The Hepatic Reductase Null (HRN™) and Reductase Conditional Null (RCN) mouse models as suitable tools to study metabolism, toxicity and carcinogenicity of environmental pollutants[§]

Volker M. Arlt,[*1] Colin J. Henderson,[2] C. Roland Wolf,[2] Marie Stiborová,[3] and David H. Phillips[1]

[1]Analytical and Environmental Sciences Division, MRC-PHE Centre for Environment and Health, King's College London, 150 Stamford Street, London SE1 9NH, United Kingdom

[2]Division of Cancer Research, Medical Research Institute, Jacqui Wood Cancer Centre, University of Dundee, Dundee DD1 9SY, United Kingdom

[3]Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic

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Abstract

The cytochrome P450 (P450) superfamily consists of a large number of haem-containing mono-oxygenases that play a pivotal role in the metabolism of many drugs and carcinogens. A number of gene knockout and transgenic mice have been developed to study the role of specific P450 isoenzymes in xenobiotic metabolism, but the functional redundancy inevitably found in the P450 gene superfamily make it difficult to determine the *in vivo* role of these enzymes in xenobiotic metabolism as a whole. Over ten years ago the Hepatic Reductase Null (HRN™) model was developed to overcome these limitations. In these mice cytochrome P450 oxidoreductase (POR), the electron donor to P450 enzymes, is deleted specifically in hepatocytes, resulting in the loss of essentially all hepatic P450 function. While the HRN model uses an hepatocyte-specific albumin promoter to drive POR deletion during mouse development to adulthood, adult Reductase Conditional Null (RCN) mice are entirely normal with respect to POR (and thus P450 function) until hepatic POR deletion is driven via a *CYP1A1* promoter which is induced by treatment with the polycyclic aromatic hydrocarbon 3-methylcholanthrene, essentially recapitulating the phenotype of the HRN mouse. The HRN and RCN models have been used to study the metabolism and genotoxicity of a variety of environmental carcinogens including benzo[*a*]pyrene, aristolochic acid, 3-nitrobenzanthrone and 2-amino-1-methyl-6-phenylimiazo[4,5-*b*]pyridine and the results obtained are summarized in this review. For example, investigations of BaP metabolism in the HRN and RCN mice revealed an apparent paradox, whereby hepatic P450 enzymes appeared to be more important for detoxification of BaP *in vivo*, despite being involved in its metabolic activation *in vitro*. Cytochrome b_5 is a microsomal haemoprotein which provides electrons (from cytochrome b_5 reductase) to P450 enzymes; however, its role in carcinogen metabolism is still enigmatic. Therefore, a mouse line was generated with a conditional hepatic deletion of cytochrome b_5 (HBN, Hepatic cytochrome b_5 Null) that was also crossed with HRN mice to create a double conditional mutant, HBRN (Hepatic cytochrome b_5/P450 Reductase Null), in which both enzymes are deleted specifically in the liver. The combination of the HRN, RCN, HBN and HBRN models provide a powerful approach to study the P450-dependent metabolism and (geno)toxicity of environmental carcinogens.

Introduction

Cytochrome P450 (P450) (EC 1.14.14.1) is a superfamily of haemoproteins distributed widely throughout nature, involved in metabolism of a broad variety of substrates and catalyzing a variety of interesting chemical reactions.[1, 2] P450 enzymes are major catalysts involved in the oxidation of xenobiotic chemicals, a significant focus of scientists working in the areas of toxicology and pharmacology.[1] These oxidation reactions can result in poor drug bioavailability and various acute and chronic toxicities, including adverse drug interactions and cancer susceptibility.[1] The metabolism and especially bioactivation of carcinogens are dominated by P450 enzymes.[3] Often the P450-mediated metabolism of the parent compound to excreted hydrophilic conjugated products leads to the formation of reactive oxygenated intermediates which can cause genotoxicity and mutation by covalently binding to DNA and proteins.[4] Damage to the structural integrity of DNA through covalent binding of chemicals is referred to as DNA adduct formation. Such DNA damage is generally considered to be causative and directly related to tumour formation.[5, 6] Thus, information on which P450 enzymes are involved in the metabolism of known or putative human carcinogens is critical for hazard identification and risk assessment. Gene knock-out and transgenic mice have been developed to study the role of specific P450 enzymes in drug metabolism [7, 8] and these mouse lines are increasingly used to study carcinogen metabolism.[9]

In this review we focus on the Hepatic Reductase Null (HRN™) and Reductase Conditional Null (RCN) mouse models and their applicability to study the metabolism, toxicity and carcinogenicity of environmental pollutants. We start with a brief overview on the role of P450 enzymes in environmental carcinogenesis. Next, we discuss the creation of the HRN and RCN mouse and their advantages over *Cyp*-knockout mouse models. Finally we describe our experiences using the HRN and RCN model to study the metabolism of a variety of different environmental and nutritional carcinogens. In addition, we discuss related mouse models used by others to study carcinogen metabolism. Beyond the scope of the review is the potential application of the HRN and RCN mouse in drug discovery, which has been reviewed previously.[10]

The role of cytochromes P450s in carcinogen metabolism

P450 enzymes are one component of a mixed function oxidase system located in the membrane of the endoplasmic reticulum that beside the P450s also contains other enzymes such as NADPH:cytochrome P450 oxidoreductase (POR),[11] and cytochrome b_5 accompanied

by its NADH:cytochrome b_5 reductase.[12] Via the activation of molecular oxygen, this multienzyme system catalyzes the monooxygenation of a variety of substrates. The oxygen is activated in the active center of P450s by two electrons transferred from NADPH and/or NADH by means of POR and/or cytochrome b_5, respectively. Whereas POR is an essential constituent of the electron transport chain towards P450,[11] the role of cytochrome b_5 in carcinogen metabolism is still quite enigmatic.[13-15]

Mammals appear to use a set of P450 enzymes (e.g. CYP5, 8, 11, 17, 19, 21, 24, 26, and 27) for the synthesis of important steroids and eicosanoids, aside from their function in the catabolism of natural products.[1, 2] Of the remainder of the mammalian P450s, a relatively small set of these enzymes accounts for most of metabolism of drugs (i.e. human CYP1A2, 2C9, 2C19, 2D6, and 3A4). It should be emphasized that ~75% of enzymatic reactions converting drugs are catalyzed by P450 enzymes and that ~90% of these P450 reactions can be accounted for by the set of human P450s mentioned above (i.e. CYP1A2, 2C9, 2C19, 2D6, and 3A4).[3, 16] The largest proportion of P450 reactions are catalyzed by P450 enzymes of the 3A subfamily, particularly CYP3A4.[3] Another small set of human P450s (i.e. human CYP1A1, 1A2, 1B1, 2A6, 2E1, and 3A4) is involved in the metabolism of most pro-toxicants and pro-carcinogens that are P450 substrates.[1, 3]

The majority of human P450 enzymes involved in carcinogen metabolism are most abundant in the liver, except for CYP1A1 and 1B1 which are mainly extrahepatic enzymes.[2, 3, 17, 18] However, many of these P450 enzymes are also found in some extrahepatic tissues and P450-mediated bioactivation of carcinogen within a target tissue may be more important, particularly if a generated reactive product is not stable enough to migrate out of the cell in which it is formed.[17] Many P450 enzymes are inducible by chemicals; the inducer may also be a substrate but this is not necessarily the case.[18]

The participation of individual P450 enzymes in carcinogen metabolism has been widely investigated, and the roles of numerous human P450s in carcinogen activation have been characterized.[2, 4, 19] Recently, it has been reviewed by Rendic and Guengerich [3] that of the human P450 enzymes involved in the metabolism of carcinogens, a major role for P450s might be attributable predominantly to the three P450 enzymes of Family 1 (CYP1A1, 1A2, 1B1) and CYP2A6, 2E1, and 3A4. CYP1A1, 1A2, 1B1, 2A6, 2E1, and 3A4 enzymes account for ~80% of the reported carcinogen activations.[3] It can be exemplified that of these P450s the enzymes of Family 1 are most important in the activation of polycyclic aromatic hydrocarbons (PAHs),[20] aromatic amines,[21] heterocyclic aromatic amines (HAAs),[22] and a

variety of other compounds. CYP2A6 and 2E1 play a crucial role in the metabolism of *N*-nitrosamines [23] and CYP3A4 activates the mycotoxin aflatoxin B_1 (AFB_1).[24]

Beside the P450 enzymes contributing to carcinogen activation, many of them also catalyse also detoxification of these compounds. Moreover, a single enzyme can generate different products from the same substrate; among them several are the detoxification metabolites but others are (geno)toxic. For instance, CYP3A4-catalysed oxidation of AFB_1 to aflatoxin Q1 (i.e. 3-hydroxylation) is a detoxification reaction, while the oxidation of this carcinogen by the same P450 to AFB_1-8,9-epoxide represents the activation pathway.[24] A similar situation can be found in the metabolism of the human carcinogen benzo[*a*]pyrene (BaP), where CYP1A1 and 1B1 are the most important P450 enzymes in its activation,[20, 25] in combination with microsomal epoxide hydrolase (mEH) (**see Figure 1A**). These P450 enzymes oxidize BaP to an epoxide that is then converted to a dihydrodiol by mEH (i.e. BaP-7,8-dihydrodiol). Further bioactivation by CYP1A1 and 1B1 leads to the ultimate reactive species BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) which can react with DNA.[26] However, BaP is also oxidized to other metabolites such as the other BaP-dihydrodiols, BaP-diones and hydroxylated BaP metabolites.[20] Another example of the dual role of P450 enzymes (toxication *versus* detoxification) in the metabolism of environmental carcinogens is the P450-mediated conversion of the plant carcinogen aristolochic acid I (AAI) (**see Figure 1B**).[27, 28] A detailed description of the role of P450 enzymes in BaP and AAI metabolism is given below.

Deletion of P450 function in the liver

The advent of embryonic stem (ES) cell technology in the early 1990s, and in particular the isolation of mouse ES cells and the ability to grow these in the laboratory in a manner in which they retain their pluripotency, allowed the development of gene targeting by which it was possible to delete all or part of a gene, or introduce mutations to affect gene function(s).[29] It is important to note that the first murine ES cells were isolated from a 129/Sv genetic background; the mice generated from early gene targeting experiments were thus a mixture of genetic backgrounds – 129 and whatever other line was used in the breeding process – yielding an often confusing number of combinations, some of which affected the phenotype arising from the gene targeting,[30] and necessitated an extensive breeding programme to generate a line on a 'defined' genetic background. It is only relatively recently that ES cells from other mouse genetic backgrounds, i.e. C57BL/6, have been successfully isolated and used routinely in genetic manipulations. By the early 2000s, a number of P450s had been

deleted in mice and the phenotypic effects studied. These generally fell into two camps whereby deletion of P450s involved in 'housekeeping' reactions tended to lead to embryonic lethality or perinatal mortality, whereas those models in which 'drug or xenobiotic metabolising' P450s were deleted often resulted in little or no overt change in phenotype, unless the mice were challenged with a compound, the metabolism of which the missing P450 was involved in. [reviewed in [31, 32]]. The yield of information from such experiments was thus relatively limited, although a number of combined deletions were generated by cross-breeding individual knockout lines, for example those involving Cyp1a1, 1a2 and 1b1 and BaP metabolism.[9, 33]

Creation of the Hepatic Reductase Null (HRN™) mouse

The liver is considered to be the major organ involved in drug metabolism; although it is recognized that other tissues, particularly the gut, have a role to play in the overall disposition of drugs, particularly those administered orally. However, dissecting the relative role of different tissues *in vivo* is necessarily fraught with difficulties. In order to study the role of the P450 system in drug metabolism and disposition in more detail, and to determine the role of specific tissues in this process, it can be desirable to be able to delete the function of a greater number of *CYP* genes simultaneously. To achieve this, a targeting strategy was devised that would inactivate the function of all P450s, with the added bonus of being able to do so in a conditional manner. All P450s are supplied with reducing equivalents from NADPH via a single electron transfer protein, POR; thus, by deleting POR, all P450s can be inactivated.

