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TITLE PAGE

MINIREVIEW

Oral Benzo[*a*]pyrene: Understanding Pharmacokinetics, Detoxication and Consequences––*Cyp1* Knockout Mouse Lines as a Paradigm

Daniel W. Nebert, Zhanquan Shi, Marina Gálvez-Peralta,

Shigeyuki Uno, and Nadine Dragin

Department of Environmental Health, and the Center for Environmental Genetics (**CEG**)

University Cincinnati Medical Center, Cincinnati OH 45267-0056

(D.W.N., Z.S., M.G.-P., S.U., N.D.)

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RUNNING TITLE PAGE

Running Title: "CYP1A1-Mediated Detoxication of Oral Benzo[*a*]pyrene"

Correspondence should be addressed to**:** Department of Environmental Health, University of Cincinnati Medical Center, P.O. Box 670056, Cincinnati OH 45267-0056, Tele 513-821-4664; Fax 513-558-4397; email dan.nebert@uc.edu

Pages of Text = 18

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Words in **Abstract** = 253

Words in **Introduction** = 221

Words in **Discussion** = 5,018

Abbreviations : AHH, aryl hydrocarbon hydroxylase**;** AHR, aryl hydrocarbon receptor**;** BaP, benzo[*a*]pyrene**;** CYP1A1, enzyme encoded by the mouse *Cyp1a1* gene**;** CYP1A2, enzyme encoded by the mouse *Cyp1a2* gene**;** CYP1B1, enzyme encoded by the mouse *Cyp1b1* gene**;** *Cyp1a1(–/–)*, *Cyp1a1* knockout mouse**;** *Cyp1a2(–/–)*, *Cyp1a2* knockout mouse**;** *Cyp1b1(–/–)*, *Cyp1b1* knockout mouse**;** GI, gastrointestinal**;** PGD, preputial gland duct**;** PSI, proximal small intestine (first 5 cm from pyloric valve includes duodenum and proximal jejunum)**;** SCC, squamous cell carcinoma**;** TCDD or dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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ABSTRACT

Benzo[*a*]pyrene (BaP) is a prototypical polycyclic aromatic hydrocarbon (PAH)**;** this ubiquitous environmental carcinogenic agent is found in tobacco smoke, charcoal-grilled foods, and PAH-contaminated surfaces of roofs, playgrounds and highways. Cytochrome P450 1 wild-type, *Cyp1a2(–/–)*, *Cyp1b1(–/–)*, or *Cyp1a2/1b1(–/–)* knockouts, and mice with *Cyp1a1* expression deleted in hepatocytes, are able to ingest large oral BaP doses (125 mg/kg/day) without apparent toxicity. *Cyp1a1(–/–)* and *Cyp1a1/1a2(–/–)* knockouts, and mice with *Cyp1a1* expression deleted in gastrointestinal (GI) tract epithelial cells, develop immunotoxicity and die within 32 days, indicating that GI tract inducible CYP1A1 is absolutely required for detoxication of oral BaP. *Cyp1a1/1b1(–/–)* and *Cyp1a1/1a2/1b1(–/–)* mice are rescued from immunosuppression and early death, due to absent metabolic activation of BaP by CYP1B1 in immune cells. Ten-fold lower oral BaP doses result in adenocarcinoma of proximal small intestine (PSI) in *Cyp1a1(–/–)* mice**;** *Cyp1a1/1b1(–/–)* double-knockout mice show no PSI cancer, but develop squamous cell carcinoma of preputial gland duct (PGD). BaP-metabolizing CYP1B1 in PSI, and CYP3A59 in PGD, are most likely candidates that participate in tumor initiation in epithelial cells of these two tissues**;** oncogenes and tumor-suppressor genes up- and down-regulated during tumorigenesis are completely different between these tissues. This "oral BaP *Cyp1*" mouse paradigm represents a powerful teaching tool, showing that gene-environment interactions depend on route-of-administration**:** the same oral, but not intraperitoneal, BaP exposure leads to dramatic differences in target-organ toxicity and tumor type, as a function of dose and *Cyp1* genotype.

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INTRODUCTION

Studies of dietary polycyclic aromatic hydrocarbons (PAHs) in laboratory animals have been underappreciated. It is well established that, in cigarette and cigar smokers, considerably greater amounts of PAHs are swallowed and enter the gastrointestinal (GI) tract, compared with amounts entering lung (Järup, 2003; Rozman and Klaassen, 2007). Furthermore, those eating charcoal-grilled meat, tar roofers, and those inhaling dust from heavily PAH-contaminated playgrounds and highways, all ingest substantial amounts of PAHs.

In laboratory animals PAH-induced lung tumor formation is common (Rubin, 2001), whereas PAH-caused GI tract cancer is rare. A common question over many decades has been**:** if more PAHs are swallowed than inhaled, why isn't GI tract cancer in smokers more prevalent than lung cancer? One answer has been**:** rapid turnover of GI tract epithelial cells might prevent tumors from forming. However, head-and-neck epithelial cells as well as cells of the skin, lung, immune system, and bone marrow also turn over rapidly**;** PAH-induced malignancies commonly occur in these cell types—in both lab animals and humans.

The present Minireview addresses this problem, focusing on benzo[*a*]pyrene (BaP) as a prototypic PAH. Over four decades, our laboratory has demonstrated that highly induced cytochrome P450 1A1 (CYP1A1) in GI tract is protective by way of oral BaP detoxication. This discovery seems to contradict the long-held concept that CYP1A1 should always be regarded as detrimental.

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RESULTS and DISCUSSION

History

Following the 1950s landmark discovery (Conney et al., 1956; Conney et al., 1957) that rat liver drug- or xenobiotic-metabolizing enzyme (DME, XME) activities are inducible by PAHs, Wattenberg proposed in 1962 that Phase I oxidative enzymes are primarily essential for detoxifying PAHs such as oral BaP (Wattenberg et al., 1962). Six years later, this concept was convincingly challenged when XME-mediated metabolism was shown unequivocally to generate reactive oxidative PAH intermediates capable of binding covalently to nucleic acids and proteins (Sims and Grover, 1968). Thus began more than three decades of a belief held by many, that Phase I XMEs and DMEs are fundamentally harmful because they can metabolically activate relatively inert xenobiotic substrates, including drugs, to reactive intermediates—capable of initiating toxicity, birth defects, oxidative stress, mutations and cancer (Phillips et al., 1978; Nebert, 1989; Wogan et al., 2004). In contrast, Phase II DMEs are usually regarded as helpful because they conjugate reactive oxidative intermediates, thereby leading to excretion of detoxified, innocuous products (Talalay, 2000).

Fig. 1 lists four (individual or classes of) BaP reactive oxygenated intermediates and four (individual or classes of) nonreactive products of detoxication. The concepts of "metabolic activation" vs "detoxication" of BaP, as well as any other PAH, are detailed further in the Fig. 1 legend.

In parallel with these events, striking genetic differences in PAH-inducible aryl hydrocarbon hydroxylase (AHH) activity were described among inbred strains of mice (Nebert and Gelboin, 1969). AHH inducibility was subsequently found to be inherited principally as a Mendelian trait—C57BL/6N (B6) expressing dominance over DBA/2N (D2) mice, which showed lack of PAH-

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inducible AHH activity as an autosomal recessive trait (Nebert et al., 1971; Gielen et al., 1972)**;** an independent study reported this same mode of inheritance (Thomas et al., 1972).

Early work in rat liver had shown that PAH treatment caused a shift in the CO-reduced cytochrome P450 spectrum from 450 nm to 448 nm (Alvares et al., 1967; Kuntzman et al., 1968). Subsequently, AHH induction in *Ah*-responsive but not *Ah*-nonresponsive mouse liver was demonstrated to be associated with a hypsochromic spectral shift in CO-reduced cytochrome P450, indicating formation of a new form of induced P450 protein (Nebert, 1970; Gielen et al., 1972).

