Evidence That the Capacity of Nongenotoxic Carcinogens to Induce Oxidative Stress Is Subject to Marked Variability

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ABSTRACT

Many drugs and environmental chemicals which are not directly mutagenic have the capacity to increase the incidence of tumors in the liver and other tissues. For this reason, such compounds are known as nongenotoxic carcinogens. The mechanisms underlying their effects remain uncertain; however, their capacity to induce oxidative stress is considered to be a critical step in the carcinogenic process, although the evidence that this is actually the case remains equivocal and sparse. We have exploited a novel heme oxygenase-1 reporter mouse to evaluate the capacity of nongenotoxic carcinogens with different mechanisms of action to induce oxidative stress in the liver in vivo. When these compounds were administered at doses reported to cause liver tumors, marked differences in activation of the reporter were observed. 1,4-Dichlorobenzene and nafenopin were strong inducers of oxidative stress, whereas phenobarbital, piperonyl butoxide, cyproterone acetate, and WY14,643 were, at best, only very weak inducers. In the case of phenobarbital and thioacetamide, the number of LacZ-positive hepatocytes increased with time, and for the latter also with dose. The data obtained demonstrate that although some nongenotoxic carcinogens can induce oxidative stress, it is not a dominant feature of the response to these compounds. Therefore in contrast to the current models, these data suggest that oxidative stress is not a key determinant in the mechanism of nongenotoxic carcinogenesis but may contribute to the effects in a compound-specific manner.

Key words: nongenotoxic carcinogens; heme oxygenase-1; oxidative stress; transgenic mice
Now generally accepted that oxidative stress is a key feature of the mechanism of action of NGCs (Block and Gorin, 2012; Klaunig and Kamendulis, 2004; Mena et al., 2009). Environmental agents, including NGCs, can directly generate or indirectly induce increases in cellular reactive oxygen species (ROS) concentrations as a consequence of increased oxidative phosphorylation, cytochrome P450 (P450) activity, peroxisomal metabolism, and inflammatory cell activation. Such increases in ROS concentrations can lead to genotoxicity (via the formation of oxidative DNA adducts) and alter gene expression via interactions with growth factor receptors, signaling pathways, and transcription factors. Direct interactions with DNA can induce changes in methylation patterns or oxidative adduct formation. The consequences include cell proliferation, apoptosis, or necrosis depending on the insult sustained.

Oxidative stress leading to continuous release of ROS, possibly associated with P450 induction and activation of nuclear receptors such as the constitutive androstane receptor (CAR), has been identified as a central feature in the mechanism of action of NGCs (Kobliakov, 2010). Indeed, uncoupled oxidation by P450 oxidoreductase and P450s may be a major source of ROS. Cellular ROS concentrations are tightly regulated by endogenous defense systems including catalase, superoxide dismutase, and heme oxygenase-1 (HO-1), the latter being one of the proteins most highly regulated in vivo and in vitro by the oxidative stress pathway and a key participant in heme metabolism (Son et al., 2013).

The nature of oxidative stress and the fact that in vivo, it may be transient make it very difficult to measure. To circumvent this problem, we have created a novel oxidative stress reporter mouse where the HO-1 promoter is used to drive the expression of LacZ. We have exploited this system to establish the capacity of a range of NGCs with different mechanisms of action to induce oxidative stress in vivo and demonstrate that, although certain of these compounds have this capacity, it is not a universal feature.

**MATERIALS AND METHODS**

*Chemicals.* Nafenopin (NAF) and WY14,643 (WY) were kind gifts from Bettina Grasl-Kraupp, Medical University of Vienna, and Michael Schwarz, University of Tübingen, respectively. All other chemicals were purchased from Sigma-Aldrich (Poole, UK).