Early gene targeting technology was limited to relatively simple genetic manipulations, i.e. introducing a mutation into a gene, or globally deleting the function of a single gene. The advent of site-specific recombination strategies introduced the possibility of gene deletion in a spatially and/or temporally controlled fashion. Perhaps the best known system to achieve this is the Cre-loxP system, whereby the gene of interest (or key parts of it) are flanked by loxP sites ('floxed'), small pieces of DNA which are recognized by Cre recombinase and the DNA between such sites is then deleted.[34] From a single targeting construct,[35] it was thus possible to globally delete POR in all tissues, an event that predictably led to embryonic lethality [36] but also yielded clues to functions of POR (and P450s) at an early stage in development.[37] By crossing floxed POR mice with a line carrying Cre recombinase under control of the hepatocyte-specific albumin promotor,[38] it was possible to create a model in which POR was deleted, and P450 function thus inactivated, specifically in

the liver (**see Figure 2A**).[35] The Hepatic Reductase Null mouse (HRN) is crossed onto a C57BL/6 background and shows no overtly changed phenotype, develops and breeds normally, and has a lifespan indistinguishable from wild-type (WT) mice. However, on closer investigation HRN mice have been found to have steatotic liver, significantly lowered blood lipids and virtually no bile acid production,[35] as a consequence of non-functioning of essential 'housekeeping' P450s involved in cholesterol and bile acid metabolism. Furthermore, as a consequence of POR deletion the expression of essentially all hepatic P450s are substantially elevated, including those involved in drug metabolism which are normally only induced following exposure to particular chemicals, drugs or toxicants. This phenomenon was further investigated using a variant of the HRN mouse (RCN - see below). Of note, other groups have also used a similar strategy to develop such a mouse [39] and some of these studies have also been reviewed below. HRN mice have been extensively used to investigate the role of hepatic metabolism in drug disposition;[40-47] this aspect is beyond the scope of the present review, which focuses on carcinogen metabolism

Creation of the RCN mouse

While the HRN mouse has extensive utility in the investigation of hepatic drug metabolism, the use of the albumin promoter to drive Cre expression has limitations, mainly centred around the inability to control or regulate gene deletion, since the albumin promoter switches on soon after birth, and in the case of the HRN line, drives POR deletion to completion by adulthood. A refinement of the targeting strategy was introduced whereby floxed POR mice were crossed with a mouse line in which Cre recombinase was expressed from the tightly regulated promoter of rat *CYP1A1* gene (**see Figure 2B**). The use of this promoter to drive expression of heterologous genes had previously been validated using β-galactosidase [48] and used to determine expression of Cyp1a1 in embryonic development.[49] The new random transgenic model, Reductase Conditional Null (RCN), is entirely normal with respect to POR function (and thus P450), until the mice are treated with compounds to induce the *CYP1A1* gene (**see Figure 2B**). Characterisation of the RCN line established that when treated with the PAH 3-methylcholanthrene (3MC), POR deletion was liver-specific and essentially recapitulated the phenotype of the HRN mouse.[50] Interestingly, by applying a second compound (i.e. the PAH β-naphthoflavone) it was possible to delete POR in both liver and small intestine, thus allowing the contribution of the gut to drug metabolism and disposition to be assessed.[50] The RCN model was also instrumental in further characterisation of one of

the more intriguing phenotypes of this and the HRN model, namely the profound, cross-family induction of hepatic P450 expression observed in the absence of POR. Finn *et al.* [51] determined that by placing RCN mice on a fat-deficient diet for 4 weeks prior to inducing POR deletion by treatment with 3MC, the elevated hepatic P450 levels could be attenuated. By systematically adding back different classes of lipids to the fat-deficient diet, they found that unsaturated fatty acids, and specifically linoleic acid, mediated the induction of hepatic P450 expression in POR null RCN mice, facilitated at least partly by the nuclear receptor CAR (constitutive androstane receptor) and to a lesser extent PXR (pregnane X receptor).[51]

Investigating metabolism and (geno)toxicity of environmental carcinogens in the HRN and RCN mouse models

As outlined above, distinguishing the role of individual P450 isoenzymes in activation *versus* detoxification of carcinogens is an important challenge. Models like the HRN and RCN mouse provide a powerful approach to distinguish the role of P450s in hepatic *versus* extrahepatic bioactivation of environmental carcinogens as a whole. Over the past 10 years we have gained extensive experience in using the HRN and RCN mouse models to study the metabolism and (geno)toxicity of environmental pollutants. Thus far, we have examined four environmental carcinogens using the HRN and RCN mouse model: (*i*) BaP; (*ii*) AAI; (*iii*) 3-nitrobenzanthrone (3-NBA); and (*iv*) 2-amino-1-methyl-6-phenylimiazo[4,5-*b*]pyridine (PhIP).[15, 26, 52-57]

(i) Benzo[a]pyrene (BaP)

BaP is a PAH that has been classified as human carcinogen (Group 1) by the International Agency for Research on Cancer (IARC).[58] BaP and other PAHs are produced mainly by incomplete combustion of organic matter but are also present in tobacco smoke and the diet.[59] Many studies using mammalian cellular and subcellular systems have demonstrated that PAHs, including BaP, are metabolically activated by P450s.[60] BaP is a ligand for the aryl hydrocarbon receptor (AHR), which up-regulates expression of several phase I and II xenobiotic metabolising genes, including CYP1A1 and 1B1, which metabolically activate BaP to reactive intermediates (**see Figure 1A**). The major pathway of activation of BaP leading to DNA adduct formation is via a bay-region diol-epoxide, BaP-7,8-dihydrodiol-9,10-oxide (BPDE), to 10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydro-BaP (dG-N^2-BPDE).[61]

In order to evaluate the role of hepatic *versus* extra-hepatic metabolism of BaP and its pharmacokinetics we used initially the HRN mouse model.[26] HRN and WT mice were treated i.p. with 125 mg/kg body weight (bw) BaP daily for up to 5 days. We hypothesized that the HRN mice would form fewer adducts in the liver than WT mice, but might form higher levels of adducts in extrahepatic tissues. DNA adduct levels were measured by ^{32}P-postlabelling analysis with structural confirmation of the formation of dG-N^2-BPDE by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis.

BaP-DNA adduct levels were indeed elevated in several extrahepatic tissues (by 1.7-2.6-fold) (**see Figure 3A**). However, adduct levels in the livers of the HRN mice were considerably higher (up to 13-fold) than in the WT mice, despite the absence of P450 activity in that tissue, and in marked contrast to our prediction. We verified that metabolic activation of BaP was via the diol-epoxide pathway by confirming the formation of dG-N^2-BPDE in DNA in both the HRN and WT mice. Pharmacokinetic analysis of BaP in blood revealed no differences in e.g. clearance, terminal half-life, and AUC in HRN relative to WT mice. Hepatic microsomes isolated from BaP-treated and untreated mice were also incubated with BaP and DNA *in vitro*. BaP-DNA adduct formation was up to 7-fold lower with the microsomes from HRN mice than with those from WT mice (**see Figure 3B**). Most of the hepatic microsomal activation of BaP *in vitro* was attributable to Cyp1a. Thus these results revealed a paradox: P450 enzymes appear to be more important for detoxification of BaP *in vivo*, despite being involved in its metabolic activation *in vitro*.

In part, these findings are comparable those obtained in mice with systemic knock-out of Cyp1a1 function. When BaP was administered i.p. to *Cyp1a1(-/-)* mice at high dose, higher adduct levels were formed in liver.[62] However, this was accompanied by slower clearance of BaP, unlike the result with HRN mice.[26] While repeated high i.p. doses of BaP were less toxic to the *Cyp1a1(-/-)* mice than to *Cyp1a1(+/-)* animals, BaP was more toxic to *Cyp1a1(-/-)* mice than WT mice when administered orally.[63] The rate of clearance of BaP was still slower, and the levels of adducts in several tissues, including liver, were still higher in the knock-out mice. Subsequent studies with *Cyp1a1(-/-)* and *Cyp1b1(-/-)* mice, as well as with the double knock-out mouse (i.e. null for both genes), produced similar results.[64]

Experiments with *Ahr(-/-)* mice have also demonstrated the complexities of BaP carcinogenicity. Depending on the route of administration, the non-responsive mice can be more susceptible to toxicity and tumour formation (e.g. after oral administration) [65] or at lower risk (by topical application or subcutaneous administration).[66] However, total hepatic

BaP-DNA adduct levels were very similar in *Ahr(-/-)* and *Ahr(+/+)* mice after a single i.p. injection, although qualitative differences in adduct profiles were observed.[67] Further studies on *Ahr(-/-)* mice again demonstrated the important influence of route of administration: when BaP was administered orally by gavage, there were increased levels of BaP-DNA adducts in liver and lung, and protein binding by BaP was higher in several tissues;[68, 69] but when exposure was by topical application, protein adduct levels in the exposed skin were lower in *Ahr(-/-)* mice.[69] These studies demonstrate not only differences in uptake and distribution of BaP in the presence and absence of Ahr, but also provide evidence of a mechanism of BaP biotransformation that is Ahr-independent.

Because it is apparent from these studies that the response of mice to BaP is dependent on both the route of administration and the dose, we carried out further studies in HRN and RCN mice to examine these potential sources of influence.[52] We compared high and low doses of BaP (12.5 or 125 mg/kg bw), administered either orally or i.p. The levels of adducts formed in liver were consistently higher (up to 10-fold) in the HRN mice than in the WT mice. Similar results were obtained with RCN mice (**see Figure 4**). We tested whether differences between hepatocytes and non-hepatocytes in P450 activity may underlie the increased liver BaP-DNA binding in HRN mice. Cellular localisation by immunohistochemistry of BaP-DNA adducts showed that HRN mice have ample capacity for formation of BaP-DNA adducts in liver, indicating that the process does not involve generation of a reactive species different from that formed in WT mice.[52] However, increased protein expression of cytochrome b_5 in hepatic microsomes of HRN relative to WT mice suggests that cytochrome b_5 may modulate the P450-mediated bioactivation of BaP in HRN mice, partially substituting the function of POR.[52]

In order to investigate further the potential role of cytochrome b_5 in BaP biotransformation we conducted some further *in vitro* experiments using hepatic microsomes from control and BaP-pretreated HRN mice and reconstituted systems with CYP1A1, POR, cytochrome b_5, and epoxide hydrolase (mEH) in different ratios.[15] In microsomes from BaP-pretreated mice, in which Cyp1a1 was induced, higher levels of BaP metabolites were formed, mainly of BaP-7,8-dihydrodiol. At a low POR:CYP1A1 ratio of 0.05:1 in the reconstituted system, the amounts of BaP diones and BaP-9-ol formed were essentially the same as at an equimolar ratio, but formation of BaP-3-ol was ~1.6-fold higher. Only after addition of mEH were BaP dihydrodiols found. Two BaP-DNA adducts were formed in the presence of mEH, but only one when CYP1A1 and POR were present alone.[15] At a ratio of POR:CYP1A1 of 0.05:1, addition of cytochrome b_5 increased CYP1A1-mediated BaP

oxidation to most of its metabolites indicating that cytochrome b_5 participates in the electron transfer from NADPH to CYP1A1 required for enzyme activity of this CYP. BaP-9-ol was formed even by CYP1A1 reconstituted with cytochrome b_5 without POR. Our results suggested that in livers of HRN mice Cyp1a1, cytochrome b_5 and mEH can effectively activate BaP to DNA binding species, even in the presence of very low amounts of POR.[15] Further *in vivo* experiments using cytochrome *b5*-knockout mouse lines (i.e. HBN and HBRN; see below) will undoubtedly shed light on these possible mechanisms of BaP biotransformation.

(ii) Aristolochic acid I (AAI)

Plants of the *Aristolochia* genus contain aristolochic acid (AA), which is used as a herbal drug. AA is a mixture of nitrophenanthrene carboxylic acids, of which 8-methoxy-6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid (aristolochic acid I, AAI) (**see Figure 1A**) and 6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid (AAII) are the main components.[70] AA has been shown to be the cause Chinese herbs nephropathy,[71] now referred to as aristolochic acid nephropathy (AAN).[72, 73] AAN is a rapidly progressive renal fibrosis with a high risk for patients of developing upper urothelial tract carcinoma (UUC) and, subsequently, bladder urothelial carcinoma.[73, 74] AA has recently been classified as a Group I carcinogen by the IARC.[75]

Exposure to AA has also been found to be the cause of a similar type of renal disease, Balkan endemic nephropathy (BEN) and its associated occurrence of urothelial malignancy.[76, 77] This disease is endemic in certain rural areas of Balkan countries that are located near the tributaries of the Danube river.[78]

Characteristic AA-DNA adducts in renal tissue of AAN and BEN patients are biomarkers of exposure to AA even long after AA exposure, the 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI) adduct being the most abundant adduct formed and the most persistant.[74, 78-80] This deoxyadenosine adduct causes characteristic A to T transversion mutations and such mutations were found in the *TP53* tumour suppressor gene in tumours from AAN and BEN patients [77, 81] and in immortalized Hupki (human *TP53* knock-in) mouse fibroblasts (HUFs) exposed to AAI,[82] indicating a molecular mechanism of AA-mediated carcinogenesis.[76, 83] More recently, these A to T transversion mutations were also observed in other loci by whole-genome and exome sequencing analysing AA-associated UUC and AAI-treated HUFs.[84-86]

Bioactivation by nitro-reduction is responsible for the carcinogenic properties of AAI. Such nitro reduction leads to the formation of *N*-hydroxylated aristolactam I which converts to a reactive cyclic acylnitrenium ion generating DNA adducts (**see Figure 1B**).[72] The product of AAI oxidation, 8-hydroxyaristolochic acid I (aristolochic acid Ia, AAIa), is formed by *O*-demethylation of the methoxy group, and is a detoxication product of this carcinogen, which is excreted either in its free form or conjugated.[87, 88]

A variety of enzymes are involved in the metabolism of AAI. Most information on the enzymes metabolizing AAI has been obtained *in vitro*.[28] These studies showed that NAD(P)H:quinone oxidoreductase (NQO1) is the most efficient cytosolic nitroreductase activating AAI. In human and rodent hepatic microsomes AAI is activated by CYP1A2 and, to a lesser extent, by CYP1A1; POR also plays a minor role.[28] In addition, human and rodent CYP1A1 and 1A2 are the main enzymes catalyzing the oxidative detoxification of AAI to AAIa *in vitro*.[28] It is difficult to evaluate exactly the significance of microsomal and cytosolic nitroreductases for AAI metabolism solely based on *in-vitro* experiments. Therefore, we looked also at the *in vivo* situation.[27, 53, 87, 88]

Using HRN and WT mice treated orally with 10 or 50 mg/kg bw of AAI,[53] we demonstrated that the P450-mediated metabolism of AAI in the liver not only influenced AAI-DNA adduct formation in this organ, but also in extrahepatic organs (**see Figure 5A**). Because hepatic P450-catalysed demethylation of AAI to AAIa was reduced in the livers of HRN mice relative to WT mice, higher amounts of AAI circulated to extrahepatic tissues resulting in higher AAI-DNA adduct levels in these organs including the target organ kidney.[53] Others showed that the higher amounts of circulating AAI also resulted in a higher sensitivity of liver-*Por*-null mice to AAI toxicity.[89]

Using selective inhibitors (e.g. α-naphthoflavone) in hepatic microsomal incubations *ex vivo* the role of Cyp1a1 and 1a2 in AAI demethylation was determined (**see Figure 5B**). A major role of these mouse P450 enzymes and their human orthologs in AAI oxidative demethylation was additionally confirmed by other studies, where *Cyp1a1/1a2* knock-out and *CYP1A1/2*-humanized mouse models were utilized.[87, 88] Thus, when AAI is not oxidatively *O*-demethylated to AAIa in the liver, it is activated by several enzymes with nitroreductase activity (reviewed elsewhere[27]) to form a cyclic acylnitrenium ion producing AAI-DNA adducts.