Next, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, "dioxin") as an inducer of AHH activity in chick embryo was established to be >30,000 times more potent than any PAH (Poland and Glover, 1974). Because of its potency, TCDD as an AHH inducer was then explored in rodents. Following TCDD treatment, hepatic AHH activity in "*Ah*-nonresponsive" D2 was inducible to levels as high as those in "*Ah*-responsive" B6 mice**;** however, the D2 dose-response curve was shifted ~20-fold to the right compared with that of B6 mice (Poland et al., 1974). The shape of the dose-response curve and its shift were predicted (Poland et al., 1974) to represent differences not in AHH, but rather a regulatory protein (later determined to be aryl hydrocarbon receptor; AHR)**;** two decades later, specific amino-acid changes in B6 vs D2 AHR responsible for differences in receptor affinity were demonstrated (Poland et al., 1994).

Thus began the research field of "genetic differences in DME- and/or XME-mediated toxicity, carcinogenesis, mutagenesis, and teratogenesis" [*reviewed in* (Nebert, 1989)]. Oxidative Phase I metabolism, especially via cytochromes P450, was generally believed to be detrimental in any cell type of every vertebrate, including microsomal studies in vitro and studies in cell culture (Conney et al., 1994; Nebert et al., 2004). In contrast, conjugative Phase II metabolism was regarded as almost always beneficial (Nebert et al., 2000; Talalay, 2000).

Target Organ Toxicity of BaP as a Function of Route-of-Administration

For studies of toxicity or cancer, xenobiotics can be administered intraperitoneally, topically, subcutaneously, intramuscularly, intravenously, orally in diet or drinking water, or injection into the stomach or trachea or eye or rectum. Largely because of convenience, intraperitoneal and topical administration have always been probably the most common means of treating laboratory animals with environmental test compounds, including drugs.

It seems likely, however, that presentation and the dose and rate of administration of any test chemical orally in the diet or drinking water, or as a stomach gavage, might lead to different pharmacokinetic responses than intraperitoneal or topical administration. One of the conclusions of this Mini-review highlights such a difference in pharmacokinetics (absorption, distribution, metabolism, excretion)**;** moreover, if the test compound is also an inducer of its own metabolism in proximal tissues, e.g. AHH activity in GI tract induced by oral BaP (Wattenberg et al., 1962) or AHH activity in skin induced by topical PAH (Schlede and Conney, 1970), this will lead to even more dramatic changes in pharmacokinetics of the test compound. Moreover, chronic administration of a chemical such as BaP which can cause both tumor initiation and promotion will cause differences in phenotype, compared with a xenobiotic not having these properties.

Intraperitoneal PAHs and dioxin at sufficiently high concentrations result in toxicity of liver, immune system, bone marrow and white cells, adipose tissue, spleen, thymus, and arterial cell wall (Nebert, 1989; Collins et al., 1991; Miller and Ramos, 2001; Bock and Kohle, 2009)**;** it has been proposed that future studies of the lymphatic system and participation of chylomicron particle release of PAHs and dioxin will provide valuable insight into understanding toxicity in these tissues (Nebert and Dalton, 2006). On the other hand, oral BaP at sufficiently high doses [*vide infra*] most

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dramatically causes immunotoxicity, whereas toxicity of liver, adipose tissue and the arterial cell wall appears to be negligible (Uno et al., 2001; Uno et al., 2004; Uno et al., 2006; Uno et al., 2008).

Apparent Paradox with Oral BaP in B6 vs D2 Mice

Dietary BaP was first studied in mice four decades ago (Robinson et al., 1975). Curiously, at high BaP doses (125 mg/kg/day), *Ah*-nonresponsive D2 mice died by 28-32 days with striking immunosuppression and toxic chemical depression of bone marrow, whereas *Ah*-responsive B6 and $B6D2F_1$ mice remained healthy. In fact, on a high-BaP chronic diet, *Ah*-responsive mice reproduced normally and even lived longer than *Ah*-responsive mice eating normal lab chow without BaP (Robinson et al., 1975)**!**

At lower oral BaP doses (12 and 6 mg/kg/day), D2 mice did not die quickly**;** instead, they exhibited higher rates of leukemia and thymoma than B6 or $B6D2F_1$ mice. Administration of 120 mg/kg/day of dietary α-naphthoflavone, a competitive inhibitor of AHH activity but not other P450 enzymes, lowered substantially this risk of malignancy (Nebert and Jensen, 1979). In mice receiving topical 3-methylcholanthrene, D2 showed more leukemia than B6 mice (Duran-Reynals et al., 1978). Therefore, we might consider that oral and topical PAH administration representing GI tract and skin are proximal tissues, whereas bone marrow and thymus might be considered as distal tissues.

After 15 years, a paradigm became apparent (Table 1). PAH administration to *Ah*-responsive mice causes in *proximal target tissues***:** AHH induction, increased cancer, mutagenesis, DNA-adduct formation, various forms of toxicity, oxidative stress, and birth defects. Consequently, lower levels of PAHs reach *distal tissues,* where one sees negligible AHH induction and lower rates of cancer, mutagenesis, DNA-adduct formation, toxicity, oxidative stress and birth defects [*reviewed in* (Nebert, 1989)]. A single oral dose of BaP to *Ah*-responsive rats was found to result in elevated blood and

tissue BaP levels, whereas giving the same BaP dose for several days (or inducing with a different PAH) leads to markedly decreased blood and tissue BaP levels (Schlede et al., 1970)**;** these results represent the same phenomenon observed with *Ah*-responsive mice receiving oral BaP (Robinson et al., 1975).

In contrast, administering the same PAH dose to *Ah*-nonresponsive mice resulted in**:** negligible AHH induction in *proximal tissues* and hence decreased risk of cancer, mutagenesis, DNA-adduct formation, toxicity, oxidative stress, and teratogenesis in these tissues. This resulted in more PAHs reaching *distal target tissues,* where we found**:** increased risk of cancer, mutagenesis, DNA-adduct formation, toxicity, oxidative stress, and birth defects in these tissues (Nebert, 1989) [*summarized in* Table 1].

Furthermore, bone marrow of *Ah*-nonresponsive mice was replaced with immune-compatible marrow from *Ah*-responsive mice**;** these animals were compared with *Ah*-responsive mice carrying bone marrow from *Ah*-nonresponsive mice (Legraverend et al., 1983). High daily oral BaP doses produced marrow toxicity in *Ah*-nonresponsive mice, regardless of the source of marrow. The conclusion was that—due to detoxication of oral BaP by induced AHH activity in GI tract and/or liver of *Ah*-responsive mice—bone marrow is protected**;** in contrast, with negligible detoxication of oral BaP in *Ah*-nonresponsive mice, much larger amounts of BaP reach the marrow, causing toxicity (Legraverend et al., 1983; Nebert, 1989).

Reexamination of The Paradigm in *Cyp1a1(–/–)* Knockout Mice

Is the observed oral BaP detoxication in B6 mice due to PAH-inducible CYP1A1, CYP1A2, or CYP1B1, or any two, or all three CYP1 enzymes? Or is another PAH-inducible PAH-metabolizing P450 involved? Also, is the inducible enzyme(s) primarily located in liver or GI tract? These

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questions could only be answered by studying conventional, as well as conditional, knockout mouse lines.