Generation of the HOD reporter line. The heme oxygenase-1 dual (HOD) reporter mouse line, distinct from that previously published (Young et al., 2010), was generated on a C57BL/6J background by random integration of 2 independent reporter transgenes constructed by recombination cloning. LacZ-SV40polyA and β-human chorionic gonadotrophin (βhCG)-SV40polyA mini-gene cassettes were engineered to contain regions homologous to exons 1 and 2 of the HO-1 gene and substituted for the endogenous HO-1 exon 1 and intron 1 sequences of a bacterial artificial chromosome (BAC) clone containing the mouse HO-1 locus (clone RPCI-23 290L07) (Fig. 1). The resulting HO-1 reporter loci contain most of the HO-1 gene together with 16.5 kb of upstream promoter and 8 kb of 3’ sequence. Correct positioning of the LacZ-SV40polyA and βhCG-SV40polyA mini-gene cassettes was confirmed by sequencing their 5’ and 3’ junction regions and the HO-1 gene sequence confirmed by sequencing their 5’ flanking regions using primers designed to amplify the SV40polyA and βhCG-SV40polyA mini-gene cassettes.

For reasons that remain unclear, the βhCG reporter was not secreted in amounts sufficient to enable detection in either blood or urine, although it was expressed in the liver (data not shown). This reporter was therefore not used in the course of this study and will not be discussed further.

All animal work was carried out in accordance with the Animals (Scientific Procedures) Act (1986) and with local ethical approval. Mice were housed in open-top cages under standard animal house conditions, with ad libitum access to standard rodent diet (RM1 Special Diet Services, Essex, UK) and water, and a 12 h light/12 h dark cycle. The HOD line bred normally exhibited no overt phenotype and yielded genotypes at the expected Mendel frequencies.

Animal experiments. Male mice aged between 8 and 22 weeks were assigned randomly to experimental and control groups (3 per group) and dosed by oral gavage with a heterogeneous set of compounds. The compounds, their Chemical Abstracts Service (CAS) numbers, vehicles, and doses are listed in Table 1. Compounds were selected after extensive discussion by the MARCAR collaborators (Eichner et al., 2013). Doses reported to induce tumors in mice in vivo were chosen from publicly available databases (eg, the Carcinogenic Potency Database: http://toxnet.nlm.nih.gov/cpdb/ and the NTP carcinogenicity database: http://ntp.niehs.nih.gov/). (Gold et al., 2005). Time-matched control groups were treated with the corresponding vehicles, PBS or corn oil (CO).

Following exposure of the appropriate duration, mice were euthanized by a rising concentration of CO2 and tissues were removed as rapidly as possible. A section of liver was incubated in 1% paraformaldehyde for 4 h at 4°C and transferred to 30% sucrose (in PBS) overnight at 4°C before being embedded in Shandon M-1 Embedding Matrix (Thermo Fisher Scientific), frozen in a dry ice-isopentane bath and stored at –80°C prior to cryosectioning for LacZ detection by histochemical staining. A further section of the median lobe was fixed in a solution of 1% paraformaldehyde and Gury (VWR International, Leicestershire, UK); the remaining liver tissue was snap frozen in liquid nitrogen and stored at –80°C for subsequent immunohistochemical analysis.

**LacZ staining.** Cryosectioning was performed using a Bright Microsystems cryostat with chamber and sample temperatures set at –30°C. Samples were allowed to equilibrate to the temperature of the cryostat for 1 h prior to sectioning. Sections (15 μm) were cut and placed on polylysine-coated microscope slides (VWR International). For the detection of β-galactosidase (β-Gal) activity by means of LacZ staining, sections were preincubated in a humidified chamber, washed twice with LacZ wash (2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% Nonidet-P40 in PBS), covered with LacZ stain (X-gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside], 25 mg/ml in dimethylformamide, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide-containing LacZ Wash), and placed in a humidified chamber at 37°C overnight. The next day they were washed in PBS, counterstained with Nuclear Fast Red, washed twice with distilled water, and dehydrated in 70% ethanol followed by 95% ethanol. Coverslips were applied using a water-based mounting gel and images were captured using a Zeiss light microscope.

