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Dibenzo[*def,p*]chrysene Transplacental Carcinogenesis in Wild-Type, *Cyp1b1* Knockout and *CYP1B1* Humanized Mice

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Abstract

The cytochrome P450 (CYP) 1 family is active toward numerous environmental pollutants, including polycyclic aromatic hydrocarbons (PAHs). Utilizing a mouse model, null for *Cyp1b1* and expressing human *CYP1B1*, we tested the hypothesis that hCYP1B1 is important for dibenzo[*def,p*]chrysene (DBC) transplacental carcinogenesis. Wild-type m*Cyp1b1*, transgenic h*CYP1B1* (m*Cyp1b1* null background), and m*Cyp1b1* null mice were assessed. Each litter had an equal number of siblings with *Ahr*^{b-1/d} and *Ahr*^{d/d} alleles. Pregnant mice were dosed (gavage) on gestation day 17 with 6.5 or 12 mg/kg of DBC or corn oil. At 10 months of age, mortality, general health, lymphoid disease, and lung tumor incidence and multiplicity were assessed. h*CYP1B1* genotype did not impact lung tumor multiplicity, but tended to enhance incidence compared to *Cyp1b1* wild-type mice ($p = 0.07$). As with *Cyp1b1* in wild-type mice, constitutive hCYP1B1 protein is non-detectable in liver but was induced with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Wild type mice were 59% more likely to succumb to T-cell Acute Lymphoblastic Leukemia (T-ALL). Unlike an earlier examination of the *Ahr* genotype in this model (Yu *et al.*, Cancer Res., 2006), but in agreement with a more recent study (Shorey *et al.*, Toxicol. Appl. Pharmacol., 2013), this

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Deceased

genotype was not associated with lung tumor incidence, multiplicity, or mortality. Sex was not significant with respect to lung tumor incidence or mortality but males exhibited significantly greater multiplicity. Lung tumor incidence was greater in *mCyp1b1* nulls compared to wild-type mice. To our knowledge this is the first application of a humanized mouse model in transplacental carcinogenesis.

Terms

PAH carcinogenesis; transplacental cancer; Cytochrome P450 1B1; CYP1B1 humanized mice

Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) as Transplacental Carcinogens

PAHs are produced following the incomplete combustion of carbon-containing materials (e.g., burning of petroleum products, coal, wood or tobacco) [1]. A number of the higher molecular weight PAHs (4 or more rings) are carcinogenic in animal models [2]. Benzo[a]pyrene (BaP), the most extensively studied PAH is rated by the International Agency for Research on Cancer (IARC) as a class 1 known human carcinogen while DBC is rated as 2A (probable human carcinogen [2]. Previous work by our laboratory employed a transplacental model of cancer utilizing DBC, also known as dibenzo[a,*h*]pyrene, and documented an aggressive T-cell acute lymphoblastic leukemia (T-ALL) in offspring beginning at 12-18 weeks of age, as well as adult onset cancers of the reproductive system, liver, and lung [3-6]. A transplacental mouse model, null for *mCyp1b1*, did not develop T-ALL, but was susceptible to lung tumors [4]. Lung tumor multiplicity in *mCyp1b1* null mice was reduced by half compared to mice possessing one or both *mCyp1b1* alleles [4].

Role of CYPs in PAH Carcinogenesis

The mouse and human CYP1 families, consisting of 1A1, 1A2, and 1B1, activate polycyclic aromatic hydrocarbons (PAHs), to epoxides, dihydrodiol-epoxides or quinones, reactive intermediates capable of DNA binding and/or redox cycling following occupational or environmental exposures [7-13]. In the case of DBC, CYP1A1 and CYP1B1, in concert with epoxide hydrolase, produce 4 enantiomers of the 11,12-dihydrodiol-13,14-epoxide with high affinity for covalent adduction to dA bases of DNA [11,12]. Phase 2 enzymes provide protection against PAH genotoxicity by conjugation. Glutathione-S-transferases react with epoxides whereas UDP-glucuronosyl transferases and sulfotransferases can conjugate phenols and quinones [8].

Role of the Ahr

The endogenous roles of Ahr appear to include cell cycle control, response to oxidative stress and apoptosis regulation [14]. Mouse strains can vary widely in response to Ahr ligands, attributable to inbred strain allelic differences. B6 mice are homozygous for the “responsive” *Ahr^{b-1}* allele, whereas D2 mice possess two copies of the less responsive *Ahr^d* allele [15-17]. The *Ahr^{b-1}* allele is dominant and *Ahr^{b-1/d}* mice are “responsive”. Human Ahr affinity toward a single substrate can vary up to 12-fold without a clear genetic polymorphic

explanation [18]. It is generally accepted that the “human like” mouse receptor is the less responsive *Ahr^d* allele, however a mouse transgenic for human *Ahr* was found to be less responsive to TCDD in liver than either wild-type *Ahr^d* or *Ahr^{b-1}* mice [19].