However, it should be emphasized that Cyp1a1/2 play a dual role in AAI metabolism. Under anaerobic conditions mouse hepatic Cyp1a enzymes can reduce AAI, thereby

activating it to DNA adduct forming species.[53, 87, 88] Indeed, while hepatic microsomes of WT mice expressing POR and Cyp1a catalysed AAI-DNA adduct formation even without pretreatment of mice with a P450 inducer (i.e. BaP) (**compare Figure 5B**), only microsomes isolated from livers of HRN mice pretreated with BaP were capable of catalysing this reaction.[26, 53] These results indicate the role of Cyp1a rather than POR in the reductive activation of AAI in mouse hepatic microsomes. The predominant role of Cyp1a in AAI-DNA adduct formation was also demonstrated in hepatic microsomes employing selective inhibitors; inhibitors of Cyp1a1/2 (i.e. α-naphthoflavone) caused a significant decrease in AAI-DNA adduct levels catalyzed by liver microsomes.[53] These results suggest that, beside the expression of Cyp1a1/2 proteins and their activities, the *in vivo* oxygen concentration in the organs determines the extent of nitroreduction and *O*-demethylation of AAI, and thus its toxicity and carcinogenicity.

The HRN model also helped us to elucidate the role of Nqo1 in AAI bioactivation *in vivo*.[53] We found that the *Por* knock-out was not the sole reason for increased AAI-DNA adduct formation in the liver and extrahepatic tissues. We found that a concomitant increase in Nqo1 activity and protein expression in the livers of HRN mice correlated with higher AAI-DNA adduct levels in *ex-vivo* incubations.[53] This was evident in experiments with cytosols of mice pretreated with the Nqo1 inducer BaP, where Nqo1 expression, activity and AAI-DNA adduct levels were approximately 3-fold higher than with incubations using hepatic cytosols from untreated HRN mice.[53] The role of Nqo1 in AAI-DNA adduct formation in hepatic cytosols of WT and HRN mice was confirmed by the effect of dicoumarol, an Nqo1 inhibitor, which decreased the levels of AAI-DNA adducts.[27, 53] These results highlight the importance of NQO1 not only for AAI activation *in vitro* but also *in vivo*.

(iii) 3-Nitrobenzanthrone (3-NBA)

3-NBA (3-nitro-7H-benz[*de*]anthracen-7-one) is a potent mutagen and suspected human carcinogen.[90] In common with other nitro-PAHs, it has been detected on the surface of ambient air particulate matter and diesel exhaust particles.[90, 91] Recently, it has been classified a possible human carcinogen by IARC (Group 2B).[91] Indeed, 3-NBA induces squamous cell carcinoma in rat lung after intratracheal instillation [92] and human exposure to 3-NBA has been demonstrated by the detection of 3-aminobenzanthrone (3-ABA), the main metabolite of

3-NBA, in urine of salt mine workers occupationally exposed to diesel emissions.[93] Thus, 3-ABA may be a suitable biomarker of diesel exposure.

The potential mechanisms for the carcinogenicity of 3-NBA have been investigated in rodents and many other experimental systems.[90] DNA adducts formed by 3-NBA represent premutagenic lesions and seem to be critical for the high mutagenic potency of 3-NBA.[94, 95] 3-NBA requires metabolic activation via reduction of the nitro group in order to form DNA adducts.[96, 97] The predominant DNA adducts detected *in vivo* in rodents after treatment with 3-NBA are 2-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone (dG-N^2-3-ABA) and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-*N*-3-ABA),[97, 98] and these adducts are most probably responsible for the G to T transversion mutations induced by 3-NBA in a variety of mutation assays.[94, 99]

A variety of phase I and phase II enzymes are involved in the bioactivation of 3-NBA. Initial nitroreduction to *N*-hydroxy-3-aminobenzanthrone (*N*-OH-3-ABA) is catalysed primarily by cytosolic nitroreductases, such as xanthine oxidase and NQO1 (**see Figure 1C**).[54, 96, 100, 101] *N*-OH-3-ABA is further activated by conjugation by *N*-acteyltransferases (NAT1 and NAT2) and sulfotransferases (SULT1A1 and SULT1A2).[54, 102, 103] However, microsomal POR is also able to metabolically activate 3-NBA by simple nitroreduction.[104] The comparison of 3-NBA-DNA adduct levels formed by human hepatic microsomes and cytosols revealed that the cytosolic enzyme systems were much more effective in the reductive activation of 3-NBA than microsomes. Nevertheless, it was difficult to evaluate exactly the significance of microsomal and cytosolic nitroreductases solely based on *in-vitro* experiments. Therefore, we looked at the *in vivo* situation. To evaluate the importance of hepatic POR in the reductive activation of 3-NBA *in vivo* compared to by cytosolic reduction *in vitro*, we treated HRN mice and WT littermates i.p. with 0.2 or 2 mg/kg bw of 3-NBA.[54] As shown in **Figure 6A** no difference in DNA binding by 3-NBA was observed in any of the tissue examined including liver, lung and kidney at both doses. These results indicated that POR does not contribute significantly to the reductive activation of 3-NBA *in vivo*. Future studies may need to focus on the tissue-specific contribution of NATs and SULTs in the bioactivation of 3-NBA *in vivo*.

The reductive metabolite of 3-NBA identified in humans is 3-ABA.[93] 3-ABA was also the main metabolite of 3-NBA formed in human bronchial cells.[105] It forms the same DNA adducts as 3-NBA *in vitro* and *in vivo* indicating that *N*-OH-3-ABA is the reactive intermediate.[103, 106, 107] Consequently, both 3-NBA and 3-ABA have been shown to be mutagenic in transgenic mouse mutation assays.[94, 95] 3-ABA-derived DNA adducts are

formed by *N*-oxidation, for which CYP1A1 and 1A2 have been identified as the main microsomal enzymes **(see Figure 1D)**.[103, 107] Nevertheless, a number of peroxidases including myeloperoxidase and prostaglandin H synthase are also effective in activating 3-ABA *in vitro*.[55] In order to understand the role of hepatic P450 enzymes in the bioactivation of 3-ABA *in vivo* we treated HRN and WT littermates i.p. with a single dose of 0.2 or 2 mg/kg bw of 3-ABA.[55] Among the tissues investigated the main target organ for DNA adduct formation by 3-ABA was the liver. In the high dose group, DNA adduct levels in the livers were reduced by up to 80% in HRN mice relative to WT mice (**see Figure 6B**); at the low dose 3-ABA-DNA adduct formation was detectable only in WT animals in the liver. No significant change in DNA binding was observed in any of the extra-hepatic tissues investigated (lung, kidney, bladder, and colon). It is noteworthy that *ex-vivo* studies showed that hepatic microsomes from HRN and WT mice were able to reduce 3-NBA to 3-ABA;[56] lower amounts of 3-ABA (~2-fold) were formed in hepatic microsomes of HRN mice. Collectively, the results obtained in the HRN mouse model confirmed the importance of CYP1A1 and 1A2 in the metabolic activation of 3-ABA *in vivo*. CYP1A1 is expressed in the human respiratory tract and both 3-NBA and 3-ABA are capable of inducing CYP1A1,[108, 109] providing a mechanism whereby 3-ABA can enhance its genotoxic potential.

(iv) 2-Amino-1-methyl-6-phenylimiazo[4,5-b]pyridine (PhIP)

Heterocyclic aromatic amines (HAAs) are carcinogenic compounds formed in grilled meat under normal cooking conditions.[110] PhIP is one of the most abundantly formed of more than 20 HAA and IARC has classified it as possibly carcinogenic to humans (Group 2B).[111] PhIP induces tumours in the colon. Cancer of the colon is a diet-related cancer most common in Western countries.[112] As many other HAAs, PhIP forms DNA adducts after metabolic activation and P450 enzymes are the most important in the initial oxidation leading to the formation of the reactive intermediate *N*-hydroxy-PhIP (*N*-OH-PhIP) (**see Figure 1E**). Based on *in vitro* experiments, CYP1A2 has been identified to have high catalytic activity for PhIP *N*-hydroxylation, but CYP1A1 and 1B1 are also able to catalyse this reaction.[110, 113] Subsequent metabolism by NATs or SULTs converts *N*-OH-PhIP into esters capable of reacting with DNA.[114] *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (dG-C8-PhIP) is the major DNA adduct detected in experimental animals and humans exposed to PhIP.[110]

In rodents the liver is the main tissue where P450-mediated *N*-oxidation of PhIP takes place. Thus, we used the RCN mouse model to determine the effect of diminished hepatic

P450 activity on DNA adduct formation by PhIP and mass spectrometry to detect and quantify dG-C8-PhIP.[57, 115] RCN mice were dosed by oral gavage for 5 days with 50 mg/kg bw of PhIP. Pretreatment of the mice with 3MC 2 weeks prior to PhIP administration resulted in hepatic POR loss (**see Figure 2B**) while PhIP-treated RCN mice without 3MC pre-treatment were used for comparison. Interestingly, as shown in **Figure 7A**, hepatic deletion of POR had no significant effect on PhIP-DNA adduct formation in the liver. In contrast, levels of dG-C8-PhIP were up to ~3-fold higher in all extrahepatic tissues examined (lung, forestomach, glandular stomach, small intestine, colon, spleen, kidney, and bladder) in RCN mice lacking hepatic POR. It is noteworthy that the highest DNA binding of PhIP was observed in the colon of RCN mice without 3-MC pretreatment (~1400 adducts/10^8 deoxyribonucleosides).[57] Lack of hepatic POR reduced PhIP-DNA adducts levels in the colon by 50%.

We also determined DNA adduct formation by PhIP *ex vivo* using hepatic microsomes from RCN mice with and without 3-MC pretreatment. P450-mediated formation of dG-C8-PhIP was stimulated in these microsomal incubations (**see Figure 7B**); DNA adduct levels were ~8-fold higher uising hepatic microsomes with active POR than with hepatic microsomes from RCN mice that lacked hepatic POR. Using α-naphthoflavone as an inhibitor most of the microsomal enzyme activity was attributed to Cyp1a1 and 1a2 (**see Figure 7B**); DNA adduct formation by PhIP was reduced by 60% in microsomal incubations with active POR. Enzyme activities of Cyp1a1/2 (measured as 3-cyano-7-ethoxycoumarin activity) correlated with PhIP-DNA adduct levels observed *ex vivo*.[57]

The lack of alteration in the livers of PhIP-treated RCN mice with and without 3MC pretreatment is puzzling as the mechanism by which DNA-binding species are generated by PhIP in the liver of RCN mice lacking POR is still unknown. However, it is clear that the process does not involve the generation of reactive species different from that formed in WT mice. *Cyp1a2*-null mice have also been used to study PhIP metabolism and carcinogenesis. One study showed that PhIP-DNA adduct levels were significantly lower in *Cyp1a2*-null mice relative to WT animals confirming the importance of Cyp1a2 in the bioactivation of PhIP *in vivo*.[116] However, another study found that PhIP induced liver carcinogenesis in neonatal *Cyp1a2*-null mice, indicating a mechanism independent of Cyp1a2 expression.[117] Our study in the RCN model show that oral administration of PhIP results in hepatic *N*-oxidation of PhIP and that *N*-OH-PhIP (and possibly *N*-acetoxy-PhIP after further hepatic *O*-acetylation) can be circulated via the blood to extrahepatic tissues where it can form dG-C8-

PhIP adducts in DNA. Further, our data and those of others suggest that PhIP may be metabolically activated in the liver mainly by a non-P450 pathway and that hepatic Cyp1a2 expression only provides a minor contribution to PhIP-DNA adduct levels in the liver. Elucidating the exact mechanism of DNA adduct formation by PhIP in the livers of RCN mice will require further investigation.