**Immunohistochemistry and hematoxylin and eosin staining.** Samples for immunohistochemical analysis and hematoxylin and eosin
(H&E) staining were fixed in a solution of 1% paraformaldehyde and Gurr®. After overnight fixation at room temperature, they were stored in 70% ethanol. When required, they were processed using a Shandon Citadel 2000 tissue processor (Thermo Scientific) and embedded in paraffin wax in a Shandon HistoCentre 3 embedding center (Thermo Scientific). Sections (5 μM) were cut using a Shandon Finesse 325 microtome (Thermo Scientific). The DakoCytomation EnVision® Dual Link System-HRP (DAB++) kit (Dako Ltd, High Wycombe, UK) was used to carry out immunohistochemical analysis of 5-μM sections according to manufacturer’s instructions. Sections were stained using antibodies against β-Gal (Promega z3781) or HO-1 (AbCam, ab13243) at a dilution of 1:100, and counterstained with hematoxylin.

For H&E staining, 5-μM liver sections were deparaffinized in xylene, rehydrated in decreasing alcohol concentrations, and stained with hematoxylin and eosin (H&E).
stained with H&E, dehydrated in increasing alcohol concentrations, and mounted using DPX mounting media (Sigma), all according to standard procedures. The sections were photographed under bright field conditions on a Zeiss Axioplan microscope; the resulting images were processed with AxioVision software (Zeiss).

Preparation of microsomes. Microsomal fractions were prepared from frozen liver tissue according to standard procedures. Briefly, tissue samples were homogenized in 10-mM potassium phosphate buffer (pH 7.4) and centrifuged 3 times (twice at 11 000 rpm, 4 °C for 20 min then once at 41 000 rpm, 4 °C for 80 min), collecting the supernatant into a fresh tube each time. A sample of the supernatant from the first centrifugation step (lysate) was retained. The supernatant from the ultracentrifugation step (cytosolic fraction) was retained and the microsomal pellet was resuspended in 10-mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose. Protein content was determined using the Bradford protein assay using bovine serum albumin as standard and all fractions were stored at –80 °C until required.

Immunoblotting. Lysates and microsomal fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes according to standard procedures. Membranes were probed with commercial antibodies against β-Gal (Promega z3781), HO-1 (AbCam, ab13243), or in-house polyclonal antisera against rat P450s, CYP1A1, CYP2B1, CYP3A1, and CYP4A1. These have previously been shown to cross-react specifically with the murine counterparts of their target P450s. The antibody for Nqo1 was obtained from Abcam (ab2346); the antibody for Gsta1/2 was a kind gift from Professor John Hayes (Kelly et al., 2000; O’Connor et al., 1999). Commercial antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma G9545), Lactate dehydrogenase (LDH) (Abcam, ab2101), and calrecticulin (AbCam, ab2907) were used as loading controls. Immunoreactive bands were visualized on x-ray film developed using an Xograph Compact X4 automatic film processor (Xograph Imaging Systems, Gloucestershire, UK).

RNA extraction and quantitative real-time polymerase chain reaction. Snap-frozen liver tissue (50–100 mg) was homogenized in 1 ml of Trizol reagent and RNA was prepared according to the manufacturer’s instructions (Invitrogen, Paisley, UK). The resulting RNA (1 μg) was treated with DNase I and complementary DNA (cDNA) synthesis was carried out using the ImProm-II™ Reverse Transcription System (Promega, Southampton, UK) according to the manufacturer’s protocol. Expression of HO-1 messenger RNA (mRNA) and 18S rRNA = ribosomal RNA (rRNA) was detected using Taqman Gene Expression primers and probes Mm00516006_m1 and Hs00306361_g1, respectively (Applied Biosystems, Warrington, UK). Reactions were carried out in triplicate on a 7500 real-time polymerase chain reaction machine (Applied Biosystems) and HO-1 gene expression was calculated in relative to 18S rRNA using the comparative Ct method (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041435.pdf).

Biochemical analysis of plasma. Blood was harvested at necropsy by cardiac puncture into heparinized tubes. Plasma was prepared according to standard procedures, snap frozen, and stored at –80 °C. Biochemical analysis for creatinine, alanine aminotransferase, total bilirubin, glucose, and lactate dehydrogenase was conducted at the Clinical Pathology Service Laboratory, Medical Research Council, Harwell, Oxford, UK (http://www.har.mrc.ac.uk/services/pathology/clinical-pathology) according to standard procedures.