PAHs, as with other Cyp1 substrates, are capable of up-regulating their own metabolism by acting as Ahr ligands [20]. Previous studies on PAH-dependent transplacental carcinogenesis have shown that susceptibility is greatest with a maternal “non-responsive (*Ahr^{d/d}*)” allele and a fetal responsive allele (*Ahr^{b-1/d}*) [21,22]. The hypothesis is that a non-responsive dam does not metabolize PAHs as readily, thus parent PAH is more available to the fetus. A responsive Ahr in the fetus is thought to enhance risk as the ratio of bioactivation to detoxication is higher than in adults (phase two detoxication enzymes such as UGT, SULF and glutathione-S-transferase (GST) are not yet fully developed) [23].

Transgenic hCYP1B1 Mice

Previously, *hCYP* transgenic (“humanized”) mice have been valuable resources in assessing the contribution of hCYP3A4 and hCYP2D6 in drug metabolism *in vivo* [24,25]. A trans-activator-controlled *hCYP1B1* transgenic mouse, also expressing mouse Cyp1b1, was developed to assess response to anti-androgens [26]. A transgenic humanized *CYP1B1* mouse model, null for mCyp1b1 expression, was developed and successfully used to assess obesity and the role of CYP1B1 and SCD1 [27]. We hypothesized that expression of hCYP1B1 in mCyp1b1 null mice would be capable of inducing T-ALL mortality following exposure to DBC *in utero* and is the genesis of this study. The translational impact associated with these findings are potentially high given the demonstrated expression of CYP1B1 in a number of potential target tissues (including thymus) in the fetus and neonate [23].

Constitutive Expression and Endogenous Role of hCyp1B1

hCYP1B1 is expressed constitutively in human extrahepatic organs in both the adult and fetus [7,28-32]. There is a significant intra-individual variation in expression in the same tissue as well as between tissues of the same individual [33]. hCYP1B1 contributes to ocular development and differentiation, and mutations in *hCYP1B1* are associated with primary congenital glaucoma [34]. Among endogenous substrates, hCYP1B1 is active in the 4-hydroxylation of 17 β -estradiol [35-37], a metabolite linked to estrogen-dependent carcinogenesis [37,38]. hCYP1B1 and polymorphisms of hCYP1B1 have been linked to several hormone-induced cancers, including prostate, breast, endometrial, and ovarian [35,37-39].

Materials and Methods

Chemicals and Reagents

DBC was purchased from Midwest Research Institute (Kansas City, MO), TCDD was from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). Biolase™ buffers, DNA polymerase, and Polymate™ were from Biotline (Taunton MA), Trizol reagent, proteinase K, Superscript III reverse transcriptase kit, Novex™ TBE gels, and dNTPS were purchased from Life Technologies (Grand Island, NY). DirectPCR® Lysis Reagent was from Viagen

Biotech (Los Angeles, CA) SYBER green polymerase master mix was from Qiagen (Valencia, CA). Western Lightning® Plus ECL was from PerkinElmer (Richmond, CA.). Zymoclean™ DNA Gel Recovery kit was from Zymo Research (Irvine, CA) Commercial antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX) All other reagents were purchased through VWR Scientific (Radnor, PA)

Animal Care and Housing

Animal subject research was approved by the Oregon State University Institutional Animal Care and Use Committee (IACUC), in accordance with Oregon State University's Association for the Assessment and Accreditation of Laboratory Care (AAALAC) International accreditation. Animal housing rooms were maintained on a 6:30 AM-6:30 PM 12 hour light/dark cycle, 21-23°C temperature, 30-70% humidity, utilizing CareFresh bedding. Mice were fed AIN93G diet from mating through pup weaning 21 days postpartum, at which time pups were separated by sex and housed up to 5 siblings per micro isolator cage. Pups remained on AIN93G until 3 months of age at which time diet was changed to AIN93M; all diet and water was *ad libitum*. Animals were checked twice daily for health until termination of the study. At signs of distress, such as lethargy, or heavy breathing, mice were preemptively euthanized. The euthanization method was asphyxiation by CO₂, followed by a cervical dislocation, in accordance with AAALAC guidelines.