Oral vs intraperitoneal BaP was first assessed in wild-type vs *Cyp1a1(–/–)* knockout mice**;** striking differences between mice with different genotypes occurred via oral, but not intraperitoneal, route-of-administration (Uno et al., 2001). At high oral BaP doses (125 mg/kg/day), *Cyp1a1(–/–)* mice on >99.8% B6 genetic background died at 28-32 days (Uno et al., 2001), which was virtually identical to that found previously in D2 mice (Robinson et al., 1975). Significant anemia, methemoglobinemia, and elevated plasma enzymes indicative of damage of various tissues occurred in *Cyp1a1(–/–)* mice at 125 and 12.5 mg/kg/day doses (Uno et al., 2004). Following oral BaP gavage, *Cyp1(+/+)* wild-type mice were shown to clear BaP from blood at least 4 times more rapidly than *Cyp1a1(–/–)* mice (Uno et al., 2004). Moreover, BaP-DNA adduct levels in liver were 4-fold greater in *Cyp1a1(–/–)* than *Cyp1(+/+)* mice (Uno et al., 2001; Uno et al., 2004). In fact, at BaP doses of 12.5 mg/kg/day, elevated BaP-DNA adducts were observed 18 days later in *Cyp1a1(–/–)* liver, GI tract, spleen and bone marrow. Even at BaP doses of 1.25 mg/kg/day, significantly elevated BaP-DNA adducts were detectable in *Cyp1a1(–/–)* spleen (Uno et al., 2004).

Studies of All Three CYP1 Enzymes

In epithelial cells of duodenum, jejunum, ileum and colon—we determined that maximally inducible CYP1A1 mRNA and protein levels were ~3-10 times greater than CYP1B1, which in turn were ~3-10 times greater than CYP1A2 (Uno et al., 2008). Whereas ablation of *Cyp1a2* or *Cyp1b1* gene expression made little difference in CYP1 mRNA or protein levels, when the *Cyp1a1* gene was deleted, CYP1B1 mRNA and protein levels in liver, but especially proximal small intestine (PSI),

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were strikingly increased, compared with that in wild-type animals (Uno et al., 2006)**;** this effect in the GI tract is believed to be a compensatory response due to the absence of CYP1A1.

To address this point, we studied *Cyp1a1(–/–)* (Dalton et al., 2000), *Cyp1a2(–/–)* (Liang et al., 1996) and *Cyp1b1(–/–)* (Buters et al., 1999) single-knockout lines and *Cyp1a1/1b1(–/–)* and *Cyp1a2/1b1(–/–)* double-knockout mouse lines (Uno et al., 2006). Generation of *Cyp1a1/1a2(–/–)* knockout mice was problematic because the two genes were separated by only 13,954 base pairs. More than 1,100 pups from the *Cyp1a1(–/–)* × *Cyp1a2(–/–)* genetic cross were genotyped but failed to achieve any double-knockout crossover pups**;** subsequently, a Cre recombinase-mediated inter-strand excision via two *loxP* sites located 26,173 bp apart was successful (Dragin et al., 2007). This made it possible to include oral BaP studies on *Cyp1a1/1a2(–/–)* double-knockout as well as *Cyp1a1/1a2/1b1(–/–)* triple-knockout lines (Dragin et al., 2008).

Table 2 summarizes data for the highest dose (125 mg/kg/day) of oral BaP. *Cyp1a2(–/–)* and *Cyp1b1(–/–)* single-knockout and *Cyp1a2/1b1(–/–)* double-knockout mice behave similarly to wild-type mice. Severe effects of oral BaP occur in both *Cyp1a1(–/–)* and *Cyp1a1/1a2(–/–)* mice**;** those life-threatening effects were largely alleviated in *Cyp1a1/1b1(–/–)* double-knockout, as well as in *Cyp1a1/1a2/1b1(–/–)* triple-knockout mice. Hence, all data implicate CYP1A1 as the enzyme which, when missing, leads to oral BaP-induced immunosuppression and early death. Moreover, our data show that, when CYP1B1 is also missing, severe effects of oral BaP on the immune system are mollified.

Fig. 2 illustrates the paradigm that explains our data. In wild-type mice, as well as those missing a functional *Cyp1a2* or *Cyp1b1* gene, oral BaP-treated CYP1A1 quickly becomes massively induced in liver and/or GI tract—thereby leading to enhanced detoxication and excretion of BaP metabolites**;** the result is rapid clearance of blood BaP, with only small amounts of BaP reaching distal tissues such as

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bone marrow and immune tissue.

In oral BaP-treated *Cyp1a1(–/–)* knockout mice (Fig. 2A, *left*), with or without functional *Cyp1a2*, absence of CYP1A1 in liver and/or GI tract results in negligible detoxication**;** this leads to ~25-fold higher blood BaP levels than those in wild-type, with much more BaP reaching distal tissues such as bone marrow and immune tissue—causing marrow toxicity, immunosuppression, and early death.

In *Cyp1a1/1b1(–/–)* mice (Fig. 2B, *left*), with or without a functional *Cyp1a2* gene, absence of CYP1A1 in liver and/or GI tract results in negligible detoxication**;** however, the additional absence of CYP1B1 in marrow and immune cells leads not only to 75-fold higher blood BaP levels than those in wild-type and greater amounts of BaP reaching distal tissues (Fig. 2B, *left*), but lack of CYP1B1—known to participate in PAH metabolism in bone marrow and immune tissue (Galván et al., 2005; Galván et al., 2006; N'jai et al., 2010)—results in diminished metabolic activation of BaP by CYP1B1. Therefore, the ultimate outcome is a substantial reversion to wild-type phenotype, *i.e.* relatively healthy mice, despite even 3-fold more BaP body burden than that of immunosuppressed *Cyp1a1(–/–)* mice. This is a pharmacologic example in which total body burden, or rate of clearance, of a foreign chemical is not necessarily correlated with specific target-organ toxicity or clinical outcome.

Studies with *Cyp1a1(–/–)* Conditional Knockout Mice

Liver is regarded as the principal organ of metabolic activation as well as detoxication. Is CYP1A1 located in liver, or CYP1A1 located in GI tract, more important in detoxication of oral BaP? Or are hepatic and GI tract hepatic CYP1A1 equally important? Answers to these questions can only be resolved by using conditional *Cyp1a1(–/–)* knockout lines. The *Alb>Cre>Cyp1a1(f/f)* line was

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thus created in which CYP1A1 is specifically ablated from albumin-expressing hepatocytes, whereas CYP1A1 is present and inducible in the rest of the animal including GI tract (Shi et al., 2010a). The *Vil>Cre>Cyp1a1(f/f)* line was also developed in which CYP1A1 function is deleted from villin-expressing epithelial cells of GI tract (also renal epithelial cells, which are of no consequence in these experiments), meaning that CYP1A1 is present and inducible in the rest of the animal including hepatocytes (Shi et al., 2010a).

Fig. 2C summarizes data with these two *Cyp1a1* conditional knockout mice. The response of *Alb>Cre>Cyp1a1(f/f)* mice to daily oral BaP was similar to that of wild-type *Cyp1(+/+)* mice. Although CYP1A1 expression is absent in liver, CYP1A1 becomes massively induced by BaP in GI tract (Fig. 2C, *right*), which in turn results in enhanced detoxication and excretion of BaP metabolites, rapid clearance of blood BaP levels, and no detectable immunotoxicity because only small amounts of BaP reach distal tissues.

On the other hand, response of *Vil>Cre>Cyp1a1(f/f)* mice to daily oral BaP was similar to that of *Cyp1a1(–/–)* mice. Absence of CYP1A1 in GI tract, rather than hepatocytes, results in negligible detoxication (Fig. 2C, *left*)**;** this leads to severely impaired clearance of BaP total body burden, large amounts of BaP reaching distal tissues such as bone marrow and immune tissue, and resultant CYP1B1-mediated bone marrow toxicity, immunosuppression and death. These two conditional knockout mouse models were supported with further pharmacokinetics parameters, blood and plasma enzyme measurements, and histology (Shi et al., 2010a). It can be unequivocally concluded that GI tract inducible CYP1A1, but not CYP1B1 or CYP1A2, can detoxify enormous amounts of oral BaP.

Studies with a 10-Fold Lower Dose of Oral BaP

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We had reported previously that lower daily oral BaP doses given to D2 mice resulted in higher rates of leukemia and thymoma, compared with no malignancies in B6 or $B6D2F_1$ mice (Nebert and Jensen, 1979). In other words, if mice do not die quickly after high toxic doses of daily oral BaP, they are able to live sufficiently long to develop cancer.