Statistical analysis. Numerical data were analyzed using Microsoft® Office Excel® 2007 (12.0.6683.5002) SP3 MSO (12.0.6683.5000) and are expressed as mean ± standard deviation based on the entire population (Excel® STDEVP function). Statistical significance was assessed using Student’s t test in 2-tailed, 2-sample unequal variance mode and p values are expressed as *p ≤ .05, **p ≤ .01, ***p ≤ .001.

RESULTS

Compounds Which Induce Oxidative Stress Induce HO-1 Reporter Expression in the Liver

To validate the utility of the model, we investigated whether compounds which induce oxidative stress activated the HO-1 reporter. The compounds chosen were cadmium chloride (CdCl2) and ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline, EQ). The heavy metal cadmium has been reported to produce ROS via a Fenton-like reaction leading to the generation of superoxide and hydroxyl radicals resulting in molecular damage and altered homeostasis (Mena et al., 2009). Although EQ is usually considered an antioxidant, it is metabolized to a toxic electrophile by the cytochrome P450 system (Burka et al., 1996) and can exert pro-oxidant effects, including cytotoxicity and oxidative DNA damage, as a result of redox cycling and generation of free radicals (Skolimowski et al., 2010). The oral dose of CdCl2 employed (4 mg/kg ip in PBS) was similar to doses administered to mice in published studies indicating the induction of oxidative stress at biochemical and transcriptional levels (Abu-Bakar et al., 2005) and the dose of EQ used (350 mg/kg po in CO) was similar to those previously administered to rodents (Buckley and Klaassen, 2009). Expression of the HO-1 reporter was visualized by histochemical staining for LacZ activity and confirmed by immunohistochimical staining for the expressed β-Gal enzyme; expression of the endogenous HO-1 enzyme was also visualized immunohistochemically and morphologically effects were visualized by H&E staining. EQ and CdCl2 induced high levels of LacZ activity in the liver (Fig. 2A). This was substantiated by immunohistochemical staining (Fig. 2A) and immunoblotting (Fig. 2B) for β-Gal. Quantitation of β-gal mRNA expression indicated that CdCl2 and EQ increased β-Gal mRNA expression compared with PBS (by 15 ± 2- and 10 ± 3-fold, respectively). Endogenous HO-1 protein and mRNA were also increased by CdCl2 and EQ treatment (Fig. 2 and Supplementary Table S1). Zonal expression of HO-1 reporter expression was observed: Both LacZ staining and expression of HO-1 protein were more evident in centrilobular regions of the liver after EQ treatment, and more generally distributed following administration of CdCl2. The CdCl2-induced changes in expression occurred in the absence of any evidence of hepatotoxicity. Indeed, plasma alanine aminotransferase (ALT) levels were unchanged after CdCl2 treatment (Supplementary Table S2). In the case of EQ, a slight increase in ALT was observed, as well as some evidence of hepatocellular necrosis (Fig. 2A and Supplementary Table S2). Together, these data demonstrate that the expression of the LacZ reporter mirrors that of the endogenous HO-1 gene, both in terms of mRNA and protein and reporter activity can precede overt toxicity. In unpublished experiments, we have also demonstrated that the HO-1 reporter is functional in a number of other tissues such as kidney and brain (Sharkey et al., unpublished data).
Having demonstrated induction of the HO-1 reporter by CdCl₂ and EQ, we used a panel of 7 NGCs which induce tumors via different mechanisms to characterize their capacity to induce oxidative stress. The doses of NGCs were chosen following extensive literature searches, consultation of databases, and expert opinion within the MARCAR program on the basis that they induce liver tumors in long-term carcinogenicity studies, and were the same doses as used in other published studies using C57BL/6 mice (Braeuning et al., 2014). No LacZ staining was observed in liver sections from mice treated with vehicle (PBS or CO) (Figs. 3 G and H). The common features of the response to NGC treatment were that hepatocytes appeared to