Other environmental pollutants studied in mouse models with liver-specific deletion of POR

Several mouse models have been developed in which the *Por* gene is deleted in a tissue-specific fashion.[35, 39, 50, 118-120] For our investigations we used the HRN and RCN mouse model as described above, but others have developed similar models with liver-specific deletion of POR.[39, 121, 122] These designated liver-*Por*-null mouse models have also been used to examine the metabolism, (geno)toxicity and carcinogenicity of environmental pollutants. Some of the results obtained in liver-*Por*-null mice are now summarized below. In some of these studies lung-*Por*-null [118] or proximal tubule-*Por*-null mice [123] have been used for comparison.

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is one of the most potent carcinogens identified in tobacco smoke and has been associated with lung cancer in humans [124]. IARC recently classified NNK as a human carcinogen (Group 1).[125] A variety of P450s including the CYP1A, CYP2A and CYP3A sub-families have been shown to catalyse α-hydroxylation of NNK.[23] This leads to the formation of a range of reactive intermediates like methyldiazonium ions that are capable of forming DNA adducts including O^6-methylguanine (O^6-mG).[23] For mutation analysis liver-*Por*-null mice were crossed with *gpt* delta mice and treated i.p. with 100 mg/kg bw of NNK for four consecutive days.[126] Whereas in the liver NNK-induced mutant frequencies (MFs) were similar to spontaneous liver MFs, they were ~3-fold higher in the lungs of NNK-treated mice than in controls.[126] Thus, P450-mediated activation of NNK is required to induce gene mutations in the liver. Further, hepatic P450s protect the lungs against NNK-induced mutagenesis as they are involved in the systemic clearance of NNK.[126] NNK carcinogenesis was studied both in liver-*Por*-null and lung-*Por*-null mice.[118] Lung tumour formation was induced with a single i.p. injection of 10 µmol NNK/mouse for liver-*Por*-null mice and 20 µmol NNK/mouse for lung-*Por*-null mice, and animals were sacrificed 2 months later. An increase in the formation of NNK-induced lung tumours was found in liver-*Por*-null mice relative to WT animals while tumour formation in

lung-*Por*-null mice was lower.[118] In lung-*Por*-null animals O^6-mG adduct levels were lower in lungs relative to WT and remained unchanged in the liver.[118] Similarly, adduct levels of O^6-mG were lower in the liver of liver-*Por*-null animals relative to WT while no alterations were observed in the lung.[118] Immunohistochemistry analysis of the lung tumours in lung-*Por*-null mice showed that they were positive for POR expression, indicating that the tumours in the lungs did not originate from *Por*-null cells.[118]

Pyrrolizidine alkaloid-containing plants are widely distributed around the world.[127] They are among the most common poisonous plants affecting livestock, wildlife and humans. Pyrrolizidine alkaloids are also of great concern due to their carcinogenicity. One representative of this class is monocrotaline (MCT) which can be found in plants of the genus *Crotalaria*. MCT is toxic in multiple organs of rats including liver, lung and kidney and hepatic P450s, such as the CYP3A, 2B and 2C sub-families, are involved in its metabolic activation.[128, 129] Liver-*Por*-null mice were treated with single i.p. injections of 300, 400 or 500 mg/kg bw of MCT and hepato- and nephrotoxicity were examined after 24 hours.[129] Histopathology revealed that a dose of 500 mg/kg bw of MCT caused severe liver injury and moderate kidney injury in WT mice while these histological abnormalities were absent in liver-*Por*-null mice.[129] These results indicated that hepatic P450s play an important role in the metabolic activation of MCT which subsequently contributes to both MCT-induced hepato- and nephrotoxicity.

Chloroform is a halogenated hydrocarbon which is linked to hepato- and nephrotoxicity in a number of species through P450-dependent metabolism.[130] CYP2E1 has been shown to be a key P450 isoenzyme in the metabolic activation of chloroform to phosgene, which can react with cellular macromolecules to initiate toxicity.[131] In order to investigate the dependence of chloroform-induced renal toxicity on hepatic P450-mediated metabolism, liver-*Por*-null mice were treated with single oral doses of 150 or 300 mg/kg bw of chloroform for 24 hours.[132] Severe signs of nephrotoxicity were found in liver-*Por*-null mice while only mild histological lesions were observed in WT animals.[133] These results indicated that loss of P450-mediated bioactivation of chloroform in the liver did not protect against chloroform-induced nephrotoxicity. P450 expression in kidney plays a crucial role in the toxicity of chloroform in the kidney.[130, 132] In further studies proximal tubule-*Por*-null mice were employed to investigate chloroform-induced nephrotoxicity. These mice were treated with a single oral dose of 200 mk/kg bw of chloroform and renal injury was assessed 24 hours later.[123] The severity of renal toxicity was less in proximal tubule-*Por*-null mice

than in WT animals, confirming results obtained in the liver-*Por*-null mice that local P450-dependent bioactivation of chloroform is important for chloroform-induced nephrotoxicity.[123]

Azoxymethane (methyl-methylimino-oxidoazanium [AOM]) is a colon carcinogen in rodents.[134, 135] It requires metabolic activation by P450s, primarily CYP2E1, and hydroxylation of AOM leads to the formation of methylazoxymethanol (MAM). MAM subsequently decomposes to formaldehyde and a highly reactive methyldiazonium ion capable of forming DNA adducts, including O^6-methylguanine (O^6-mG) and *N*-7-methylguanine (*N*-7-mG). In order to study hepatic and extrahepatic metabolism of AOM, liver-*Por*-null mice were treated with a single injection of AOM at 14 mg/kg bw (s.c.) for 6 hours.[136] To differentiate between intestinal and extra-gut metabolism this study also employed intestinal epithelium-*Por*-null mice. In liver-*Por*-null mice the adduct levels of O^6-mG and N^7-mG in the liver were ~60% lower compared to WT mice, while in the intestine (i.e. duodenum, jejunum, ileum, proximale colon, and distal colon) O^6-mG and *N*-7-mG levels increased up to 2-fold.[136] In intestinal epithelium-*Por*-null mice O^6-mG and *N*-7-mG levels did not change in the liver relative to WT mice but adduct levels were often lower in the intestine; these changes were dependent on the section of the intestine analysed.[136] The tissue-specific loss of Por in liver-*Por*-null mice and intestinal epithelium-*Por*-null mice on microsomal AOM metabolic activity correlated with the rate of formaldehyde formation *in vitro*.[136] AOM induces colonic aberrant crypt foci (ACF) which are preneoplastic lesions. To study ACF formation liver-*Por*-null mice and intestinal epithelium-*Por*-null mice were treated once weekly for 3 weeks with 7.5 mg/kg bw of AOM and animals were sacrificed 6 weeks later.[136] The incidence of ACF did not change among the strains and the authors concluded that these results were largely consistent with the DNA adduct data indicating that other factors besides hepatic or intestinal P450 enzymes contribute to AOM-induced DNA damage and subsequent ACF formation in the colon.[136]

Creation of the new mouse lines refining the HRN and RCN models

Cytochrome b_5 is a microsomal haemoprotein involved in a number of cellular reactions including lipid desaturation and methaemoglobin reduction, as well as providing electrons (from cytochrome b_5 reductase) to cytochrome P450s.[137] For more than four decades, the role of cytochrome b_5 in P450 catalysis has been controversial, and based entirely on *in vitro* data, which showed that cytochrome b_5 could inhibit or stimulate P450 activity dependent on a number of variables including P450 isozyme, substrate and cytochrome b_5 concentration.[138] In order to define the role of cytochrome b_5 in P450 function *in vivo*, a mouse line has been

generated with a conditional hepatic deletion of cytochrome b_5 (HBN, Hepatic cytochrome b_5 Null). HBN mice are overtly normal with no obviously altered phenotype and show reduced NADPH-dependent metabolism of a number of substrates, including drugs such as midazolam, metoprolol and tolbutamide, demonstrating for the first time the effects of cytochrome b_5 *in vivo*.[139] One potential confounding issue previously identified with the HRN line is the presence of low but detectable (~5-10%) residual mono-oxygenase activity, even in the absence of POR.[35] To determine whether this phenomenon is due to cytochrome b_5/cytochrome b_5 reductase acting as sole electron donors to the P450 catalytic cycle, HRN mice were crossed with the HBN line to create a double conditional mutant, HBRN (Hepatic cytochrome b_5 /P450 Reductase Null), in which both enzymes are deleted specifically in the liver.[140]

The HBRN phenotype has been found to be similar to that of the HRN mouse, having enlarged, fatty livers with significantly increased hepatic P450 expression, yet HBRN mice also breed and developed normally. *In vitro* metabolic studies with hepatic microsomes from WT, HRN, HBN and HBRN mice showed that the deletion of POR alone resulted in a greater reduction in metabolism of a range of probe substrates (e.g. benzyloxyresorufin, methoxyresourufin) or drugs (e.g. midazolam, metoprolol and tolbutamide) than cytochrome b_5 alone. However, removal of both enzymes had an additive effect for most substrates, leading to near-complete abolition of mono-oxygenase activity in HBRN mice, although for metoprolol and tolbutamide loss of POR alone was sufficient to abolish metabolism, indicating a lack of dependency on cytochrome b_5 for these drugs.[140] The finding of cytochrome b_5 involvement *in vitro* was also demonstrated *in vivo*, with midazolam pharmacokinetic parameters altered to a greater extent in HBRN than in either HRN or HBN alone. Further *in vitro* work with WT and HRN microsomes and titration of NADH or NADPH as co-factors provided strong evidence that in the absence of POR, cytochrome b_5/cytochrome b_5 reductase are capable of supplying electrons for P450 catalytic function.[140]

A subsequent variant of the RCN mouse has been made, where Cre recombinase has been cloned into the mouse *Cyp1a1* locus, replacing one copy of the *Cyp1a1* gene. This model, termed Endogenous Reductase Locus (ERL), has the advantage that deletion in liver or liver and gut is inducible by differential dosing of a single agent (i.e. 3MC) at significantly lower doses than used in the RCN model, allowing the relative contribution of liver and gut metabolism to drug oral bioavailability to be determined more easily[141]

Concluding remarks

Metabolic activation of environmental pollutants is often a critical determinant in chemical carcinogenesis and the HRN and RCN mouse models provide a powerful tool to assess to role of hepatic *versus* extrahepatic P450s in carcinogen metabolism as a whole. These models developed at the University of Dundee over the last 10 years have proved their applicability in drug disposition and in the present review we have shown that both models have also been successfully used to study carcinogen metabolism. These studies in the HRN and RCN mice show that extrapolation from *in vitro* data to *in vivo* pharmacokinetics is not always possible confirming the need for these mouse models. Some of the limitations observed in the HRN model have been overcome with the RCN and ERL models which even allow the relative contribution of P450-mediated metabolism in both liver and gut to be studied. The HBN and HBRN mouse models can permit study of the potential role of cytochrome b_5 in hepatic P450-mediated metabolism. The role of cytochrome b_5 in carcinogen metabolism is still quite enigmatic, but these models, for the first time, will allow investigating the contribution of this enzyme to P450-mediated carcinogen metabolism. In conlision, the use of the HRN, RCN, ERL, HBN and HBRN models, alone and in combination, provide a powerful approach to study the metabolism, toxicity and carcinogenicity of environmental pollutants.