Transgenic CYP1B1 Mice

We incorporated the hCYP1B1 transgenic mouse into the *in utero* DBC exposure model, previously found to be susceptible to T-ALL and lung cancer [3-6,40]. The transgenic hCYP1B1 mouse was generated by pronuclear injection of ovulated mouse eggs on a mCyp1b1 null background with a CYP1B1 bacterial artificial chromosome (BAC) clone as previously described [27]. hCYP1B1 transgenic hemizygous C57BL6 dams on a mCyp1b1 null background were shipped to Oregon State University (OSU) from the colony maintained by one of the authors (FJG) at the National Cancer Institute in order to establish a breeding colony for this study. Incoming transgenic dams were bred with matched null male mice from a colony at OSU (original breeding pairs from Dr. Gonzalez). For rederivation, following natural birth, offspring were cleaned and placed with foster dams as part of an OSU-mandated procedures when importing mice from non-commercial facilities. All subsequent mating for colony maintenance was limited to C57BL6 mCyp1b1 null mice where at least one parent had at least one copy of the transgene (Suppl. Figure 1).

mCyp1b1 is located on chromosome 17 [41] whereas hCYP1B1 is located on chromosome 2 at the 2p21-22 region [28,42]. Pronuclear injection is not location- or copy number-specific, therefore the hCYP1B1 transgenic mouse contains an unknown copy number and chromosomal location. The mCyp1b1 and hCYP1B1 genes have the same number of exons and introns and open reading frames (ORFs) and sequence similarity is quite high (Suppl. Figure 2) [41,42]. Both ORFs start in the second exon and continue into the third exon. mCyp1b1 exons 1, 2, and 3 (Suppl. Figure 2, blue boxes) are 371, 1042, and 3780 bp, respectively, whereas hCYP1B1 exons 1, 2, and 3 are 371, 1044, and 3707 bp, respectively. mCyp1b1 introns 1 and 2 (Suppl. Figure 2, orange) are 376 and 2591 bp in length compared to 390 and 3032 bp, respectively for hCYP1B1 [28,40-42]. Both mRNAs are 5.2 kb and both

proteins are 543 amino acids (AA) in length [39,40]. The six substrate responsive sites (SRS) of the enzyme have a similar AA sequence and alignment as follows: SRS1, AA 116-139, 81%; SRS2, AA 224-236, 100%; SRS3, AA 259-263, 80%; SRS4, AA 330-343, 100%; SRS5, AA 392-401, 90%; SRS6, AA 503-513, 90% [39,40] (Suppl. Figure 2).

Breeding of Experimental Animals and Dosing Dams with DBC

We modified our previous transplacental DBC exposure model [3-6,40] to include a transgenic *hCYP1B1* group. All experimental transgenic null (C57BL6) and null mice (C57BL6 and 129S genetic background) were maintained and bred at OSU. One generation was needed to produce null B6129F1 dams and a second generation was needed to produce the offspring from DBC-treated B6129F1 dams (Suppl. Figure 1). Control *mCyp1b1* B6129F1/J dams and 129S/J sires were purchased from Jackson. On gestation day 17, dams were dosed by gavage with 0, 6.5 or 12 mg/Kg DBC b.w. in 5 ml/Kg b.w. corn oil.

Tumor Induction and Histopathology

Health checks were conducted twice daily for general health. T-ALL mortality was evident beginning at 12 weeks of age and continued through the study termination at 10 months of age. At the first sign of distress, animals were euthanized and accounted in the mortality curve. Gross morphological observations were made of the entire body, with close focus on thymus, spleen, lymph nodes of the throat and chest, lungs, liver gonad, and spleen. These tissues were fixed in 10% neutral buffered formalin. Histopathology and gross anatomy were utilized to determine cause of death. Lung tumor multiplicity was compared between animals surviving to the 10 month terminal endpoint by fixing in 10% neutral buffered formalin. At gross necropsy, using a dissecting microscope, lung tumors were identified and counted over the entire surface of the lung and confirmed, along with identification of tumor type by histopathology of the fixed tissue as previously described [3].

Genotyping for CYP1B1 and AHR alleles

hCYP1B1^(-/-) and *hCYP1B1*^(+/-) F₁ pups were genotyped in each litter (Suppl. Figure 3). Ear punches, 0.2 cm, were digested in 49 µL Direct PCR Lysis Reagent with 1 µL Proteinase K (20 mg/mL) overnight at 55°C. Primers were diluted to 5 µM in Tris buffer and utilized at a 1:1 ratio of forward and reverse primers. Each PCR reaction mix included: 2 µL 10x Biolase buffer, 0.8 µL 50 mM MgCl₂, 0.4 µL 10 mM dNTPs, 1.6 µL *hCYP1B1* mix, 1.6 µL *mFMO1* primer mix, 0.8 µL BiolaseTM polymerase (5 U/µL), 10 µL PolymateTM and 1 µL 200 ng tissue lysate. PCR conditions were: Step 1, 95°C for 5 min; Step 2, (95°C for 30 sec → 55 °C for 30 sec → 72 °C for 45 sec) × 35 cycles followed by Step 3, 72°C for 10 min. Gel separation was achieved in a 2% agarose/1x TAE gel spiked with 4 µL Gel RedTM (10,000x) per 100 mL gel. Wells were loaded with 18 µL amplicon and 3 µL loading dye and electrophoresis conducted at 130 V for 45 min, then 100 V for 15 min. The *hCYP1B1* product is 201 bp in length while the *mFmo1* product (positive control) is 99 bp. The *hCYP1B1* primer sequences were:

hCYP1B1-f CCA ACC TGC CCT ATG TCC T

hCYP1B1-r CTG GAT CAA AGT TCT CCG GG

The forward and reverse primers for mFmo1 were:

mFmo1-197f AAG TGA GTT TGC ATG GCG CAG C

mFmo1-198r CCC TTT AGC CCC TTC CCT CTG

Ahr^{b-1/d} and *Ahr^{d/d}* genotyping (Suppl. Figure 4), was modified from a method previously described [3]. Lysates of 0.2 cm ear punches were digested overnight at 55°C in 49 µL Direct PCR Lysis Reagent with 1 µL Proteinase K (20 mg/mL). *Ahr* primers were diluted to 5 µM in Tris buffer and utilized at a 2:1:2 mix of *Ahr*546-f:*Ahr*596^{b-1}-r:*Ahr*596^d-r. Each PCR reaction mix included: 1 µL 10x BiolaseTM buffer, 0.5 µL 50 mM MgCl₂, 0.2 µL 10 mM each dNTPs, 2.0 µL primer mix, 0.2 µL BiolaseTM enzyme, 5.1 µL PolymateTM and 1 µL 200 ng tissue lysate. PCR conditions were as follows: Step 1, 95 °C for 10 min; Step 2, (95 °C for 45 sec → 58 °C for 45 sec → 68 °C for 1 min) (repeat 14 times); Step 3, (95 °C for 45 sec → 58 °C for 45 sec → 68 °C for 2 min) (repeat 17 times); Step 4, 68 °C for 10 min. Gel separation was performed with 0.4 µL PCR amplicon, 1.6 µL water, and 2 µL loading dye per well in an 8% acrylamide, 1xTBE gel at 200 V for 54 min prior to imaging with gel red stain. The *Ahr^{b-1}* product is 158 bp and the *Ahr^d* product is 148 bp. The primers sequences were:

*Ahr*546-f 5'-GAA GCA TGC AGA ACG AGG AG

*Ahr*596^{b-1}-r 5'-caa gct tat aTG CTG GCA AGC CGA GTT CAG

*Ahr*596^d-r 5'- TG CTG GCA AGC GGA GTT CAT

Constitutive mRNA Expression

Measurement of hCYP1B1 and mCyp1b1 mRNA Transcripts by qPCR—Both *hCYP1B1* and *mCyp1b1* mRNA expression were assessed at the 3 month time point via qRT-PCR in lung, thymus, liver, and gonad tissues collected from F₁ progeny and snap frozen prior to storage at -80°C. Male mice, genotyped as *Ahr^{b-1/d}* or *Ahr^{d/d}*, were chosen for analysis of lung, liver, and thymus. Analysis of gonads included both male and female *Ahr^{b-1/d}* and *Ahr^{d/d}* tissues. For all tissues and both genotypes, analysis was performed on tissues of 3 pups from 3 separate litters.

Total RNA was isolated using TRIzol®. RNA isolate was analyzed via Bioanalyzer (Agilent Technologies, Santa Clara, CA), with a minimum RNA Integrity Number (RIN) of 8. cDNA was synthesized with a Superscript III First-Strand cDNA Synthesis Kit per manufacturer's instructions. Each reaction included 240 ng RNA, 1 µL 50 mM poly dT oligo, 1 µL annealing buffer, 10 µL 2X first strand reaction mix, and 2 µL Rnase OUT in a reaction volume of 8 µL. PCR conditions were 50 min 50°C, 5 min 85°C, hold 20°C.