**FIG. 2.** Validation of heme oxygenase-1 (HO-1) reporter using compounds which induce oxidative stress. Adult male heme oxygenase-1 dual mice (n = 3) were treated with PBS (ip), corn oil (po), cadmium chloride (4 mg/kg ip in PBS), or ethoxyquin (350 mg/kg po in corn oil) as described in Materials and Methods and Table 1 A. Histological appearance of liver sections from treated mice: Histochemical staining for LacZ (cryosections); immunohistochemical staining for β-galactosidase (β-Gal) (paraffin-embedded sections); immunohistochemical staining for HO-1 (paraffin-embedded sections), and hematoxylin and eosin staining (paraffin-embedded sections)—all stained as described in Materials and Methods. Representative photomicrographs are shown. B, Immunoblotting of pooled liver cell lysates or microsomal samples for β-Gal and HO-1, respectively; loading controls GAPDH (cell lysate) and calreticulin (microsomal fraction).
be the only cell population affected and the induction of oxidative stress was observed, with one exception (thioacetamide, TAA), occurred in the absence of toxicity (data not shown).

Each NGC treatment yielded distinct patterns of LacZ reporter expression, both in terms of intensity and localization within the liver. The most marked induction of LacZ was observed in response to 1,4-dichlorobenzene (DCB). Liver:body weight ratios remained unchanged after DCB treatment (Supplementary Table S1) and H&E staining was normal (data not shown). Administration of this compound resulted in strong zonal induction of the HO-1 promoter (Fig. 3A); centrilobular hepatocytes stained intensely, while the staining in periportal hepatocytes was much weaker. Expression of β-Gal and the endogenous HO-1 protein was profoundly induced by DCB (Fig. 3I) and the endogenous HO-1 mRNA level was elevated 6.3-fold in DCB-treated mice compared with CO-treated controls (Supplementary Table S1). The weak induction of Cyp3a would also be consistent with this (Fig. 3J, long exposure). This has been subsequently confirmed by demonstrating the absence of Cyp2b10 induction in Car null mice (unpublished).

In addition to DCB, the only other compound to induce a marked level of HO-1 reporter expression at a dose equivalent to that used in long-term carcinogenicity studies was NAF (Fig. 3B). Both NAF and WY act through the same nuclear receptor, peroxisome proliferator-activated receptor α (PPARα). The livers of HOD mice treated with NAF exhibited a distinctive centrilobular pattern of reporter expression (Fig. 3B), while only a few hepatocytes were positive following WY administration (Fig. 3C). Both NAF and WY induced immunochemically detectable β-Gal and endogenous HO-1 protein (Fig. 3I), but there was no significant change in HO-1 mRNA expression with either compounds (Supplementary Table S1). This could be accounted for by the observation that induction was observed in only a relatively small proportion of hepatocytes. Alternatively, the increases in mRNA expression could well be transient (Young et al., 2010). In relation to their mode of action, both WY and NAF caused markedly induced Cyp4a expression, consistent with the activation of PPARα. Also, in the case of WY, an increased liver:body weight ratio was observed (Supplementary Table S1).

Treatment of HOD mice with the antiandrogen cyproterone acetate (CPA) or the pesticide synergist piperonyl butoxide (PBO) resulted in no or very weak LacZ staining in individual cells (Figs. 3D and E). This agrees with the absence of HO-1 mRNA/
protein induction (Fig. 3I and Supplementary Table S1). Consistent with reports that CPA is a pregnane X receptor (PXR) ligand, Cyp3a and Cyp2b10 induction was observed in HOD mice after short-term CPA treatment (Fig. 3 J). None of the P450s examined was affected by PBO. TAA treatment (20 mg/kg) also resulted in sporadic LacZ-positive hepatocytes (Fig. 3F).

To study the effects of the classical NGC phenobarbital (PB), we investigated changes in reporter activity in a time-dependent manner. PB was administered at 0.05% (wt/vol) in the drinking water, which is the same dose we recently published induces liver tumors in C57BL/6 mice (Braeuning et al., 2014). Very few LacZ-positive hepatocytes were observed 4 days after exposure, but a greater number was detected at 28 days. This change was not further increased, ie, after 56 days (Fig. 4). As expected, the liver:body weight ratio was increased at all time points studied and no changes in ALT were observed.