Therefore, we decreased the daily oral BaP dose of 125 mg/kg/day to 12.5 mg/kg/day**;** this dose allowed *Cyp1a1(–/–)* mice to live beyond 20 weeks of age, instead of dying at 28-32 days when given the higher BaP dose (Shi et al., 2010b). Adenocarcinoma of PSI developed in *Cyp1a1(–/–)* mice between 8 and 12 weeks of oral BaP (12.5 mg/kg/day). Interestingly, in *Cyp1a1/1b1(–/–)* double-knockout mice between 8 and 12 weeks on this same BaP oral dose, no GI tract cancer occurred**;** however, squamous cell carcinoma (SCC) of the preputial gland duct (PGD) appeared (Shi et al., 2010b) (Table 3).

In addition to characterizing the tumors histologically, we performed cDNA microarray analyses of PSI and PGD during formation of the malignancies (Shi et al., 2010b; Gálvez-Peralta et al., 2013). Zero vs 4-week-interval time-points of oral BaP (12.5 mg/kg/day) were compared between relevant genotypes. Greatest attention was given to XME-related genes and cancer-related genes that were most highly up- and down-regulated as the cancer process began (4 and 8 weeks of oral BaP) and then progressed (8 and 12 weeks and beyond).

Adenocarcinoma of the PSI

Many of the top-ranked up- and down-regulated genes during PSI adenocarcinoma formation are discussed in detail in (Shi et al., 2010b). Genes involved in inflammation and acute phase response were among those strikingly up-regulated by daily oral BaP after 4 weeks. Although PSI

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adenocarcinomas showed no immunohistochemical evidence of being lymphatic in origin, paradoxical over-expression of a large number of immunoglobulin kappa and heavy chain variable genes was observed (Shi et al., 2010b)**;** this has previously been reported, with studies showing *Igk* and *Igh* gene expression paradoxically expressed in various malignancies of epithelial origin (Hu et al., 2008).

It is noteworthy that oral BaP-treated *Cyp1a1(–/–)* mice exhibit markedly increased CYP1B1 mRNA levels in the GI tract (Uno et al., 2006; Shi et al., 2010a; Shi et al., 2010b). Association of elevated CYP1B1 in the same epithelial cells (Uno et al., 2008) that develop the adenocarcinoma strongly suggests that metabolic activation of oral BaP by CYP1B1 might be pivotal during the process of tumor initiation. Hence, PSI adenocarcinoma occurs in oral BaP-treated *Cyp1a1(–/–)* mice which have highly induced CYP1B1 present in GI tract—whereas no GI tract cancer arises in oral BaP-treated *Cyp1a1/1b1(–/–)* mice which have CYP1B1 deleted. This is additional evidence that CYP1B1-mediated metabolic activation of BaP is likely an important factor in GI tract tumorigenesis.

The putative oncogenes and tumor-suppressor genes in PSI that are most strikingly up- and down-regulated as a function of time of oral BaP exposure and adenocarcinoma formation (especially at the 4- and 8-week time-points) include**:** up-regulation of the *Xist* gene, suggesting epigenetic silencing**;** up-regulation of the *Rab30* oncogene**;** and down-regulation of the *Nr0b2* tumor-suppressor gene during adenocarcinoma development (Shi et al., 2010b). This mouse model may be relevant to human PAH-induced or inflammation-induced epithelial cancers of the GI tract.

SCC of the PGD

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Intriguingly, between 8 and 12 weeks SCC formation occurred in PGD of *Cyp1a1/1b1(–/–)* double-knockout mice receiving daily oral BaP (12.5 mg/kg/day). One might ask**:** "Of all tissues, why should we see cancer in *preputial gland duct*?" Actually, PGD tumors are well known to occur commonly in male rodents during toxicity and cancer testing with many different environmental chemicals (Mitsumori and Elwell, 1988). PAHs commonly cause hyperkeratosis of PGD epithelium**;** indeed, among the best examples of an AHR ligand causing hyperkeratosis is dioxin-induced chloracne in humans (*reviewed in* Bock and Kohle, 2006). It seems likely that BaP-induced keratin-plugging of secretions from the PGD could lead to pruritus**;** the mouse's response by scratching might set up secondary infections. BaP-induced hyperkeratinization, and BaP as a well-known tumor promoter and cause of inflammation (Bock and Kohle, 2006), might therefore combine to cause both initiation and promotion of SCC in the PGD.

Many of the most dramatically up- and down-regulated genes during SCC formation in the PGD are described in detail (Gálvez-Peralta et al., 2013). If both CYP1A1 and CYP1B1 are absent, and these two enzymes are well-known metabolic activators of BaP, what enzyme might compensate and take their place when both CYP1 genes are missing? Various CYP2C (Meehan et al., 1988; Yun et al., 1992; Bauer et al., 1995) and CYP3A (Yun et al., 1992; Bauer et al., 1995; Sun et al., 1995; Koley et al., 1997; Fukuhara et al., 1999; James et al., 2005) enzymes, some of which are PAH-inducible, have been shown in various vertebrates to metabolize PAHs such as BaP. Interestingly, microarray data revealed by far the most striking increases in CYP3A59 mRNA up-regulation which peaked at 8 weeks of oral BaP**;** these data strongly suggest—but do not prove—that CYP3A59 is the most likely BaP-inducible candidate responsible for initiation of BaP-induced SCC (Gálvez-Peralta et al., 2013).

Future studies might include Western immunoblot data to confirm that CYP3A59 protein is

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indeed highly induced in PGD of oral BaP-treated *Cyp1a1/1b1(–/–)* mice—if an antibody specific for mouse CYP3A59 can be made. CYP3A59 cDNA expression studies in cell culture or bacteria would also be informative, to confirm that CYP3A59 does indeed specifically metabolize BaP and what the metabolite profile might be. Specifically knocking out the *Cyp3a59* gene, and finding no SCC in PGD of oral BaP-treated *Cyp1a1/1b1(–/–)* mice, would provide the ultimate means to prove CYP3A59 is responsible for SCC initiation. [And it is also possible in an oral BaP-treated *Cyp1a1/1b1/3a59(–/–)* triple-knockout that a new P450 might arise to take the place of CYP3A59 in the PGD epithelium**!**]

It is worth noting that oral BaP-treated *Cyp1a1/1b1(–/–)* double-knockout, but not oral BaP-treated *Cyp1a1(–/–)* single-knockout, mice develop SCC of the PGD. This suggests that CYP1B1 in PGD of oral BaP-treated *Cyp1a1(–/–)* mice might function in the role of BaP detoxication**;** in other words, when CYP1B1, as well as CYP1A1, is absent, only then does BaP result in SCC formation. And with both CYP1B1 and CYP1A1 missing, this would lead to higher accumulation of BaP in PGD**;** consequently, the compensatory response is CYP3A59 induction, and then presumably CYP3A59-mediated metabolic activation of BaP, become crucial in initiating SCC tumorigenesis.

Twenty-six cancer-related genes plus eight *Serpin* genes were up- and down-regulated as a function of time during oral BaP exposure and development of SCC (especially at the 4- and 8-week time-points). Among these 26 genes, eight were RAS-related oncogenes—which have often been associated with PAH-induced cancers (Gálvez-Peralta et al., 2013).

Inflammation-associated genes were also highly up-regulated at the 4- and 8-week time-points**;** thus, specific mechanisms by which cancer-related genes are responsible for SCC tumor progression in the PGD remain to be elucidated. This mouse model of SCC in the PGD may be relevant to human PAH-induced, as well as inflammation-mediated, epithelial cell cancers.

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Conclusions

This oral BaP mouse paradigm represents an exciting example of "gene-environment interactions" in which the same chronic exposure of a PAH carcinogen results in dramatic differences in target-organ toxicity and tumor type, as a function of zero, vs one *Cyp1* gene missing, vs two *Cyp1* genes missing. With an intact genome in *Cyp1(+/+)* wild-type mice, enormous amounts of BaP can be consumed without apparent toxicity to the animal, because CYP1A1 in GI tract epithelial cells quickly becomes highly induced and functions to detoxify oral BaP very efficiently.