Real-time Quantitative PCR: Twenty µL reactions were run for each of the three genes containing 240 ng cDNA; each respective forward primer (*hCYP1B1*: AAGTTCTTGAGGCACTGCGAA, 150 nM) (*mCyp1b1*: TTG ACC CCA TAG GAA ACT GC, 75 nM) (GAPDH: TCT CCC TCA CAA TTT CCA TCC CAG, 75 nM), reverse primer (*hCYP1B1*: GGCCGGTACGTTCTCCAAAT, 150 nM) (*mCyp1b1*: GCT GTC TCT TGG TAG GAG GA, 75 nM) (GAPDH: GGG TGC AGC GAA CTT TAT TGA TGG, 75 nM), 10 µL 2X SYBER Green master mix (containing buffers, DNA polymerase, and dNTPs) and

3.6 μ L of water. Amplifications were done on a BioRad iQ5 thermocycler (Hercules, CA) with the following conditions: 95°C 10 minutes, 40 cycles of 95°C for 15 sec, to denature, followed by 58.8°C for 1 min, melt curve cycles, 10°C hold. The primer efficiency cut off was 90%. Standards were generated by PCR amplification of pooled cDNA using gene-specific primer pairs. Products were separated by gel electrophoresis and purified with a Zymoclean™ Gel Recovery Kit. Using a relative quantitation method, CT values were related to starting quantity of mRNA via standard curve.

The quality control for instrument and technical consistency included a CT value standard error cutoff of 0.5 between biological replicates and among the average GAPDH values across genotypes of a specific tissue type (i.e., all *Ahr*, *mCyp1b1* and *hCYP1B1* groups were 0.5 standard error within a tissue type).

Measurement of hCYP1B1 Protein Levels in Transgenic Mice and Induction by TCDD

—To assess the induction of the *hCYP1B1* transgenic protein, 12 mg/kg DBC, 0.1 μ g/kg TCDD, or 6.5 mL/kg corn oil were administered by oral gavage to the transgenic mice once daily for 3 days prior to euthanasia 24 hours post final gavage. Microsomes from control, DBC- and TCDD-treated *hCYP1B1* transgenic mice, 6 weeks of age, were isolated from liver homogenate by centrifugation at 100,000g. The positive control was *hCYP1B1* supersomes (BD Gentest) and the negative control was a from a mouse hepal cell homogenate treated with TCDD for induction of *mCyp1a1* (generously donated by Edward O'Donnell, Oregon State University). A 4-12% gradient SDS-PAGE gel (Nupage) was loaded with 40 μ g of liver microsomal protein. The lanes containing liver microsomes from TCDD-treated mice contained 20 μ g of protein. A *hCYP1B1*-specific antibody, previously described [43], was donated by Dr. Craig Marcus (Oregon State University) and utilized at a 1:1000 dilution. Goat anti-rabbit IgG secondary antibody (Santa Cruz) was utilized at a 1:2000 dilution with Western Lightning Plus® ECL chemiluminescent detection with a BioRad ChemiDoc® imaging system (Hercules, CA).

Statistics

Biostatistics were assessed for the outcomes of mortality/morbidity, lung tumor incidence and lung tumor multiplicity across the experimental parameters of sex, *Ahr* and *hCYP1B1* or *mCyp1b1* genotype, as well as DBC dose. Pairwise comparisons of probability were used to assess experimental factors in the probability of disease outcome using a proportional hazards model [44] and significance was attributed to p values <0.05. Due to the few numbers of animals remaining in some treatments groups, a Monte Carlo test for independence [45] was conducted instead of Chi-squared approximation, thus the usual Pearson's Chi-squared Test for independence could not be used to assess a possible litter effect. Based on the Monte Carlo test, the litter was found to have a statistically significant effect on tumor incidence (p <0.001). The litter effect was included as a random effect in the following models.

T-All mortality results were verified with analysis of variance, the Wald chi-square test statistics and p values for the main effects, based on the Type III sums of squares. The estimated odds ratios for the comparisons and p values were adjusted for multiple

comparisons using the single-step method [46]. The assessment of influence by multiple factors (sex, *Ahr* status, *mCyp1b1* and *hCYP1B1* genotype) was assessed by Cox proportional hazards regression parameter estimates. Multiplicity data was assessed with a reduced Poisson model based on the Type III sums of squares from the 6.5 and 12 mg/kg DBC groups. The 0 mg/kg dose control was excluded due to insufficient numbers of samples containing tumors.