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References

1. F. P. Guengerich, *Chem. Res. Toxicol.*, 2001, **14**, 611-650.
2. F. P. Guengerich, *Chem. Res. Toxicol.*, 2008, **21**, 70-83.
3. S. Rendic and F. P. Guengerich, *Chem. Res. Toxicol.*, 2012, **25**, 1316-1383.
4. D. W. Nebert and T. P. Dalton, *Nature Rev.*, 2006, **6**, 947-960.
5. A. Luch, *Nature Rev.*, 2005, **5**, 113-125.
6. D. H. Phillips, *Mutat. Res.*, 2005, **577**, 284-292.
7. F. J. Gonzalez and S. Kimura, *Mutat. Res.*, 2001, **477**, 79-87.
8. C. J. Henderson, D. M. Otto, A. W. McLaren, D. Carrie and C. R. Wolf, *Drug Metab. Rev.*, 2003, **35**, 385-392.
9. D. W. Nebert, Z. Shi, M. Galvez-Peralta, S. Uno and N. Dragin, *Mol.Pharmacol.*, 2013, **84**, 304-313.
10. C. J. Henderson, G. J. Pass and C. R. Wolf, *Toxicol. Lett.*, 2006, **162**, 111-117.
11. D. S. Riddick, X. Ding, C. R. Wolf, T. D. Porter, A. V. Pandey, Q. Y. Zhang, J. Gu, R. D. Finn, S. Ronseaux, L. A. McLaughlin, C. J. Henderson, L. Zou and C. E. Fluck, *Drug Metab. Disp.*, 2013, **41**, 12-23.
12. T. D. Porter, *J. Biochem. Mol. Toxicol.*, 2002, **16**, 311-316.
13. V. Kotrbova, B. Mrazova, M. Moserova, V. Martinek, P. Hodek, J. Hudecek, E. Frei and M. Stiborova, *Biochem. Pharmacol.*, 2011, **82**, 669-680.
14. M. Stiborova, R. Indra, M. Moserova, V. Cerna, M. Rupertova, V. Martinek, T. Eckschlager, R. Kizek and E. Frei, *Chem. Res. Toxicol.*, 2012, **25**, 1075-1085.
15. M. Stiborova, M. Moserova, V. Cerna, R. Indra, M. Dracinsky, M. Sulc, C. J. Henderson, C. R. Wolf, H. H. Schmeiser, D. H. Phillips, E. Frei and V. M. Arlt, *Toxicology*, 2014, **318**, 1-12.
16. L. C. Wienkers and T. G. Heath, *Nature Rev. Drug Dis.*, 2005, **4**, 825-833.
17. F. P. Guengerich and D. C. Liebler, *Crit. Rev. Toxicol.*, 1985, **14**, 259-307.
18. S. Rendic and F. J. Di Carlo, *Drug Metab. Rev.*, 1997, **29**, 413-580.
19. F. P. Guengerich and T. Shimada, *Chem. Res. Toxicol.*, 1991, **4**, 391-407.
20. W. M. Baird, L. A. Hooven and B. Mahadevan, *Environ. Mol. Mutagen.*, 2005, **45**, 106-114.
21. Y. Tsuneoka, T. P. Dalton, M. L. Miller, C. D. Clay, H. G. Shertzer, G. Talaska, M. Medvedovic and D. W. Nebert, *J. Natl. Cancer Inst.*, 2003, **95**, 1227-1237.
22. C. Cheung, X. Ma, K. W. Krausz, S. Kimura, L. Feigenbaum, T. P. Dalton, D. W. Nebert, J. R. Idle and F. J. Gonzalez, *Chem. Res. Toxicol.*, 2005, **18**, 1471-1478.
23. S. S. Hecht, *Chem. Res. Toxicol.*, 1998, **11**, 559-603.
24. V. Dohnal, Q. Wu and K. Kuca, *Arch. Toxicol.*, 2014, **88**, 1635-1644.
25. H. Hamouchene, V. M. Arlt, I. Giddings and D. H. Phillips, *BMC Genomics*, 2011, **12**, 333.
26. V. M. Arlt, M. Stiborova, C. J. Henderson, M. Thiemann, E. Frei, D. Aimova, R. Singh, G. Gamboa da Costa, O. J. Schmitz, P. B. Farmer, C. R. Wolf and D. H. Phillips, *Carcinogenesis*, 2008, **29**, 656-665.
27. M. Stiborova, E. Frei, V. M. Arlt and H. H. Schmeiser, *Xenobiotica*, 2014, **44**, 135-145.
28. M. Stiborova, V. Martinek, E. Frei, V. M. Arlt and H. H. Schmeiser, *Curr. Drug Metab.*, 2013, **14**, 695-705.
29. B. H. Koller and O. Smithies, *Annu. Rev. Immunol.*, 1992, **10**, 705-730.
30. M. Harvey, M. J. McArthur, C. A. Montgomery, Jr., A. Bradley and L. A. Donehower, *FASEB J.*, 1993, **7**, 938-943.
31. C. J. Henderson, N. Scheer and C. R. Wolf, *Expert Rev. Clin. Pharmacol.*, 2009, **2**, 105-109.

32. C. J. Henderson, C. R. Wolf and N. Scheer, in *Handbook of Drug Metabolism*, eds. P. G. Pearson and L. C. Wienkers, Informa Healthcare, 2008, pp. 637-658.
33. N. Dragin, Z. Shi, R. Madan, C. L. Karp, M. A. Sartor, C. Chen, F. J. Gonzalez and D. W. Nebert, *Mol. Pharmacol.*, 2008, **73**, 1844-1856.
34. Y. Le and B. Sauer, *Methods in Molecular Biology (Clifton, N.J*, 2000, **136**, 477-485.
35. C. J. Henderson, D. M. Otto, D. Carrie, M. A. Magnuson, A. W. McLaren, I. Rosewell and C. R. Wolf, *J. Biol. Chem.*, 2003, **278**, 13480-13486.
36. D. M. Otto, C. J. Henderson, D. Carrie, M. Davey, T. E. Gundersen, R. Blomhoff, R. H. Adams, C. Tickle and C. R. Wolf, *Mol. Cell. Biol.*, 2003, **23**, 6103-6116.
37. V. Ribes, D. M. Otto, L. Dickmann, K. Schmidt, B. Schuhbaur, C. Henderson, R. Blomhoff, C. R. Wolf, C. Tickle and P. Dolle, *Dev. Biol.*, 2007, **303**, 66-81.
38. C. Postic, M. Shiota, K. D. Niswender, T. L. Jetton, Y. Chen, J. M. Moates, K. D. Shelton, J. Lindner, A. D. Cherrington and M. A. Magnuson, *J. Biol. Chem.*, 1999, **274**, 305-315.
39. J. Gu, Y. Weng, Q. Y. Zhang, H. Cui, M. Behr, L. Wu, W. Yang, L. Zhang and X. Ding, *J. Biol. Chem.*, 2003, **278**, 25895-25901.
40. K. Pickup, A. Gavin, H. B. Jones, E. Karlsson, C. Page, K. Ratcliffe, S. Sarda, T. Schulz-Utermoehl and I. Wilson, *Xenobiotica*, 2012, **42**, 195-205.
41. K. Pickup, J. Wills, A. Rodrigues, H. B. Jones, C. Page, S. Martin, S. Sarda and I. Wilson, *Xenobiotica*, 2014, **44**, 164-173.
42. C. Kunne, M. de Graaff, S. Duijst, D. R. de Waart, R. P. Oude Elferink and C. C. Paulusma, *Lab. Invest.*, 2014 [Epub ahead of print].
43. C. Kunne, A. Acco, S. Hohenester, S. Duijst, D. R. de Waart, A. Zamanbin and R. P. Oude Elferink, *Hepatology*, 2013, **57**, 1509-1517.
44. C. Kunne, A. Acco, S. Duijst, D. R. de Waart, C. C. Paulusma, I. Gaemers and R. P. Oude Elferink, *Biochim. Biophys. Acta*, 2014, **1842**, 739-746.
45. J. W. Boggs, C. E. Hop, E. McNamara, Y. Deng, K. Messick, K. West and E. F. Choo, *Mol. Pharm.*, 2014.
46. M. Stiborova, V. M. Arlt, C. J. Henderson, C. R. Wolf, V. Kotrbova, M. Moserova, J. Hudecek, D. H. Phillips and E. Frei, *Toxicol. Appl. Pharmacol.*, 2008, **226**, 318-327.
47. M. Stiborova, V. Cerna, M. Moserova, V. M. Arlt and E. Frei, *Neuro. Endocrinol. Lett.*, 2013, **34 Suppl 2**, 43-54.
48. S. J. Campbell, F. Carlotti, P. A. Hall, A. J. Clark and C. R. Wolf, *J. Cell Sci.*, 1996, **109 (Pt 11)**, 2619-2625.
49. S. J. Campbell, C. J. Henderson, D. C. Anthony, D. Davidson, A. J. Clark and C. R. Wolf, *J. Biol. Chem.*, 2005, **280**, 5828-5835.
50. R. D. Finn, A. W. McLaren, D. Carrie, C. J. Henderson and C. R. Wolf, *J Pharmacol. Exp. Ther.*, 2007, **322**, 40-47.
51. R. D. Finn, C. J. Henderson, C. L. Scott and C. R. Wolf, .*Biochem. J.*, 2009, **417**, 43-54.
52. V. M. Arlt, M. C. Poirier, S. E. Sykes, K. John, M. Moserova, M. Stiborova, C. R. Wolf, C. J. Henderson and D. H. Phillips, *Toxicol. Lett.*, 2012, **213**, 160-166.
53. K. Levova, M. Moserova, V. Kotrbova, M. Sulc, C. J. Henderson, C. R. Wolf, D. H. Phillips, E. Frei, H. H. Schmeiser, J. Mares, V. M. Arlt and M. Stiborova, *Toxicol. Sci.*, 2011, **121**, 43-56.
54. V. M. Arlt, M. Stiborova, C. J. Henderson, M. R. Osborne, C. A. Bieler, E. Frei, V. Martinek, B. Sopko, C. R. Wolf, H. H. Schmeiser and D. H. Phillips, *Cancer Res.*, 2005, **65**, 2644-2652.
55. V. M. Arlt, C. J. Henderson, C. R. Wolf, H. H. Schmeiser, D. H. Phillips and M. Stiborova, *Cancer Lett.*, 2006, **234**, 220-231.

56. M. Stiborova, T. Cechova, L. Borek-Dohalska, M. Moserova, E. Frei, H. H. Schmeiser, J. Paca and V. M. Arlt, *Neuro. Endocrinol. Lett.*, 2012, **33 Suppl 3**, 8-15.
57. V. M. Arlt, R. Singh, M. Stiborova, G. Gamboa da Costa, E. Frei, J. D. Evans, P. B. Farmer, C. R. Wolf, C. J. Henderson and D. H. Phillips, *Drug Metab. Dispos.*, 2011, **39**, 2169-2173.
58. IARC, *IARC Monogr. Eval. Carcinog. Risk Hum.*, 2010, **92**.
59. D. H. Phillips, *Mutat. Res.*, 1999, **443**, 139-147.
60. A. Luch and W. M. Baird, in *The Carcinogenic Effects of Polycyclic Aromatic Hydrocarbons*, ed. A. Luch, Imperial College Press, London, 2005, pp. 19-96.
61. D. H. Phillips, in *The Carcinogenic Effects of Polycyclic Aromatic Hydrocarbons*, ed. A. Luch, Imperial College Press, London, 2005, pp. 137-170.
62. S. Uno, T. P. Dalton, H. G. Shertzer, M. B. Genter, D. Warshawsky, G. Talaska and D. W. Nebert, *Biochem. Biophys. Res. Comm.*, 2001, **289**, 1049-1056.
63. S. Uno, T. P. Dalton, S. Derkenne, C. P. Curran, M. L. Miller, H. G. Shertzer and D. W. Nebert, *Mol. Pharmacol.*, 2004, **65**, 1225-1237.
64. S. Uno, T. P. Dalton, N. Dragin, C. P. Curran, S. Derkenne, M. L. Miller, H. G. Shertzer, F. J. Gonzalez and D. W. Nebert, *Mol. Pharmacol.*, 2006, **69**, 1103-1114.
65. D. W. Nebert, *Critical reviews in toxicology*, 1989, **20**, 153-174.
66. Y. Shimizu, Y. Nakatsuru, M. Ichinose, Y. Takahashi, H. Kume, J. Mimura, Y. Fujii-Kuriyama and T. Ishikawa, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 779-782.
67. S. R. Kondraganti, P. Fernandez-Salguero, F. J. Gonzalez, K. S. Ramos, W. Jiang and B. Moorthy, *Int. J. Cancer*, 2003, **103**, 5-11.
68. C. Sagredo, S. Ovrebo, A. Haugen, Y. Fujii-Kuriyama, R. Baera, I. V. Botnen and S. Mollerup, *Toxicol. Lett.*, 2006, **167**, 173-182.
69. C. Sagredo, S. Mollerup, K. J. Cole, D. H. Phillips, H. Uppstad and S. Ovrebo, *Chem. Res. Toxicol.*, 2009, **22**, 584-591.
70. V. M. Arlt, M. Stiborova and H. H. Schmeiser, *Mutagenesis*, 2002, **17**, 265-277.
71. J. L. Vanherweghem, M. Depierreux, C. Tielemans, D. Abramowicz, M. Dratwa, M. Jadoul, C. Richard, D. Vandervelde, D. Verbeelen, R. Vanhaelen-Fastre and et al., *Lancet*, 1993, **341**, 387-391.
72. H. H. Schmeiser, M. Stiborova and V. M. Arlt, *Curr. Opin. Drug Dis. Develop.*, 2009, **12**, 141-148.
73. M. R. Gokmen, J. P. Cosyns, V. M. Arlt, M. Stiborova, D. H. Phillips, H. H. Schmeiser, M. S. Simmonds, H. T. Cook, J. L. Vanherweghem, J. L. Nortier and G. M. Lord, *Ann. Intern. Med.*, 2013, **158**, 469-477.
74. J. L. Nortier, M. C. Martinez, H. H. Schmeiser, V. M. Arlt, C. A. Bieler, M. Petein, M. F. Depierreux, L. De Pauw, D. Abramowicz, P. Vereerstraeten and J. L. Vanherweghem, *N. Engl. J. Med.*, 2000, **342**, 1686-1692.
75. IARC, *IARC Monogr. Eval. Carcinog. Risk Hum.*, 2012, **100A**.
76. V. M. Arlt, M. Stiborova, J. vom Brocke, M. L. Simoes, G. M. Lord, J. L. Nortier, M. Hollstein, D. H. Phillips and H. H. Schmeiser, *Carcinogenesis*, 2007, **28**, 2253-2261.
77. A. P. Grollman, S. Shibutani, M. Moriya, F. Miller, L. Wu, U. Moll, N. Suzuki, A. Fernandes, T. Rosenquist, Z. Medverec, K. Jakovina, B. Brdar, N. Slade, R. J. Turesky, A. K. Goodenough, R. Rieger, M. Vukelic and B. Jelakovic, *Proc. Natl. Acad. Sci. USA*, 2007, **104**, 12129-12134.
78. H. H. Schmeiser, J. E. Kucab, V. M. Arlt, D. H. Phillips, M. Hollstein, G. Gluhovschi, C. Gluhovschi, M. Modilca, L. Daminescu, L. Petrica and S. Velciov, *Environ. Mol. Mutagen.*, 2012, **53**, 636-641.
79. V. M. Arlt, D. Ferluga, M. Stiborova, A. Pfohl-Leszkowicz, M. Vukelic, S. Ceovic, H. H. Schmeiser and J. P. Cosyns, *Int. J. Cancer*, 2002, **101**, 500-502.