Removal of just one gene—*Cyp1a1* globally or from only the GI tract epithelium—results in immunosuppression and death within 28-32 days at high daily oral BaP doses**;** immunotoxicity is mediated by CYP1B1 metabolic activation of BaP in marrow and other immune tissues. It should be emphasized that the striking difference between wild-type and *Cyp1a1(–/–)* mice happens with oral, but not intraperitoneal, route-of-administration (Uno et al., 2001). At 10-fold lower daily oral BaP doses, absence of the *Cyp1a1* gene leads to adenocarcinoma of PSI.

Removal of two genes—*Cyp1a1* plus *Cyp1b1*—protects against CYP1B1-mediated immunosuppression and death at high daily oral BaP doses, and at 10-fold lower daily oral BaP doses leads to no PSI adenocarcinoma but rather SCC formation in PGD. This paradigm should be attractive as a teaching model emphasizing the effects of gene-environment interactions on pharmacokinetics of PAH carcinogens and toxicants, including route-of-administration, rate of administration, ultimate target-organ specificity, and whether toxicity or malignancy is the end result.

Consideration of Human Epidemiological Studies

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Human exposure to PAHs in cigarettes and charcoal-grilled meat is well known to stimulate carcinogen as well as drug metabolism (Welch et al., 1968; Welch et al., 1969; Nebert et al., 1969; Pantuck et al., 1972; Conney et al., 1976). Such human exposures to PAHs occur not only in GI tract and lung but can also be important topically**:** PAH-induced AHH activity and BaP metabolic activation and detoxication are well known to occur in human skin (Alvares et al., 1973).

It is noteworthy that the three CYP1 enzymes are very highly conserved between mouse and human. Similar PAHs and other inducers, via AHR, up-regulate these three genes in the same tissues of both species. Also, each of the three CYP1 enzymes of mouse and human handles substrates with a great deal of similarity (Nebert and Dalton, 2006). In fact, oral BaP-treated *Cyp1a1/1a2(–/–)* mice having the human *CYP1A1* and *CYP1A2* genes were shown to function the same as oral BaP-treated wild-type mice, *i.e.* human CYP1A1 in the mouse GI tract is inducible and detoxifies the oral BaP (Dragin et al., 2007). Hence, it is quite likely that our findings in mouse might be relevant to human clinical studies. For example, is it possible that eating oil slick-contaminated seafood, or coal or road tar, or BaP-contaminated dust on a playground or highway is not as dangerous to adults or children as one might think?

Innumerable epidemiological studies have attempted to show correlations between cancers of several types and various single-nucleotide variants in and near the *CYP1A1*, *CYP1A2* and/or *CYP1B1* genes [*reviewed in* (Nebert and Dalton, 2006)]. However, cancer represents a multifactorial trait involving most likely thousands of "low-effect" genes, *i.e.* each gene contributes far less than 0.5% to the trait**;** therefore, cohorts of perhaps 50,000 or more would likely be necessary to generate sufficient statistical power to show a truly significant *association*, whereas prediction of risk of cancer in the individual patient is virtually impossible (Nebert et al., 2013b). Moreover, not a single *CYP1A1* variant allele has been shown to lead to significant differences in clinical function (Nebert et

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al., 2004; Nebert and Dalton, 2006; Nebert et al., 2013a). It has already been concluded (Nebert and Dalton, 2006; Nebert et al., 2013b) that—due to lack of statistical power—no study to date shows unequivocally a clinically useful relationship between any DNA sequence variants, alone or in combination, in any of the three *CYP1* genes and prediction of a specific form of cancer.

The findings described in the present review also introduce more complexity into the relationship between "CYP1 induction and toxicity or cancer". In other words, the route-of-administration, rate of administration, and target-organ response all depend on whether the CYP1 enzyme in any specific tissue or cell type might play a predominant role in metabolic activation or in detoxication. Thus, CYP1 induction *per se* is not all good, nor is it all bad.

Future Directions

Studies of PAH exposure to humans include cooked meat at high temperatures (Sinha et al., 2005; Anderson et al., 2005; Li et al., 2007; De Stefani E. et al., 2009), an aluminum smelter cohort (Friesen et al., 2007; Gibbs et al., 2007), consumption of fried chicken (BaP ~5.4 μg/kg) and smoked dried beef (BaP ~5.5 μg/kg), smokers inhaling daily ~0.26 μg of BaP per pack of 20 cigarettes (Piccardo et al., 2010), and charcoal-broiled steak containing BaP levels of ~9.0 μg/kg (http://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=122&tid=25). Summarizing all available data leads to an extrapolated environmental dose of perhaps ~40-50 ng/kg/day. BaP concentrations have been reported as high as 19 μg/kg in smoked meat in Austria (Tiefenbacher et al., 1982) and 69 μg/kg in rape seed oil (Pupin and Toledo, 1996)**;** ingestion of these foods would increase BaP amounts to 80-380 ng/kg/day. The daily median total of ingested BaP exceeds the daily inhalation dose by ~16-fold in winter and ~120-fold in summer (Buckley et al., 1995). In New Jersey, the daily oral PAH intake from food per capita was estimated at 1.6-16 μg/day—with ~10% as BaP (Santodonato et al., 1981).

Hence, we guesstimate that the daily oral BaP doses of 125 and 12.5 mg/kg/day in mouse studies described herein might be ~7,500 and ~750 times, respectively, beyond known human exposure levels. It would be informative to see what effects might occur in this mouse *Cyp1* paradigm—if daily oral BaP were administered at 1.25 (~75-fold higher), 0.125 (~7.5-fold higher), 0.0125 (~75% of highest human exposures), and even 0.00125 mg/kg/day (~7.5% of highest known human exposures). It would also be useful to know the BaP metabolite profiles of tissues that develop toxicity or malignancy, *i.e.* do they change as a function of the above suggested regimens of dosage?

Finally, epigenetics is undoubtedly involved in gene-environment interactions leading to toxicity and neoplasia. With availability of present-day genome-wide DNA-methylation maps and assays (Boerno et al., 2010; He et al., 2011), we suggest that the role of hypermethylation vs hypomethylation in some of the oncogenes and tumor-suppressor genes described herein should be examined. With recent availability of current microRNA chips (Baer et al., 2013), we also suggest that involvement of all known miRNAs should be assessed during development of immunosuppression and toxic chemical depression of the bone marrow, as well as during PSI adenocarcinoma and PGD SCC formation. We predict additional exciting advances with this *Cyp1* paradigm in the near future.

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AUTHORSHIP CONTRIBUTIONS

Authorship contributions:

*Participated in research design***:** Nebert, Shi, Galvez, Uno, and Dragin

*Conducted experiments***:** Nebert, Shi, Galvez, Uno, and Dragin

*Contributed new reagents or analytic tools***:** Nebert, Shi, Galvez, Uno, and Dragin

*Performed data analysis***:** Nebert, Shi, Galvez, Uno, and Dragin

*Wrote or contributed to the writing of the manuscript***:** Nebert, Shi, and Galvez

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FOOTNOTES

Current affiliation of Zhanquan Shi**:** The James L. Winkle College of Pharmacy, University of Cincinnati Medical Center, Cincinnati, Ohio 45267

Current affiliation of Marina Galvez-Peralta**:** Department of Basic Pharmaceutical Sciences, West Virginia University, Morgantown, West Virginia, 26506

Current affiliation of Shigeyuki Uno**:** Division of Biochemistry, Department of Biomedical Sciences, Nihon University School of Medicine, Itabashi-ku, Tokyo 173-8610, Japan

Current affiliation of Nadine Dragin**:** Université Pierre et Marie Curie - Paris VI (U76), INSERM U974, CNRS-AIM (UMR 7215), Paris, France