Results

hCYP1B1 Genotyping, mRNA Expression and Protein Expression in Control and TCDD-Treated Hepatic Microsomes; *Ahr* Genotyping

Offspring born to *hCYP1B1*^{+/+} dams, crossed to *hCYP1B1*^{-/-} sires, were genotyped as shown in Supplemental Figure 3. Constitutive expression of *hCYP1B1* mRNA from 3 month old transgenic mice was evaluated and compared to *mCyp1b1* mRNA from wild-type mice in lung, thymus, liver, ovary and testis. The level of *mCyp1b1* mRNA expression in these tissues was about 4 orders of magnitude higher than *hCYP1B1* mRNA (Figure 1). Protein expression was examined by western blotting. The *hCYP1B1* supersome standard (Figure 2, lane 2) and *hCYP1B1* from liver homogenate of TCDD-treated *hCYP1B1* transgenic mice (Figure 2, lanes 8-10) both had an estimated MW of 55 kD. Constitutive hepatic expression of *hCYP1B1* is low to non-detectable (Figure 2, lane 4). The human gene is capable of induction in liver (lanes 8-10) from this transgenic mouse by a strong *Ahr* ligand (TCDD). Conversely, DBC (Figure 2, lanes 5-7) did not induce *hCYP1B1* levels above that of corn oil controls (lane 4). Inclusion of a *mCyp1a1* standard (Figure 2, lane 3) confirmed that there was no cross-reaction of this human CYP1B1- specific antibody with *mCyp1a1*. The *Ahr*^{b-1/d} and *Ahr*^{d/d} alleles were readily discerned (Suppl. Figure 4).

T-ALL- Induced Mortality

Dose-dependent T-ALL-induced mortality was observed for wild-type (*mCyp1b1*^{+/+}) mice treated with 6.5 and 12 mg/kg DBC (Figure 3, panels C and F). In addition to T-ALL, other diseases of lymphatic tissues were present, such as, splenomegaly, enlarged lymph nodes of the neck and axillary areas, and dysfunction of the bone marrow (anemia). The only groups significantly ($p < 0.001$) expressing diseases of lymphatic tissues were wild-type mice (Figure 3, panels C and F). Sex and *Ahr* status were not significant predictors of mortality for T-ALL. Mice that were genotyped as wild-type were 75-times more likely ($p < 0.001$) to experience mortality, relative to *mCyp1b1* null mice (Figure 3, panels A and D); the transgenic *hCYP1B1* mice did not differ significantly from *mCyp1b1* nulls (Figure 3, panels B and E).

Lung Tumor Incidence and Type

Lung tumor incidence was assessed in offspring surviving to 10 months of age. The incidence, across all genotypes, of lung tumor development was 5.5% in controls (consistent with the spontaneous lung tumor incidence at 10 months previously observed in this model [3-6,40]), 96.7% in mice born to mothers dosed with 6.5 mg/kg DBC, and 94.9% at a maternal dose of 12 mg/kg DBC (Table 1). Mice not expressing *mCyp1b1* exhibited a higher spontaneous tumor incidence at 10 months of age compared to wild-type mice. At the

highest maternal DBC dose a similar difference was observed, although it must be kept in mind that these are survivors that did not succumb to DBC-induced T-ALL mortality. *Ahr* status and sex had no significant effect on lung tumor incidence.

Excised tumors were analyzed and categorized from a subset (n=7 or 8) of mice with tumors (multiplicity of 6-9 tumors/tumor-bearing mouse). Samples from 5/7 wild-type *Cyp1b1* mice contained a single adenocarcinoma and samples from 1/7 two adenocarcinomas. Lungs from 5/8 m*Cyp1b1*^{-/-} and 3/8 h*CYP1B1* mice had two or more adenocarcinomas. All lung tumors were taken from offspring whose mother had been treated with the high (12 mg/Kg b.w.) dose.

Lung Tumor Multiplicity

Of the offspring surviving to 10 months of age, lung tumor multiplicity was increased in a dose dependent manner with mean values of 1.5, 8.1 and 12.5 for maternal doses of 0, 6.5 and 12 mg/Kg, respectively (Figure 4) which is consistent with previous studies by our laboratory [3-6,40]. In examining all mice with lung tumors, males had a slightly greater, statistically significant (p<0.05), multiplicity than females (8.5 and 7.2, respectively). Lung tumor multiplicity was not significantly different statistically with respect to h*CYP1B1*, m*Cyp1b1* or *Ahr* genotype (data not shown) which differs from our previous study where multiplicity was lower in *Cyp1b1* null mice. This may be due to the higher maternal dose (15 mg/Kg) which resulted in a higher incidence (100% in all groups) and enhanced multiplicity compared to this study and to differences between the two studies in the number of mice surviving to 10 months of age.

Discussion

Mouse *Cyp1b1* vs Human *CYP1B1*

Transgenic h*CYP1B1* mice were not susceptible to *CYP1B1* protein induction following DBC exposure, or to T-ALL mortality. Additionally, they were not significantly different in response to DBC compared to m*Cyp1b1* null mice. Potentially regulatory mechanisms responsible for constitutive (and inducible) developmental- and tissue-specific expression did not recapitulate m*Cyp1b1* in wild-type mice. As with other CYP orthologs, mouse *Cyp1b1* and human *CYP1B1* can exhibit distinct substrate specificities and enzyme kinetics although studies *in vitro* with expressed m*Cyp1b1* and h*CYP1B1* indicate similar kinetics toward most PAHs including DBC [7,8].