80. H. H. Schmeiser, J. L. Nortier, R. Singh, G. G. da Costa, J. Sennesael, E. Cassuto-Viguier, D. Ambrosetti, S. Rorive, A. Pozdzik, D. H. Phillips, M. Stiborova and V. M. Arlt, *Int. J. Cancer*, 2014, **135**, 502-507.
81. G. M. Lord, M. Hollstein, V. M. Arlt, C. Roufosse, C. D. Pusey, T. Cook and H. H. Schmeiser, *Am. J. Kidney Dis.*, 2004, **43**, e11-17.
82. T. Nedelko, V. M. Arlt, D. H. Phillips and M. Hollstein, *Int. J. Cancer*, 2009, **124**, 987-990.
83. J. E. Kucab, D. H. Phillips and V. M. Arlt, *Febs J.*, 2010, **277**, 2567-2583.
84. S. L. Poon, S. T. Pang, J. R. McPherson, W. Yu, K. K. Huang, P. Guan, W. H. Weng, E. Y. Siew, Y. Liu, H. L. Heng, S. C. Chong, A. Gan, S. T. Tay, W. K. Lim, I. Cutcutache, D. Huang, L. D. Ler, M. L. Nairismagi, M. H. Lee, Y. H. Chang, K. J. Yu, W. Chan-On, B. K. Li, Y. F. Yuan, C. N. Qian, K. F. Ng, C. F. Wu, C. L. Hsu, R. M. Bunte, M. R. Stratton, P. A. Futreal, W. K. Sung, C. K. Chuang, C. K. Ong, S. G. Rozen, P. Tan and B. T. Teh, *Sci. Trans. Med.*, 2013, **5**, 197ra101.
85. M. L. Hoang, C. H. Chen, V. S. Sidorenko, J. He, K. G. Dickman, B. H. Yun, M. Moriya, N. Niknafs, C. Douville, R. Karchin, R. J. Turesky, Y. S. Pu, B. Vogelstein, N. Papadopoulos, A. P. Grollman, K. W. Kinzler and T. A. Rosenquist, *Sci. Trans. Med.*, 2013, **5**, 197ra102.
86. M. Olivier, A. Weninger, M. Ardin, H. Huskova, X. Castells, M. P. Vallee, J. McKay, T. Nedelko, K. R. Muehlbauer, H. Marusawa, J. Alexander, L. Hazelwood, G. Byrnes, M. Hollstein and J. Zavadil, *Sci. Rep.*, 2014, **4**, 4482.
87. V. M. Arlt, K. Levova, F. Barta, Z. Shi, J. D. Evans, E. Frei, H. H. Schmeiser, D. W. Nebert, D. H. Phillips and M. Stiborova, *Chem. Res. Toxicol.*, 2011, **24**, 1710-1719.
88. M. Stiborova, K. Levova, F. Barta, Z. Shi, E. Frei, H. H. Schmeiser, D. W. Nebert, D. H. Phillips and V. M. Arlt, *Toxicol. Sci.*, 2012, **125**, 345-358.
89. Y. Xiao, M. Ge, X. Xue, C. Wang, H. Wang, X. Wu, L. Li, L. Liu, X. Qi, Y. Zhang, Y. Li, H. Luo, T. Xie, J. Gu and J. Ren, *Kid. Int.*, 2008, **73**, 1231-1239.
90. V. M. Arlt, *Mutagenesis*, 2005, **20**, 399-410.
91. IARC, *IARC Monogr. Eval. Carcinog. Risk Hum.*, 2013, **105**.
92. E. Nagy, M. Zeisig, K. Kawamura, Y. Hisamatsu, A. Sugeta, S. Adachi and L. Moller, *Carcinogenesis*, 2005, **26**, 1821-1828.
93. A. Seidel, D. Dahmann, H. Krekeler and J. Jacob, *Int. J. Hyg. Environ. Health*, 2002, **204**, 333-338.
94. V. M. Arlt, L. Zhan, H. H. Schmeiser, M. Honma, M. Hayashi, D. H. Phillips and T. Suzuki, *Environ. Mol. Mutagen.*, 2004, **43**, 186-195.
95. V. M. Arlt, J. Gingerich, H. H. Schmeiser, D. H. Phillips, G. R. Douglas and P. A. White, *Mutagenesis*, 2008, **23**, 483-490.
96. V. M. Arlt, C. A. Bieler, W. Mier, M. Wiessler and H. H. Schmeiser, *Int. J. Cancer*, 2001, **93**, 450-454.
97. V. M. Arlt, H. H. Schmeiser, M. R. Osborne, M. Kawanishi, T. Kanno, T. Yagi, D. H. Phillips and T. Takamura-Enya, *Int. J. Cancer*, 2006, **118**, 2139-2146.
98. G. Gamboa da Costa, R. Singh, V. M. Arlt, A. Mirza, M. Richards, T. Takamura-Enya, H. H. Schmeiser, P. B. Farmer and D. H. Phillips, *Chem. Res. Toxicol.*, 2009, **22**, 1860-1868.
99. J. vom Brocke, A. Krais, C. Whibley, M. C. Hollstein and H. H. Schmeiser, *Mutagenesis*, 2009, **24**, 17-23.
100. M. Stiborova, V. Martinek, M. Svobodova, J. Sistkova, Z. Dvorak, J. Ulrichova, V. Simanek, E. Frei, H. H. Schmeiser, D. H. Phillips and V. M. Arlt, *Chem. Res. Toxicol.*, 2010, **23**, 1192-1201.

101. M. Stiborova, E. Frei, H. H. Schmeiser, V. M. Arlt and V. Martinek, *Int. J. Mol. Sci.*, 2014, **15**, 10271-10295.
102. V. M. Arlt, H. Glatt, E. Muckel, U. Pabel, B. L. Sorg, H. H. Schmeiser and D. H. Phillips, *Carcinogenesis*, 2002, **23**, 1937-1945.
103. V. M. Arlt, H. Glatt, E. Muckel, U. Pabel, B. L. Sorg, A. Seidel, H. Frank, H. H. Schmeiser and D. H. Phillips, *Int. J. Cancer*, 2003, **105**, 583-592.
104. V. M. Arlt, M. Stiborova, A. Hewer, H. H. Schmeiser and D. H. Phillips, *Cancer Res.*, 2003, **63**, 2752-2761.
105. J. Borlak, T. Hansen, Y. Zhi-Xin, H. C. Sikka, S. Kumar, S. Schmidbauer, H. Frank, J. Jacob and A. Seidel, *Polycyclic. Aromatic. Compound.*, 2000, **21**, 73-86.
106. V. M. Arlt, B. L. Sorg, M. Osborne, A. Hewer, A. Seidel, H. H. Schmeiser and D. H. Phillips, *Biochem. Biophys. Res. Comm.*, 2003, **300**, 107-114.
107. V. M. Arlt, A. Hewer, B. L. Sorg, H. H. Schmeiser, D. H. Phillips and M. Stiborova, *Chem. Res. Toxicol.*, 2004, **17**, 1092-1101.
108. M. Stiborova, H. Dracinska, J. Hajkova, P. Kaderabkova, E. Frei, H. H. Schmeiser, P. Soucek, D. H. Phillips and V. M. Arlt, *Drug Metab. Dispos.*, 2006, **34**, 1398-1405.
109. M. Stiborova, H. Dracinska, M. Martinkova, J. Mizerovska, J. Hudecek, P. Hodek, J. Liberda, E. Frei, H. H. Schmeiser, D. H. Phillips and V. M. Arlt, *Mutat. Res.*, 2009, **676**, 93-101.
110. R. J. Turesky and L. Le Marchand, *Chem. Res. Toxicol.*, 2011, **24**, 1169-1214.
111. IARC, *IARC Monogr. Eval. Carcinog. Risk Hum.*, 1993, **56**.
112. K. H. Dingley, K. D. Curtis, S. Nowell, J. S. Felton, N. P. Lang and K. W. Turteltaub, *Cancer Epidemiol. Biomarkers Prev.*, 1999, **8**, 507-512.
113. T. Shimada, C. L. Hayes, H. Yamazaki, S. Amin, S. S. Hecht, F. P. Guengerich and T. R. Sutter, *Cancer Res.*, 1996, **56**, 2979-2984.
114. H. A. Schut and E. G. Snyderwine, *Carcinogenesis*, 1999, **20**, 353-368.
115. R. Singh, V. M. Arlt, C. J. Henderson, D. H. Phillips, P. B. Farmer and G. Gamboa da Costa, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, 2010, **878**, 2155-2162.
116. E. G. Snyderwine, M. Yu, H. A. Schut, L. Knight-Jones and S. Kimura, *Food Chem. Toxicol.*, 2002, **40**, 1529-1533.
117. S. Kimura, M. Kawabe, A. Yu, H. Morishima, P. Fernandez-Salguero, G. J. Hammons, J. M. Ward, F. F. Kadlubar and F. J. Gonzalez, *Carcinogenesis*, 2003, **24**, 583-587.
118. Y. Weng, C. Fang, R. J. Turesky, M. Behr, L. S. Kaminsky and X. Ding, *Cancer Res.*, 2007, **67**, 7825-7832.
119. C. Fang, J. Gu, F. Xie, M. Behr, W. Yang, E. D. Abel and X. Ding, *Drug Metab. Dispos.*, 2008, **36**, 1722-1728.
120. Q. Y. Zhang, C. Fang, J. Zhang, D. Dunbar, L. Kaminsky and X. Ding, *Drug Metab. Dispos.*, 2009, **37**, 651-657.
121. Y. Weng, C. C. DiRusso, A. A. Reilly, P. N. Black and X. Ding, *J. Biol. Chem.*, 2005, **280**, 31686-31698.
122. J. Gu, C. S. Chen, Y. Wei, C. Fang, F. Xie, K. Kannan, W. Yang, D. J. Waxman and X. Ding, *J. Pharmacol. Exp. Ther.*, 2007, **321**, 9-17.
123. S. Liu, Y. Yao, S. Lu, K. Aldous, X. Ding, C. Mei and J. Gu, *Toxicol. Appl. Pharmacol.*, 2013, **272**, 230-237.
124. S. S. Hecht, *Int. J. Cancer*, 2012, **131**, 2724-2732.
125. IARC, *IARC Monogr. Eval. Carcinog. Risk Hum.*, 2012, **100E**.
126. Y. Luan, G. Xing, X. Qi, M. Wu, C. Li, J. Yao, L. Gong, T. Nohmi, J. Gu, W. Zhou, S. Zheng and J. Ren, *Arch. Toxicol.*, 2012, **86**, 1753-1761.
127. P. P. Fu, Q. Xia, G. Lin and M. W. Chou, *Drug Metab. Rev.*, 2004, **36**, 1-55.

128. D. W. Wilson, H. J. Segall, L. C. Pan, M. W. Lame, J. E. Estep and D. Morin, *Crit. Rev. Toxicol.*, 1992, **22**, 307-325.
129. J. Yao, C. G. Li, L. K. Gong, C. C. Feng, C. Z. Li, M. Gao, Y. Luan, X. M. Qi and J. Ren, *Acta Pharmacol. Sin.*, 2014, **35**, 292-300.
130. R. V. Branchflower, D. S. Nunn, R. J. Highet, J. H. Smith, J. B. Hook and L. R. Pohl, *Toxicol. Appl. Pharmacol.*, 1984, **72**, 159-168.
131. A. A. Constan, C. S. Sprankle, J. M. Peters, G. L. Kedderis, J. I. Everitt, B. A. Wong, F. L. Gonzalez and B. E. Butterworth, *Toxicol. Appl. Pharmacol.*, 1999, **160**, 120-126.
132. C. Fang, M. Behr, F. Xie, S. Lu, M. Doret, H. Luo, W. Yang, K. Aldous, X. Ding and J. Gu, *Toxicol. Appl. Pharmacol.*, 2008, **227**, 48-55.
133. F. D. Debelle, J. L. Vanherweghem and J. L. Nortier, *Kidney Int.*, 2008, **74**, 158-169.
134. A. Papanikolaou, Q. S. Wang, D. A. Delker and D. W. Rosenberg, *Cancer Lett.*, 1998, **130**, 29-34.
135. A. Bissahoyo, R. S. Pearsall, K. Hanlon, V. Amann, D. Hicks, V. L. Godfrey and D. W. Threadgill, *Toxicol. Sci.*, 2005, **88**, 340-345.
136. V. Megaraj, X. Ding, C. Fang, N. Kovalchuk, Y. Zhu and Q. Y. Zhang, *Chem. Res. Toxicol.*, 2014, **27**, 656-662.
137. J. B. Schenkman and I. Jansson, *Pharmacol. Ther.*, 2003, **97**, 139-152.
138. H. Zhang, S. C. Im and L. Waskell, *J. Biol. Chem.*, 2007, **282**, 29766-29776.
139. R. D. Finn, L. A. McLaughlin, S. Ronseaux, I. Rosewell, J. B. Houston, C. J. Henderson and C. R. Wolf, *J. Biol. Chem.*, 2008, **283**, 31385-31393.
140. C. J. Henderson, L. A. McLaughlin and C. R. Wolf, *Mol. Pharmacol.*, 2013, **83**, 1209-1217.
141. C. J. Henderson, L. A. McLaughlin, M. Osuna-Cabello, A. W. McLaren and C. R. Wolf, *Biochem. J.*, **submitted**.