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LEGENDS for FIGURES

Fig. 1. Molecular structure of the prototypical polycyclic aromatic hydrocarbon, benzo[*a*]pyrene (BaP), with its standardized numbering system. Among 709 possible oxygenated BaP metabolites, which includes all *syn*- and *anti*-isomers, four products (or classes of products) of metabolic activation that are highly reactive intermediates are listed in the *left column***;** four products (or classes of products) of detoxication which are negligibly reactive are listed in the *right column*. Electrophilic oxides and epoxides can react with nucleophilic groups of cellular macromolecules, can rearrange to become hydroxyl products, or can become conjugated with moieties such as glucuronide, glutathione or sulfate. Phenols and the tetrol are generally nontoxic, and conjugation renders them even more hydrophilic and easy to excrete from the cell. Whereas the epoxide oxygen is derived from diatomic oxygen, addition of a second oxygen atom across the same C—C bond to form a diol comes from cellular water. [a]BaP phenols are major nonreactive metabolites and therefore represent important detoxication pathways**;** all BaP phenols have been tested for carcinogenicity and are inactive except for 2-hydroxyBaP, which is active but not a known biological metabolite. [b]Only one of the four possible optically active diol-epoxide metabolites is carcinogenic. [c]Although the 7,8-diol is not "reactive", it is highly carcinogenic because of its further metabolism. [d]Whereas BaP 4,5-oxide is chemically reactive and also mutagenic, it is not carcinogenic. In summary, some chemically reactive metabolites are not carcinogenic but can be toxic and/or mutagenic, whereas some nonreactive metabolites can be carcinogenic, toxic and/or mutagenic due to further metabolism (*reviewed in* Conney et al., 1994).

Fig. 2. Illustrations comparing response of oral BaP (125 mg/kg/day) for 18 days in mice of different genotypes. BPO denotes the first step in oxygenated BaP**;** BPO can be the reactive intermediate, as well as undergo all the detoxication possibilities, as detailed in Fig. 1. The ratio of reactive-intermediates-to-detoxified-products is most likely tissue- or cell-type-specific, depends on how "tightly coupled" phase II conjugation systems might be to the membrane-bound P450 enzymes (Nebert and Dalton, 2006), and is also

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likely to be dependent on the degree of CYP1 induction**;** the relative amounts of CYP1A1, CYP1A2 and CYP1B1 protein in each tissue or cell type and the route and rate of administration, as well as the function of time during induction by BaP, will also affect this ratio. ***A,*** *Cyp1(+/+)* wild-type (*right*) vs *Cyp1a1(–/–)* single-knockout mice (*left*). Oral BaP in *Cyp1(+/+)* mice rapidly induces intestinal CYP1A1, leading to detoxication and rapid excretion of BaP metabolites**;** absence of CYP1A1 in GI tract of *Cyp1a1(–/–)* mice (*left*) results in negligible detoxication and therefore a 25-fold greater blood BaP level than wild-type, larger amounts of BaP reaching nonhepatic tissues, which then results in BaP-induced CYP1B1-mediated immunosuppression, wasting and death. ***B,*** *Cyp1(+/+)* (*right*) vs *Cyp1a1/1b1(–/–)* double-knockout mice. Lack of CYP1B1 in marrow and immune tissues (*left*) results in negligible metabolic activation of BaP and therefore markedly diminished immunosuppression, toxicity, and prevention of early death—despite a 3-fold more BaP body burden than *Cyp1a1(–/–)* mice in Panel A. ***C,*** *Alb>Cre>Cyp1a1(f/f)* vs *Vil>Cre>Cyp1a1(f/f)* conditional knockout mice. *Alb>Cre>Cyp1a1(f/f)* mice (*right*) respond to oral BaP much like wild-type mice, whereas *Vil>Cre>Cyp1a1(f/f)* mice (*left*) respond much like *Cyp1a1(–/–)* mice**;** these experiments demonstrate unequivocally that it is the CYP1A1 in GI tract, not liver, that is most important in oral BaP detoxication and, hence, protection from immunosuppression, immunotoxicity, and early death.

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TABLE 1

Association of oral PAH-inducible AHH activity with various cellular effects in proximal vs distal tissues or organs [*reviewed in detail in* (Nebert, 1989)]

***Ahr* genotype**[a]	**PAH-induced effect**	**Proximal tissues/organs**[b]	**Distal tissues/organs**[c]
B6 and B6D2F$_1$ (*Ahr*$^{b1/b1}$, *Ahr*$^{b1/d}$)	**AHH induction**	**Increased**	**Decreased**
	Neoplasia; mutagenesis	**Increased risk**	**Decreased risk**
	DNA-adduct formation	**Increased**	**Decreased**
	Toxicity, oxidative stress	**Increased**	**Decreased**
	Teratogenesis	**Increased risk**	**Decreased risk**
	Detoxication	**Increased**	**Decreased**
D2 (*Ahr*$^{d/d}$)	**AHH induction**	**Decreased**	**Increased**
	Neoplasia; mutagenesis	**Decreased risk**	**Increased risk**
	DNA-adduct formation	**Decreased**	**Increased**
	Toxicity; oxidative stress	**Decreased**	**Increased**
	Teratogenesis	**Decreased risk**	**Increased risk**
	Detoxication	**Decreased**	**Increased**

[a]Standardized mouse genetic nomenclature states that the *Ahr* allele for B6 is *b1*, for D2 is *d* (Poland et al., 1994).

[b]"Proximal" in this context denotes tissues or organs in contact with, or in close proximity to, the incoming PAH. Interestingly, this includes in utero fetuses when a PAH is administered intraperitoneally (Nebert, 1989).

[c]"Distal" in this context denotes tissues or organs not in contact with, or in close proximity to, the incoming PAH. This includes in utero fetuses when the PAH is administered topically (Nebert, 1989).

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TABLE 2

Summary of the response to oral BaP (125 mg/kg/day) by all possible *Cyp1* genotypes

Genotypes[a]	***Cyp1(+/+)*** ***Cyp1a2(–/–)*** ***Cyp1b1(–/–)*** ***Cyp1a2/1b1(–/–)***	***Cyp1a1(–/–)*** ***Cyp1a1/1a2(–/–)***	***Cyp1a1/1b1(–/–)*** ***Cyp1a1/1a2/1b1(–/–)***
Clinical outcome	Healthy for lifetime	Dies 28-32 days	Reverts almost completely to wild-type phenotype
Blood BaP levels (ng/mL)[b]	~2.0	~50	~150
ALT, AST,[c] **hemoglobin, hematocrit, methemoglobin levels**	Normal	Abnormal	Near normal
Bone marrow, spleen	Normal	Severe hypocellularity	Slight hypocellularity
Liver, thymus	Normal	AHR activation[d]	AHR activation[d]

CYP1A1 and CYP1B1 metabolize PAHs, whereas CYP1A2 metabolism of PAHs is low but detectable**;** CYP1A2 metabolizes *N*-arylamines most efficiently (Nebert et al., 2004). Subtle differences in BaP metabolite profiles generated by CYP1A1 vs CYP1B1 vs CYP1A2 are well known (Guengerich and Shimada, 1998) and are probably tissue- and cell-type-specific.

[a]The knockout genotypes in all lines were backcrossed into C57BL/6J (B6) mice at least 8 times, rendering all animals with >99.8% B6 genetic background**;** hence, B6 inbred mice were used as *Cyp1(+/+)* controls. These data are summarized from (Uno et al., 2004; Uno et al., 2006; Dragin et al., 2007).

[b]Measured after 125 mg/kg/day oral BaP for 5 days. All other parameters were described after oral BaP at this dose was given for 18 days.

[c]ALT, plasma alanine aminotransferase levels, to assess hepatocellular injury**;** AST, plasma aspartate aminotransferase levels, also to assess liver injury but can also be a sign of cardiac and skeletal muscle damage.

[d]Activation reflects the fact that daily BaP treatment causes chronic AHR activation—which in turn leads to proliferation of the endoplasmic reticulum and increased liver weight and thymus weight**;** these effects are AHR-dependent but independent of CYP1 metabolism (Uno et al., 2006).