Ahr Genotype

Studies pioneered by Drs. Anderson and Miller utilized maternal exposure of 3-methylcholanthrene to pregnant mice, using females either *Ahr* “responsive” (B6D2F1, *Cyp1b1*^{+/+}) or “non-responsive” (D2, *Cyp1b1*^{-/-}), crossed with D2 males (resulting in litters with a 1:1 ratio of responsive versus non-responsive [21,22]. A maternal non-responsive *Ahr* phenotype enhanced the risk of the offspring for development of lung cancer (the B6D2F1xD2 cross did not develop T-ALL); conversely the risk was enhanced in responsive *Ahr* offspring irrespective of maternal *Ahr* status [22]. In previous studies we have observed both an enhanced risk and no impact of T-ALL mortality with a responsive *Ahr* offspring

phenotype [3-6,40]. Although DBC is a ligand for the Ahr, DBC is rapidly metabolized in the pregnant B6129F1 mouse ($T_{1/2}$ =5.3 hours in maternal liver and 6.3 hours in the fetus) [47]. The result may be low to no induction of Cyp1b1; constitutive expression in liver of pregnant mice was below the limits of detection [47]. Expression of constitutive mCyp1b1 mRNA in adult wild-type female mice is independent of Ahr “responsiveness” as is the case for the hCYP1B1 transgenic mice (Figure 1). Expression of constitutive CYP1B1 protein in hCYP1B1 transgenic female mice is below detection and not inducible by DBC. However, these same mice exhibit marked induction of hCYP1B1 protein in liver upon treatment with TCDD (Figure 2).

When considering DBC carcinogenic risk to humans one must also consider genetic polymorphisms of CYP1B1, such as leu432val, asn453ser, arg48gly, and ala119ser. For example, the CYP1B1*3 (leu432val) polymorphism has been linked to an increased risk of lung cancer [39]. hCYP1B1 is over-expressed in a number of human cancers [48] and has been suggested as a potential target for cancer chemoprevention [49-51].

Conclusions

In the fetus, Cyp1b1 expression is required for DBC-induction of T-ALL mortality and maximum lung multiplicity at 10 months of age [4]. Unlike maternal tissues, the ratio of DBC-11,12-dihydrodiol (the precursor to the ultimate carcinogenic metabolite (DBC-11,12-dihydrodiol-13,14-epoxide)) is higher than parent compound from 0-36 hours following maternal dosing with DBC [47]. Thus, it is likely that fetal constitutive expression of Cyp1b1 in lung is responsible for the potency of DBC as a transplacental lung carcinogen. A previous study employing high-precision rat liver and lung, as well as human liver, slices documented that, compared to BaP and other carcinogenic PAHs, DBC was a poor inducer of CYP1B1 consistent with its low binding affinity for the AHR [52]. A recent study from our laboratory (mouse dermal model) documented that DBC appears to differ from benzo[a]pyrene (BaP), and other PAHs in that DBC carcinogenesis involves pathways regulated by p53 and cMyc, whereas BaP carcinogenesis impacts genes regulated by Ahr/Arnt and Nrf2 as well as Sp1 [53]. The lack of hCYP1B1 up-regulation by DBC, compared to robust induction by TCDD, confirms that the Ahr/Arnt pathway may not be the primary pathway of DBC carcinogenesis in this transplacental model as well. BaP, at maternal doses up to 50 mg/kg, does not induce T-ALL [3]. Conversely, p53^{-/-} mice spontaneously develop T-ALL [54] with a time course and pathology remarkably similar to transplacental DBC in wild-type mice. The failure of hCYP1b1 to drive DBC-dependent transplacental T-ALL, as does mCyp1b1, prevents us from assessing the potential for hCYP1B1 as a target for cancer chemoprevention in contrast to the study of Li et al. [27] in which they demonstrated hCYP1B1 could replace mCyp1b1 in promoting obesity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AAALAC	Association for the Assessment and Accreditation of Laboratory Care
Ahr	Aryl hydrocarbon receptor
Arnt	Aryl hydrocarbon receptor nuclear translocator
BaP	benzo[a]pyrene
CYP	cytochrome P450
hCYP1B1	human cytochrome P450 1B1
hCYP3A4	human cytochrome P450 3A4