Legends to Figures

Figure 1

Major pathways in the metabolic activation and DNA adduct formation of BaP (A), AAI (B), 3-NBA (C), 3-ABA (D) and PhIP (E). R = $–SO_3H$; R = $–C(O)CH_3$.

Figure 2

Creation of the HRN (A) and RCN (B) mice. (A) The Cre^{ALB} transgene is introduced into a mouse line which both *Por* alleles are flanked by *loxP* sites ("floxed"). The resultant expression of Cre recombinase only in hepatocytes leads to excision and inactivation of POR exclusively in the liver, whereas all other cells and tissues retain normal POR function.[35] (B) Mice in which the *Por* alleles are flanked by *loxP* sites ("floxed") were crossed with a nontargeted transgenic mouse line in which an 8.5-kilobase fragment of the rat *CYP1A1* promoter was used to drive the expression of Cre recombinase (Cre^{CYP1A1}) [50]. Animals from the resulting $Por^{lox/lox}/Cre^{CYP1A1}$ line, in the absence of a chemical inducer, were phenotypically normal and indistinguishable from wild-type animals. There was also no excision of the *Por* gene in any tissue, demonstrating the absence of any background Cre recombinase activity.

Figure 3

DNA adduct formation induced by BaP in the HRN model *in vivo* (A) and *in vitro* (B). (A) HRN and WT mice were treated (i.p.) with a single dose of 125 mg/kg bw for 24 hours.[26] DNA adduct formation was determined using the nuclease P1-enrichment version of the ^{32}P-postlabelling method. Values are given as mean ± SD (n = 3); each DNA sample determined by two post-labelled analyses. Comparison was performed by t-test analysis: $^{*}P<0.01$, $^{\#}P<0.05$, different from WT. (B) DNA adduct formation by BaP activated with hepatic microsomes of untreated HRN and WT mice.[26] Values are given as mean ± range (n = 2); each DNA sample determined by two post-labelled analyses. α-NF, α-naphthoflavone.

Figure 4

DNA adduct formation induced by BaP in the HRN (A) or RCN model (B) *in vivo*. (A) HRN and WT mice were treated (i.p.) with a single dose of 125 mg/kg bw for 24 hours.[52] (B) RCN mice were treated (i.p) with 125 mg/kg bw for 24 hours without (-3MC) and with 3-methylcholanthrene (+3MC) pretreatment (single dose of 40 mg/kg bw 3-methylcholanthrene

14 days before BaP treatment).[52] DNA adduct formation was determined using the nuclease P1-enrichment version of the ^{32}P-postlabelling method. Values are given as mean ± SD (n = 3); each DNA sample determined by two post-labelled analyses. Comparison was performed by t-test analysis: *P<0.01, different from WT or different from RCN mice treated with BaP but without inducer.

Figure 5

DNA adduct formation induced by AAI in the HRN model *in vivo* (A) and *in vitro* (B). (A) HRN and WT mice were treated orally with a single dose of 50 mg/kg bw for 24 hours [53]. DNA adduct formation was determined using the nuclease P1-enrichment version of the ^{32}P-postlabelling method. Values are given as mean ± SD (n = 3); each DNA sample determined by two post-labelled analyses. Comparison was performed by t-test analysis: *P<0.01, different from WT. (B) DNA adduct formation by AAI activated with hepatic microsomes of untreated HRN and WT mice.[53] Values are given as mean ± range (n = 2); each DNA sample determined by two post-labelled analyses. α-NF, α-naphthoflavone; ND, not detected.

Figure 6

DNA adduct formation induced by 3-NBA (A) and 3-ABA (B) in the HRN model *in vivo*. HRN and WT mice were treated (i.p) with a single dose of 2 mg/kg bw of 3-NBA (A) or 3-ABA (B) for 24 hours.[53, 55] DNA adduct formation was determined using the butanol-enrichment version of the ^{32}P-postlabelling method. Values are given as mean ± SD (n = 3); each DNA sample determined by two post-labelled analyses. Comparison was performed by t-test analysis: *P<0.01, different from WT. ND, not detected.

Figure 7

DNA adduct formation induced by PhIP in the RCN model *in vivo* (A) and *in vitro* (B). (A) RCN mice were treated orally with 50 mg/kg bw for 5 days without (-3MC) and with 3-methylcholanthrene (+3MC) pretreatment (single dose of 40 mg/kg bw 3-methylcholanthrene 14 days before PhIP treatment).[57] DNA adduct formation was determined by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis. Values are given as mean ± SD (n = 3); each DNA sample determined by two independent measurements. Comparison was performed by t-test analysis: *P<0.01, different from RCN mice treated with PhIP but without inducer. (B) DNA adduct formation by PhIP

activated with hepatic microsomes of untreated RCN, without (-3MC) or with 3-methylcholanthrene (+3MC) pretreatment.[57] Values are given as mean ± range (n = 2); each DNA sample determined by two independent measurements. α-NF, α-naphthoflavone; ND, not detected.

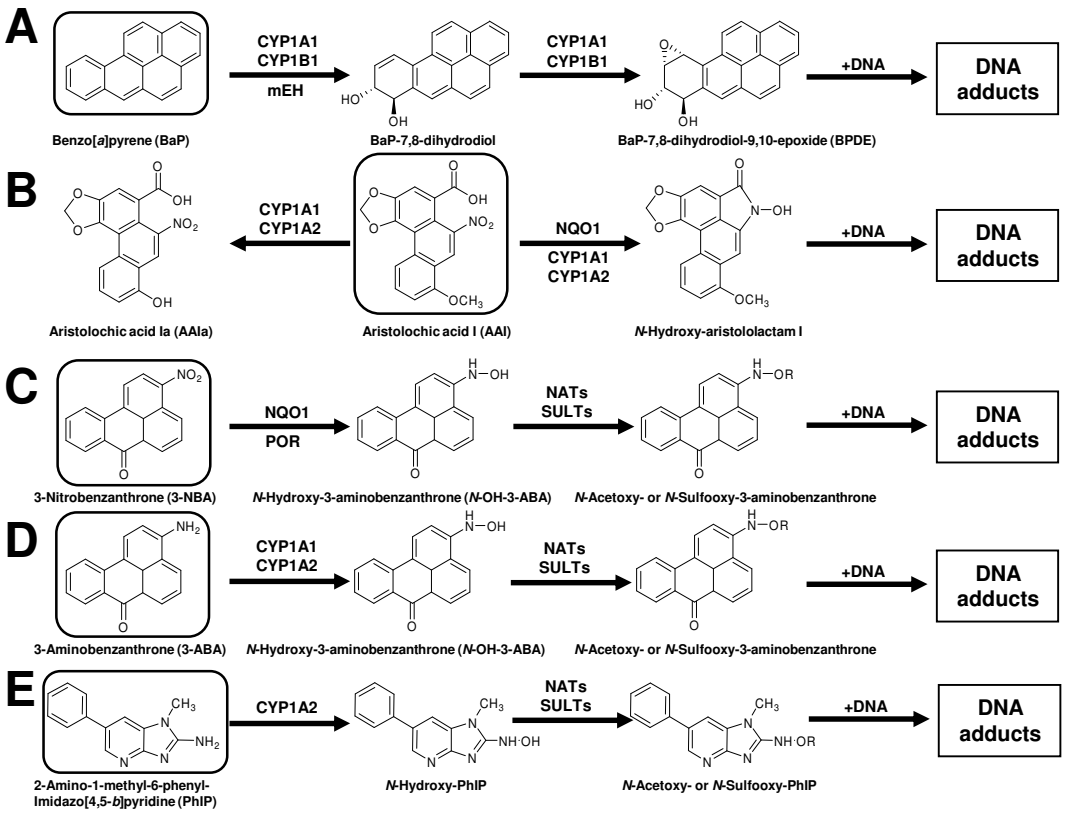
A
CYP1A1
CYP1B1
mEH
CYP1A1
CYP1B1
+DNA
DNA adducts
Benzo[a]pyrene (BaP)
BaP-7,8-dihydrodiol
BaP-7,8-dihydrodiol-9,10-epoxide (BPDE)
B
CYP1A1
CYP1A2
NQO1
CYP1A1
CYP1A2
+DNA
DNA adducts
Aristolochic acid Ia (AAIa)
Aristolochic acid I (AAI)
N-Hydroxy-aristololactam I
C
NQO1
POR
NATs
SULTs
+DNA
DNA adducts
3-Nitrobenzanthrone (3-NBA)
N-Hydroxy-3-aminobenzanthrone (N-OH-3-ABA)
N-Acetoxy- or N-Sulfooxy-3-aminobenzanthrone
D
CYP1A1
CYP1A2
NATs
SULTs
+DNA
DNA adducts
3-Aminobenzanthrone (3-ABA)
N-Hydroxy-3-aminobenzanthrone (N-OH-3-ABA)
N-Acetoxy- or N-Sulfooxy-3-aminobenzanthrone
E
CYP1A2
NATs
SULTs
+DNA
DNA adducts
2-Amino-1-methyl-6-phenyl-
Imidazo[4,5-b]pyridine (PhIP)
N-Hydroxy-PhIP
N-Acetoxy- or N-Sulfooxy-PhIP

Figure 1

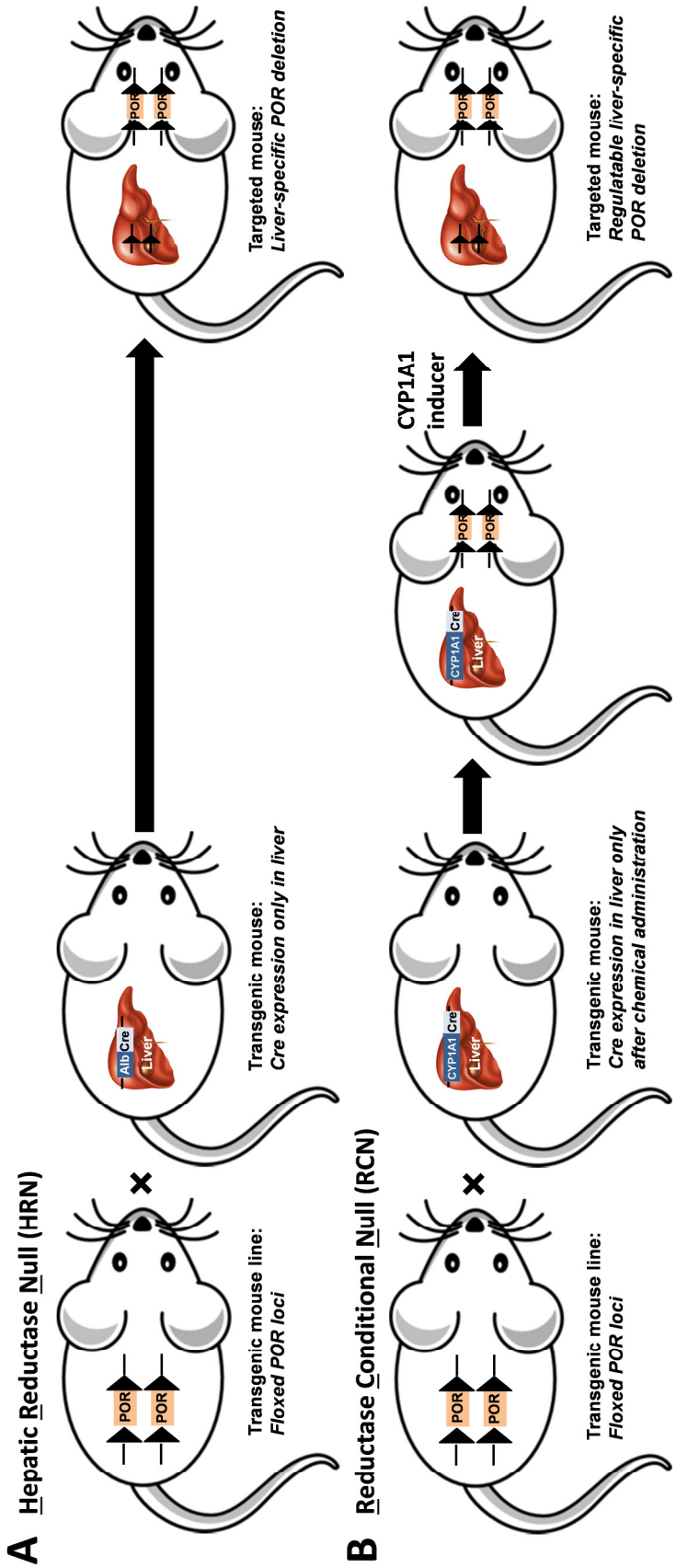
A Hepatic Reductase Null (HRN)
Transgenic mouse line:
Floxed POR loci
POR
POR
Transgenic mouse:
Cre expression only in liver
Alb Cre
Liver
Targeted mouse:
Liver-specific POR deletion
B Reductase Conditional Null (RCN)
Transgenic mouse line:
Floxed POR loci
POR
POR
Transgenic mouse:
Cre expression in liver only
after chemical administration
CYP1A1
Cre
Liver
CYP1A1 inducer
Targeted mouse:
Regulatable liver-specific
POR deletion