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TABLE 3

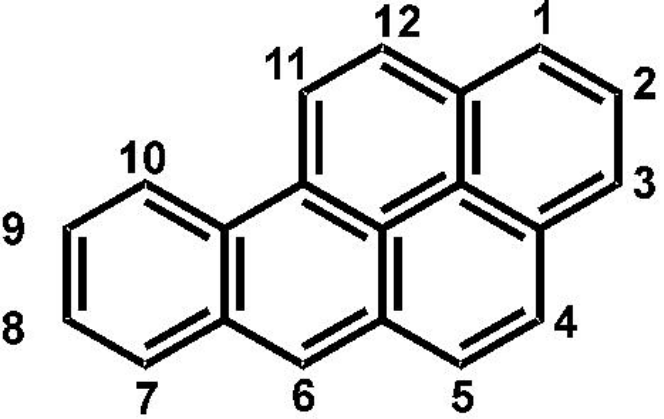
Summary of the response to oral BaP (12.5 mg/kg/day) by four genotypes

Genotypes[a]	***Cyp1(+/+)*** ***Cyp1b1(–/–)***	***Cyp1a1(–/–)***	***Cyp1a1/1b1(–/–)***
Clinical outcome	Healthy for lifetime	Adenocarcinoma of PSI (at 8-12 weeks)	Squamous cell carcinoma of preputial gland duct (at 8-12 weeks)
Blood BaP levels (ng/mL)[b]	~0.1	~4	~13
ALT, AST, hemoglobin, hematocrit, methemoglobin levels	Normal[b]	Borderline abnormal[b]	Normal[b]
Bone marrow, spleen[b]	Normal	Slight hypocellularity	Normal
Liver, thymus[b]	Normal	AHR activation[c]	AHR activation[c]

[a]Knockout genotype in all lines was backcrossed into C57BL/6J (B6) mice at least 8 times, rendering all animals with >99.8% B6 genetic background**;** hence, B6 inbred mice were used as *Cyp1(+/+)* controls. These data are summarized from (Shi et al., 2010b; Gálvez-Peralta et al., 2013).

[b]Measured after 12.5 mg/kg/day oral BaP for 4 weeks. Abbreviations are the same as in Table 2.

[c]Activation reflects the fact that daily BaP treatment causes chronic AHR activation—which in turn leads to proliferation of the endoplasmic reticulum and increased liver weight and thymus weight**;** these effects are AHR-dependent but independent of CYP1 metabolism (Uno et al., 2006).



Benzo[*a*]pyrene (BaP)

Examples of reactive metabolites: products of metabolic activation	Examples of nonreactive metabolites: products of detoxication
BaP 7,8-epoxide	3-hydroxy-BaP; other phenols[a]
BaP 7,8-diol-9,10-epoxide[b]	BaP 7,8-diol[c]
BaP 3,6-quinone; other quinones	BaP 9,10-diol
BaP 4,5-oxide[d]	BaP 7,8,9,10-tetrol

Figure 1

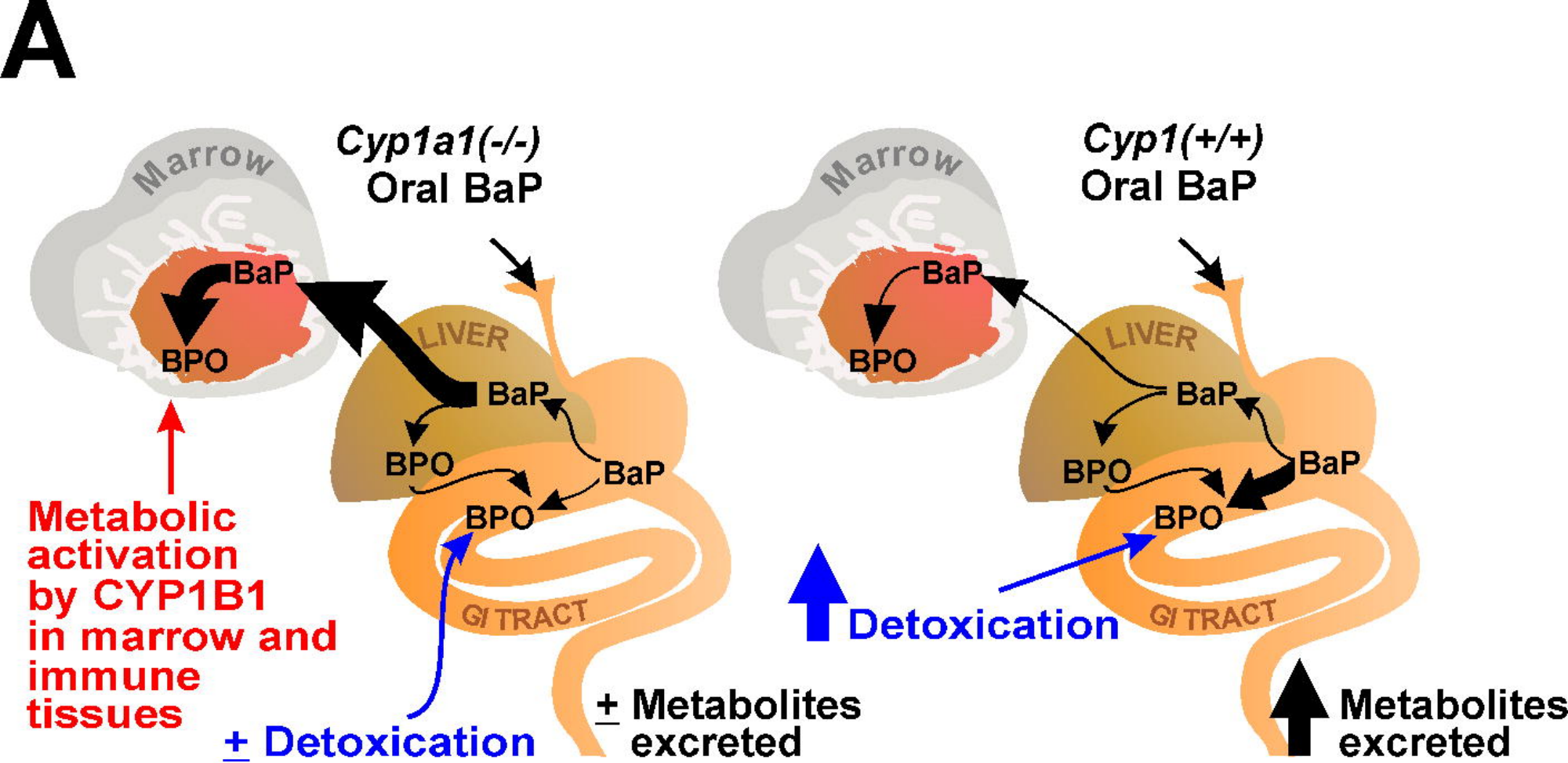

A
Cyp1a1(-/-)
Oral BaP
Marrow
BaP
BPO
LIVER
BaP
BPO
BaP
BPO
GI TRACT
Metabolic activation by CYP1B1 in marrow and immune tissues
± Detoxication
± Metabolites excreted
Cyp1(+/+)
Oral BaP
Marrow
BaP
BPO
LIVER
BaP
BPO
BaP
BPO
GI TRACT
Detoxication
Metabolites excreted