hCYP2D6	human cytochrome P450 2D6
mCyp1b1	mouse cytochrome P450 1b1
DBC	dibenzo[<i>def,p</i>]chrysene
mFMO1	mouse flavin-containing monooxygenase 1
GST	glutathione- <i>S</i> -transferase
IACUC	Institutional animal care and use committee
IARC	International Agency for Research on Cancer
NRF2	nuclear factor, erythroid 2-like 2
ORF	open reading frame
OSU	Oregon State University
PAH	polycyclic aromatic hydrocarbon
SCD1	stearoyl-CoA desaturase 1
Sp1	specificity protein 1 (a transcription factor)
SRS	substrate recognition site
SULT	sulfotransferase
T-ALL	T-cell acute lymphoblastic leukemia
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
UGT	UDP-glucuronosyl transferase

Figure 1. Constitutive Expression of *mCyp1b1* and *hCYP1B1* mRNA in F₁ Mice
RT-PCR was performed with cDNA isolated from testis, ovary, liver, lung and thymus as described (see Materials and Methods). Symbols distinguish wild-type mice (squares and triangles) and *hCYP1B1* transgenic mice (circles and squares), respectively. *Ahr* genotype had no significant impact on the constitutive expression of either mRNA.

Figure 2. Western Blot of hCYP1B1 Protein Expression in Control, DBC- and TCDD-Initiated Liver Microsomes

The treatment of transgenic *hCYP1B1* mice with corn oil vehicle, DBC or TCDD, preparation of microsomal protein and the protocol for western blotting to detect hCYP1B1 is described in Materials and Methods. The lane assignments were as follows: lane 1, molecular weight protein standards; lane 2, 20 μ g of supersomes expressing hCYP1B1; lane 3, 20 μ g of mouse hep1 cell homogenate treated with TCDD as a negative control for cross-reaction with *mCyp1a1*; lane 4, 40 μ g of microsomal protein from liver of mice treated with vehicle only; lanes 5-7, 40 μ g of microsomal protein from liver of DBC-treated *hCYP1B1* transgenic mice; lanes 8-10, 20 μ g of microsomal protein from liver of TCDD-treated *hCYP1B1* transgenic mice.

Figure 3. Survival Curves for Mice Succumbing to T-ALL as a Function of mCyp1b1, hCYP1B1, *Ahr^{b-1/d}* and *Ahr^{d/d}* Alleles

Survival curves for all F1 offspring to 10 months of age are shown. Panels A and D show that very little or no mortality was observed in the mCyp1b1 null mice as previously observed [4]. The same was true for the transgenic hCYP1B1 mice (panels B and E). Wild-type mice expressing both copies of the mCyp1b1 allele exhibited a high rate of mortality over the course of the study, beginning at around 10 weeks of age (panels C and F). Comparison of panels A-C with D-F, indicate little or no effect of the *Ahr* genotype on T-ALL mortality.

Figure 4. Lung Tumor Multiplicity at 10 Months of Age in Mice Born to Mothers Dosed with 0, 6.5 or 12 mg/kg DBC

Lung tumor multiplicity at 10 months of age is shown by a box and whisker plot; the box represents the standard deviation, the mean is the line through the box and the whiskers above and below are the range. The DBC dose to the pregnant dam on GD 17 is shown on the x axis. The data shown is from all mice surviving to 10 months of age.

Table 1
Lung Tumor Incidence in Offspring Surviving to Ten Months of Age

Maternal DBC Dose(mg/kg)	Offspring Genotype	Number of mice [†]	Incidence (%)
0	All mice	5/91	5.5
	<i>Cyp1b1</i> ^{-/-}	3/34	8.8 [‡]
	<i>CYP1B1</i>	2/38	5.3
	<i>Cyp1b1</i> ^{+/+}	0/19	0.0 [§]
6.5	All mice	89/92	96.7
	<i>Cyp1b1</i> ^{-/-}	38/39	97.4
	<i>CYP1B1</i>	33/34	97.1
	<i>Cyp1b1</i> ^{+/+}	18/19	94.7
12	All mice	112/118	94.9
	<i>Cyp1b1</i> ^{-/-}	48/49	97.0 [‡]
	<i>CYP1B1</i>	33/33	100
	<i>Cyp1b1</i> ^{+/+}	31/36	86.1 [§]

[†]The numerator is the number of mice with at least one lung tumor and the denominator is the total number of mice in the treatment group. The number of mice surviving to 10 months differs due to differential susceptibility to T-ALL mortality

^{‡/§}Values with different superscripts are statistically significant at p<0.05 [46].

Note: It should be noted that the breeding scheme (see Supplemental Figure 1) for the wild-type and *Cyp1b1* knockout mice were different which may account for differences in tumor incidence.