Figure 2

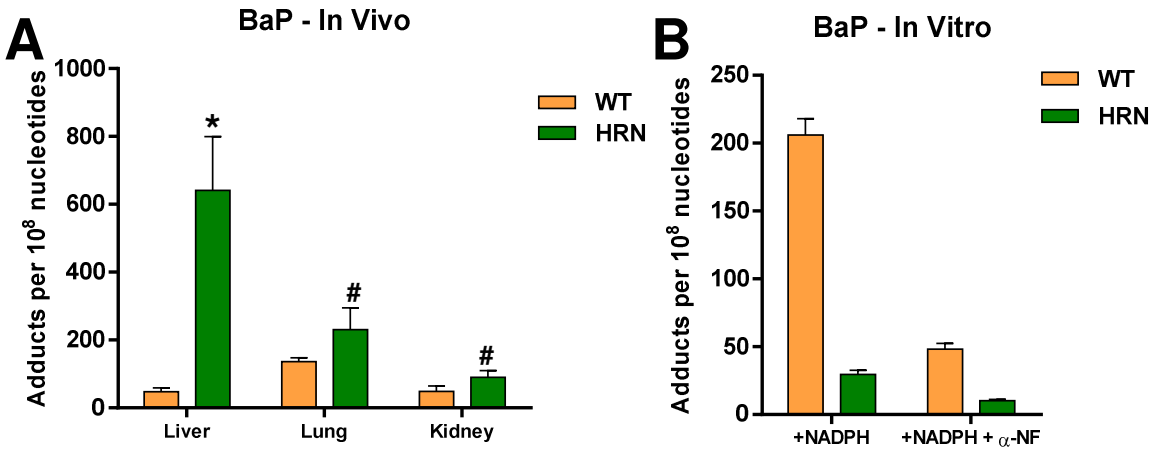
A
BaP - In Vivo
Adducts per 10^8 nucleotides
1000
800
600
400
200
0
*
#
#
Liver
Lung
Kidney
WT
HRN
B
BaP - In Vitro
Adducts per 10^8 nucleotides
250
200
150
100
50
0
+NADPH
+NADPH + α-NF
WT
HRN

Figure 3

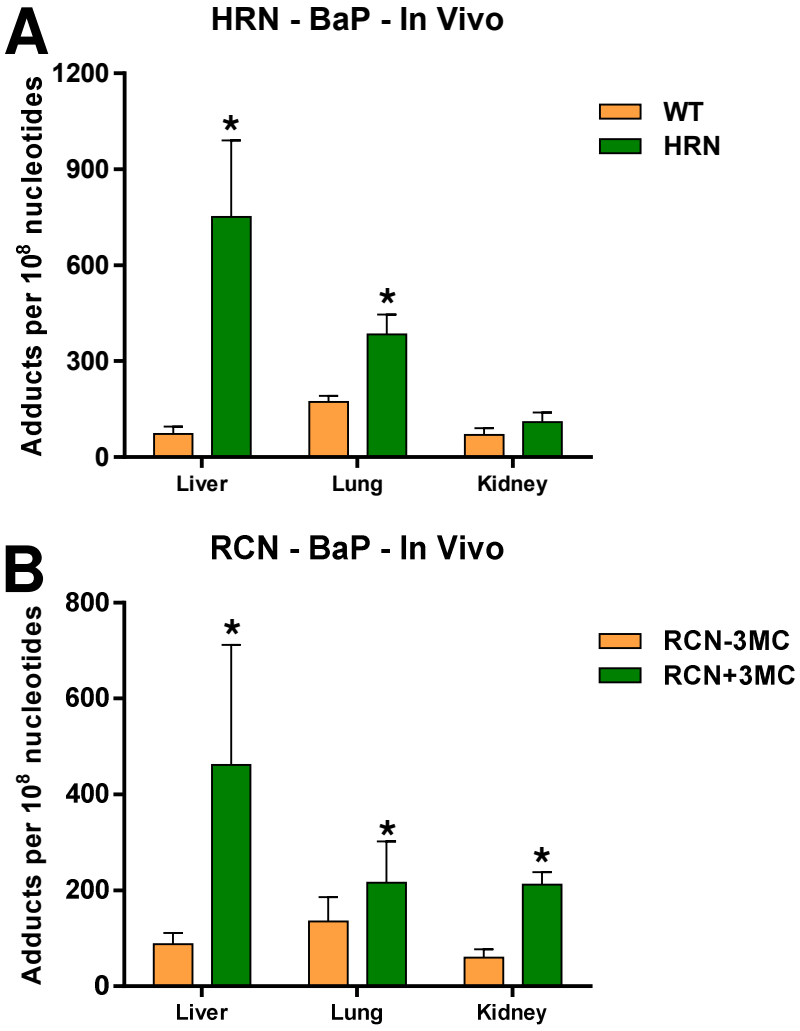
A
HRN - BaP - In Vivo
Adducts per 10^8 nucleotides
1200
900
600
300
0
*
*
WT
HRN
Liver
Lung
Kidney
B
RCN - BaP - In Vivo
Adducts per 10^8 nucleotides
800
600
400
200
0
*
*
*
RCN-3MC
RCN+3MC
Liver
Lung
Kidney

Figure 4

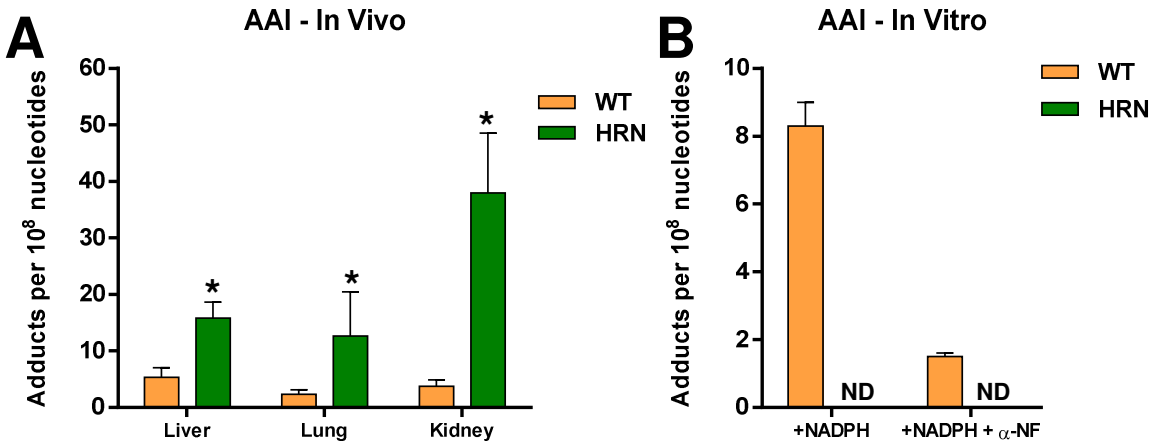
A
AAI - In Vivo
Adducts per 10^8 nucleotides
60
50
40
30
20
10
0
WT
HRN
*
*
*
Liver
Lung
Kidney
B
AAI - In Vitro
Adducts per 10^8 nucleotides
10
8
6
4
2
0
WT
HRN
ND
ND
+NADPH
+NADPH + α-NF

Figure 5

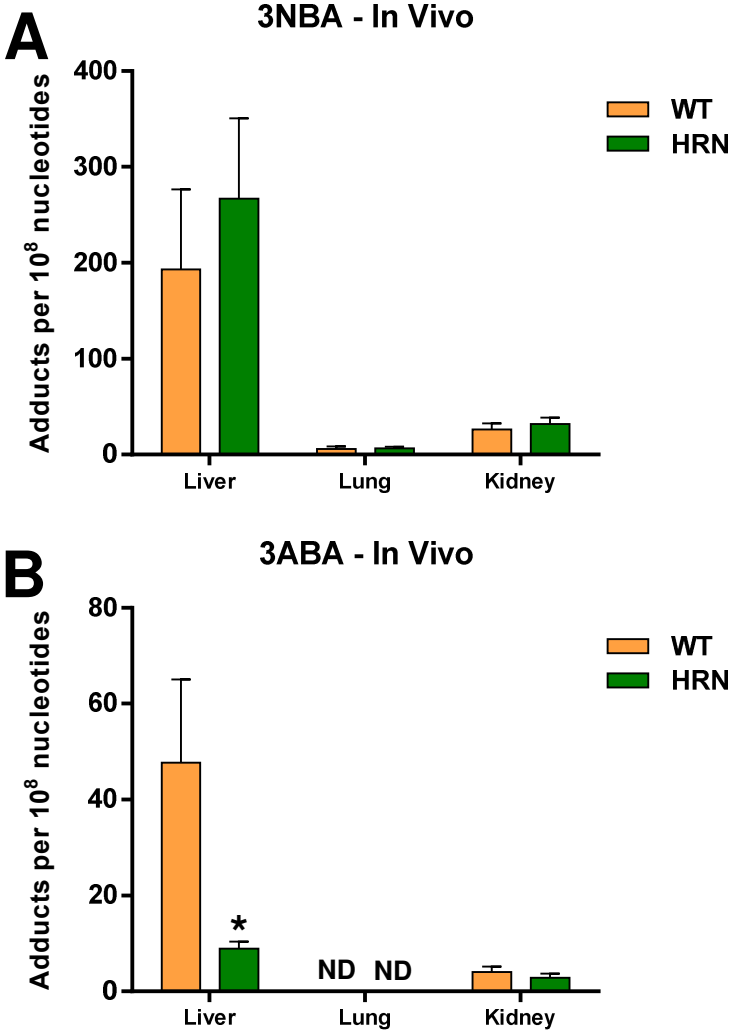
A
3NBA - In Vivo
Adducts per 10^8 nucleotides
400
300
200
100
0
Liver
Lung
Kidney
WT
HRN
B
3ABA - In Vivo
Adducts per 10^8 nucleotides
80
60
40
20
0
*
ND ND
Liver
Lung
Kidney
WT
HRN

Figure 6

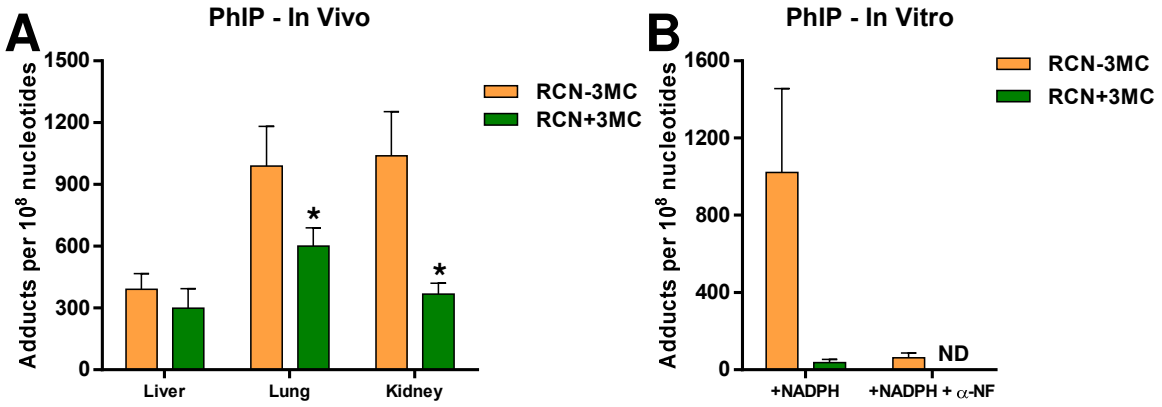
A
PhIP - In Vivo
Adducts per 10^8 nucleotides
1500
1200
900
600
300
0
Liver
Lung
Kidney
*
*
RCN-3MC
RCN+3MC
B
PhIP - In Vitro
Adducts per 10^8 nucleotides
1600
1200
800
400
0
+NADPH
+NADPH + α-NF
ND
RCN-3MC
RCN+3MC

Figure 7