Figure 2

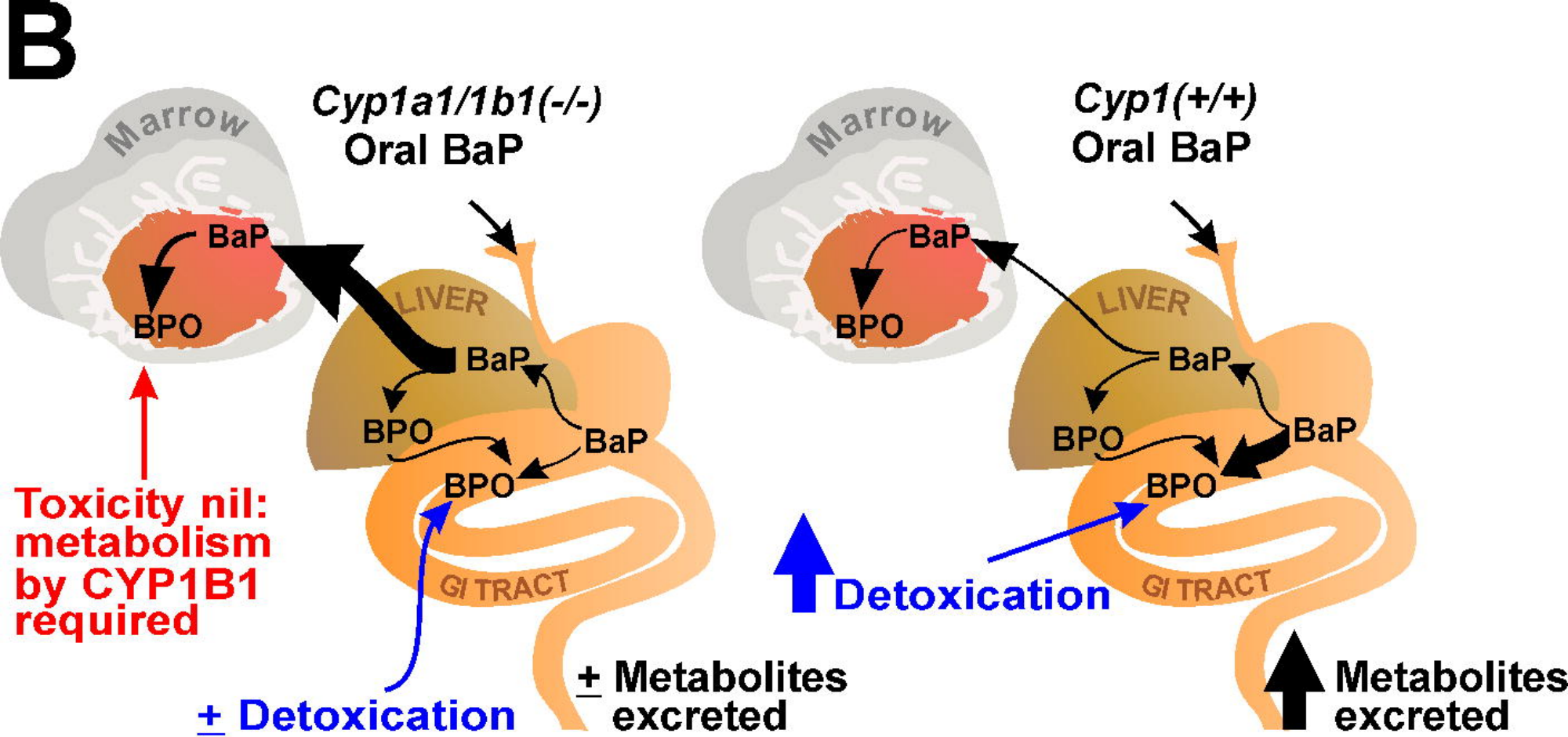

B
Cyp1a1/1b1(-/-)
Oral BaP
Marrow
BaP
BPO
LIVER
BaP
BPO
BaP
BPO
GI TRACT
Toxicity nil:
metabolism
by CYP1B1
required
± Detoxication
± Metabolites
excreted
Cyp1(+/+)
Oral BaP
Marrow
BaP
BPO
LIVER
BaP
BPO
BaP
BPO
GI TRACT
Detoxication
Metabolites
excreted

Figure 2

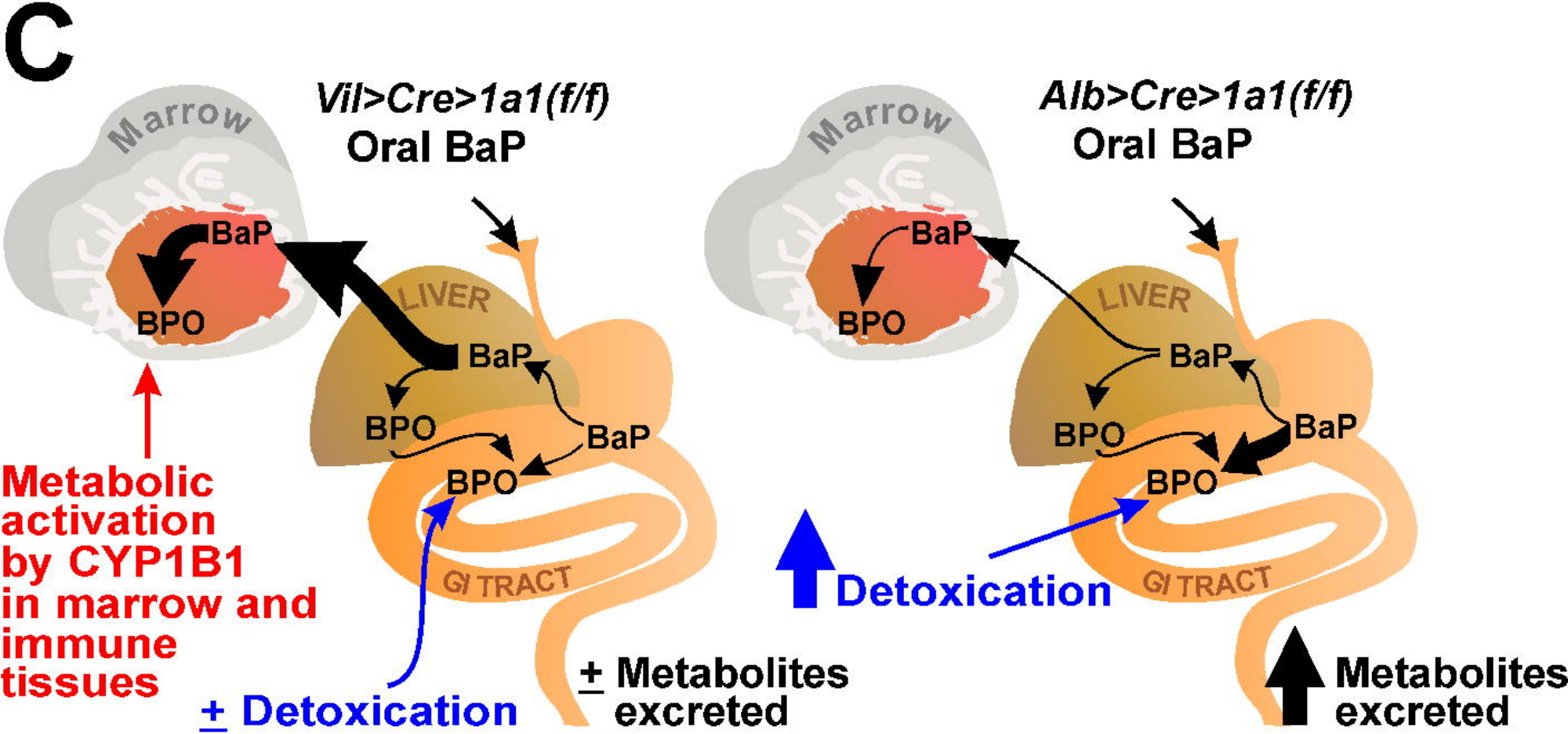

C
Vil>Cre>1a1(f/f)
Oral BaP
Marrow
BaP
BPO
LIVER
BaP
BPO
BaP
BPO
GI TRACT
Metabolic activation by CYP1B1 in marrow and immune tissues
± Detoxication
± Metabolites excreted
Alb>Cre>1a1(f/f)
Oral BaP
Marrow
BaP
BPO
LIVER
BaP
BPO
BaP
BPO
GI TRACT
Detoxication
Metabolites excreted

Figure 2