We also looked at the effects of chronic dosing on LacZ staining for CPA, PBO, and TAA, and for the latter also time, to establish whether more extensive exposure increased the level of oxidative stress. In the case of TAA, LacZ-positive hepatocytes were increased both in a dose- and time-dependent manner (Figs. 5A and B). In both experiments, this was paralleled by an increase in ALT (Figs. 5C and D). At the highest dose of TAA (125 mg/kg), a ring of LacZ-positive hepatocytes was observed which appeared to be on the periphery of an area of necrosis (Fig. 5B). This was observed in only 1 of the 3 mice, although the other 2 animals had clearly been overwhelmed by the toxicity of the TAA dose as evidenced by H&E staining, serum ALT, and pathology reports (Supplementary Fig. 1).

Measurement of Nrf2 Target Genes as a Marker of Oxidative Stress
The Nrf2 signaling cascade provides an adaptive response system to toxic electrophiles and oxidative stress. To establish whether some of the changes observed may be attributed to the activation of this pathway, we measured the expression of the Nrf2-regulated genes Gsta1/2 and Nqo1 (Fig. 7). Of the compounds tested, only DCB stimulated a response robustly and a
slight degree of activation was also observed for TAA, over the respective vehicle controls.

**DISCUSSION**

In this study, we examined the effects of a range of NGCs on oxidative stress through the application of an HO-1 reporter mouse, the measurement of endogenous HO-1 protein, and the activation of the Nrf2 signaling cascade. Our data demonstrate marked differences between compounds to activate these pathways. Using the HO-1 reporter DCB caused marked induction of oxidative stress in the majority of hepatocytes. NAF and TAA also caused an oxidative stress response, whereas the other compounds tested had only marginal effects. Our conclusion from these experiments is that the effects of oxidative stress on NGC-induced tumorigenesis is likely to be compound specific and does not represent a universal feature of this process.

The mouse liver-specific carcinogen DCB strongly induced centrilobular reporter expression, corresponding with the known localization of the enzymes responsible for its oxidative metabolism, CYP1A2 and CYP2E1 (Irie et al., 2010; Wijsman et al., 2007), and consistent with the induction of oxidative stress by its 2,5-dichloroquinone metabolite. It is interesting to note that quinones are known inducers of the Nrf2 signaling cascade and would explain the induction of Nrf2-regulated genes by this compound, including HO-1. Generation of the quinone metabolite has been associated with DNA strand breaks, an effect which is enhanced under redox cycling conditions and blocked by catalase (Muller, 2002). A carcinogenic mechanism involving metabolism to hydroquinones has therefore been proposed. However, the ability of DCB to induce oxidative stress in vivo has been unclear (Suhua et al., 2010). Our results provide strong evidence that DCB does induce oxidative stress in vivo in mouse liver.

TAA studies in rats have shown the induction of hepatotoxicity via oxidative stress; generation of ROS is an early event in pathogenesis and is associated with transcriptional effects implicating oxidative stress and lipid peroxidation (Natarajan et al., 2006). This hepatotoxicity has been associated with the expression of the metabolic activating enzyme CYP2E1 in...
Effects on hepatic heme oxygenase-1 (HO-1) reporter expression for extending the duration of exposure to cyproterone acetate or piperonyl butoxide. Adult male heme oxygenase-1 dual mice were treated with cyproterone acetate (160 mg/kg po daily for 6 days), piperonyl butoxide (600 mg/kg daily for 7 days), or vehicle (corn oil) as described in Materials and Methods and Table 1. A, Detection of LacZ in liver sections by histochemical staining; arrows indicate individual LacZ-positive cells. B, Immunoblotting for β-galactosidase (pooled liver lysates), HO-1, Cyp1a, Cyp2b, Cyp3a, or Cyp4a (hepatic microsomal samples); loading controls are GAPDH (liver lysates) and calreticulin (hepatic microsomal samples).

