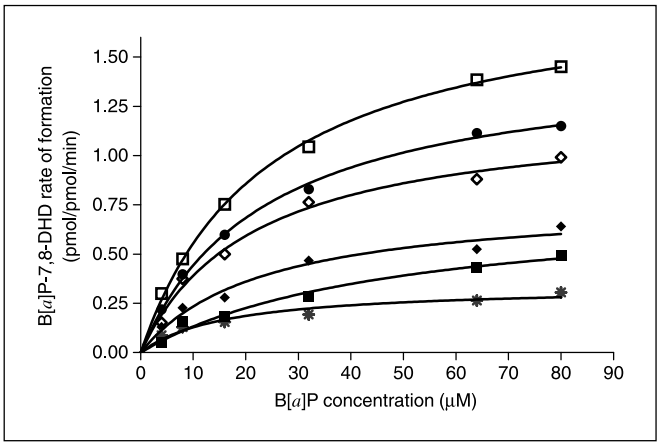

Figure 2. Michaelis-Menten plot for the formation of B[*a*]P-7,8-DHD by human CYP1B1 variant enzymes expressed in yeast. Kinetic studies were done by incubating microsomes (containing 5 pmol P450) with B[*a*]P (0, 4, 8, 16, 32, 64, and 80 μmol/L) in the presence of recombinant human microsomal epoxide hydrolase as explained under Materials and Methods. The values were estimated by fitting the one enzyme-one substrate Michaelis-Menten equation using a nonlinear regression. *Points,* mean of three incubations in triplicate. □ CYP1B1.1; ♦ CYP1B1.2; ● CYP1B1.3; ⋆ CYP1B1.4; ◇ CYP1B1.6; ■ CYP1B1.7.

expression and suggested a reduced metabolic activation of endogenous and exogenous procarcinogens by CYP1B1.4 as a mechanism to explain the recently reported lower incidence of endometrial cancer in individuals carrying the *CYP1B1*4* (47). Thus, our results regarding CYP1B1.4 are in accordance with these suggestions.

There is a pronounced difference in the distribution of *CYP1B1* haplotypes in various populations. *CYP1B1*7* and *CYP1B1*4* are more common in Black and White populations, respectively. In fact, only 1 of 150 Ethiopians and 2 of 116 Spaniards were homozygous for the wild-type allele (*CYP1B1*1*). Considerable interindividual variability has been observed in carcinogen metabolism, such as of B[*a*]P and estradiol. Large interindividual variation has been noted in the metabolic activation of B[*a*]P in human tissues as well as in measurements of levels of B[*a*]P-DNA or protein adducts in humans (33).

A thorough investigation of the haplotypes in White populations and evaluation of the kinetic properties of CYP1B1.7 towards B[*a*]P metabolism have not been previously presented. The importance of understanding linkage disequilibrium in candidate loci is becoming increasingly evident. Several epidemiologic studies have been carried out associating each individual SNP in the *CYP1B1* gene with the incidence of lung, breast, and endometrial cancer, and the findings are controversial. Our results indicate the importance of taking the entire haplotype into consideration to be able to make functionally based epidemiologic studies of *CYP1B1* genetic polymorphism and its relation to incidence of cancer.

In conclusion, our results indicate that CYP1B1 variant enzymes vary in their activity with respect to metabolism of B[*a*]P. Thus, subjects carrying the *CYP1B1*7* allele may be protected from CYP1B1-mediated metabolic activation of precarcinogenic polyaromatic hydrocarbons. Thus, the polymorphism of CYP1B1 might have a role in the link between environmental B[*a*]P, estrogens, and hormonelike substances and the interindividual risk of lung and breast cancer. We believe that the results of our present investigation will serve as a basis to further elucidate the relationship between *CYP1B1* genetic polymorphism and cancer incidence in humans.

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Characterization of Common CYP1B1 Variants with Different Capacity for Benzo[*a*]pyrene-7,8-Dihydrodiol Epoxide Formation from Benzo[*a*]pyrene

Eleni Aklillu, Steinar Øvrebø, Ingrid V. Botnen, et al.

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