FIG. 6. Effects on hepatic heme oxygenase-1 (HO-1) reporter expression for extending the duration of exposure to cyproterone acetate or piperonyl butoxide. Adult male heme oxygenase-1 dual mice were treated with cyproterone acetate (160 mg/kg po daily for 6 days), piperonyl butoxide (600 mg/kg daily for 7 days), or vehicle (corn oil) as described in Materials and Methods and Table 1. A, Detection of LacZ in liver sections by histochemical staining; arrows indicate individual LacZ-positive cells. B, Immunoblotting for β-galactosidase (pooled liver lysates), HO-1, Cyp1a, Cyp2b, Cyp3a, or Cyp4a (hepatic microsomal samples); loading controls are GAPDH (liver lysates) and calreticulin (hepatic microsomal samples).

centrilobular hepatocytes (Shirai et al., 2013). In our study, the induction of oxidative stress as a consequence of necrosis in the centrilobular hepatocytes also appears to be linked to liver damage.

Both NAF and WY activate PPARs leading to peroxisome proliferation and are potent hepatocarcinogens in rodents. The normal activity of peroxisomal enzymes generates ROS and reactive nitrogen species as byproducts of metabolism, but peroxisomes also possess mechanisms that participate in the maintenance of redox homeostasis. Increased numbers of peroxisomes do not, therefore, necessarily lead to cellular oxidative stress and the role of peroxisomes in inducing and/or preventing oxidative stress and ROS production remains the subject of ongoing research.

discussion (del Rio, 2013). In this study, acute dosing with NAF induced HO-1 reporter and protein expression in HOD mouse liver (suggesting direct induction of oxidative stress in hepatocytes), while WY had little effect on the reporter but did induce a slight increase in HO-1 protein. On this basis, a role for oxidative stress in the mechanism of action of these compounds cannot be excluded. The differences observed between these compounds on their ability to induce the HO-1 reporter could be a consequence of the different dosing regimens used, although there is currently no direct evidence for oxidative DNA damage due to WY; PPARα-dependent induction of long-patch base excision repair genes has been observed in C57BL/6j mice treated for 1 month via the diet (Rusyn et al., 2004).

CPA, PBO, and PB had minimal effects on HO-1 reporter expression. There is little evidence that CPA, which is an androgen receptor antagonist and PXR ligand, can induce oxidative stress, and this is confirmed by our study. In contrast to CPA, hepatocarcinogenesis by PBO has been linked to increased ROS production as a byproduct of increased microsomal P450 activity which is thought to occur as a result of Cyp1a1 induction (Kawai et al., 2010; Mena et al., 2009). It has been suggested that PBO generates ROS via redox cycling of quinone derivatives and/or P450 catalytic pathways, but it does not cause mutations, possibly because it concomitantly induces ROS-scavenging detoxifying enzymes, and contradictory results have been reported regarding 8-hydroxy-2′-deoxyguanosine formation due to PBO in mouse liver (Kawai et al., 2010; Tasaki et al., 2013). However, our studies do not support a role for PBO-induced oxidative stress in its mechanism of carcinogenesis.

Several of the compounds tested activated the nuclear receptors CAR or PXR (DCB, CPA, PB). These compounds exhibited marked differences in their ability to induce oxidative stress; therefore, the induction of oxidative stress is not a direct consequence of this interaction. A number of mechanisms have been proposed for the CAR-mediated carcinogenic effects of PB, including oxidative stress (Kleppeizis et al., 2013). On the basis of the extremely weak induction of oxidative stress by this compound in our studies, such a mechanism would seem implausible. It is important to note that the conditions used for our experiments were the same as those we have recently shown induce CAR activation, hepatomegaly, and liver tumors in C57BL/6 mice (Braeuning et al., 2014). As part of our European collaboration, we have carried out detailed investigations into other mechanisms of PB-induced carcinogenicity in mice (Lempiainen et al., 2011; Luisier et al., 2014). This work has identified a number of potential CAR-mediated mechanisms. Of
particular note are the epigenetic changes observed in both WT and humanized CAR/PXR mice at the Dlk3-Dio1 gene locus. These changes are associated with the increased expression of Meg3, a gene that has been shown to be involved in hepatocarcinogenesis in mice (Wang et al., 2012). Mechanisms such as this provide a much more plausible basis for the carcinogenic effects of these compounds (Lempiainen et al., 2013; Thomson et al., 2013, 2014).

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci. oxfordjournals.org/.

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