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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 unclear; 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

The identification and control of carcinogens is central to chemical safety assessment. Genotoxic carcinogens are relatively easy to distinguish by means of tests for DNA damage and mutagenesis, but nongenotoxic carcinogens (NGCs) induce tumors by various alternative mechanisms and can be difficult to identify, other than through classic rodent bioassays. Mammalian bioassays are used to identify chemical carcinogens, but are limited by their length (≥2 years) and cost in terms of time, money, animals, and resources.

We are currently engaged in a collaborative European Union (EU) program whose aim is to speed up the process for identifying NGCs by identifying biomarkers of effect, which can be detected following short-term administration. This program, the EU Innovative Medicines Initiative-funded MARCAR project

(http://www.imi-marcar.eu/), aims to identify early biomarkers for nongenotoxic carcinogenesis using a range of transcriptomic and epigenetic approaches (Braeuning *et al.*, 2014; Eichner *et al.*, 2013; Jonker *et al.*, 2009; Lempiainen *et al.*, 2011, 2013; Schaap *et al.*, 2012; Thomson *et al.*, 2012, 2013, 2014).

It is 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 HOD mouse line was maintained by random crossing with wild-type C57BL/6J mice, generating reporter hemizygotes.

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 caging 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 Mendelian 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 CO_2 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 Gurr® (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 (2mM $MgCl_2$, 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, 5mM 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

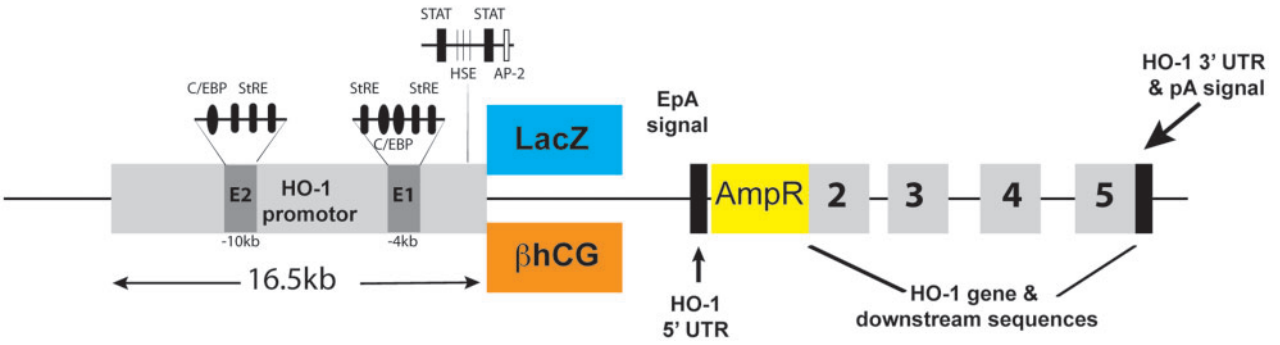

FIG. 1. Transgenic constructs for the heme oxygenase-1 (HO-1) dual reporter with β-galactosidase or β-human chorionic gonadotrophin (βhCG). Reporter genes shown - LacZ and hCG - are driven by the HO-1 promoter. Exon 1 and intron 1 of the murine HO-1 gene are replaced by an ampicillin-resistance (AmpR) cassette; 16.5 kb of 5′ untranslated sequence, exons 2–5, and 8 kb of 3′ untranslated sequence remain intact. Regulatory elements in the promoter are shown as follows: AP-2, activator protein-2; C/EBP, CAAT/enhancer binding protein binding site; HSE, heat shock element; STAT, signal transducer and activator of transcription; StRE, stress response element, structurally and functionally similar to the antioxidant and TPA response elements, responding to (among others) AP-1 and Nrf2 transcription factors; UTR, untranslated region; pA, polyadenylation; EpA signal, early polyadenylation signal; AmpR, ampicillin-resistance cassette. Adapted from: Ryter *et al.* (2006). *Physiol. Rev.* **86**, 563.

TABLE 1. Compounds and Dosing Regimens Used in This Study

Compound (CAS No.)	Dose/Route (Vehicle)	Dosing Schedule	Rationale for Dosing Regimen Used (All Studies in Mice, Except *)
$CdCl_2$ (10108-64-2)	4 mg/kg ip (PBS)	Single dose, harvest after 12 h	Induces HO-1 *in vivo* in *rats and mice (Alam, 1994)
Ethoxyquin (91-53-2)	350 mg/kg po (corn oil)	Daily for 3 days, harvest after 5 days	Activates Nrf2 as a consequence of metabolism to toxic electrophile and induces oxidative stress (Hayes *et al.*, 2000)
1,4-Dichlorobenzene (106-46-7)	600 mg/kg po (corn oil)	Daily for 2 day, harvest after 3 days	Dose used to induce tumors in NGC studies (Kossler *et al.*, 2015)
Thioacetamide (62-55-5)	20, 62.5, or 125 mg/kg ip (PBS)	Single dose, harvest after 2 days	Higher dose reflects that used to induce tumors long-term in *rats (Cascales *et al.*, 1991)
	20 mg/kg ip (PBS)	Daily for 7 or 14d, harvest d8 or d15	Lower doses were used due to overt toxicity, but used to induce tumors in NGC studies (Kossler *et al.*, 2015)
Nafenopin (3771-19-5)	125 mg/kg po (corn oil)	Single dose, harvest after day 2	Dose used to induce tumors in NGC studies (Reddy *et al.*, 1976)
WY14,643 (50892-23-4)	200 mg/kg po (corn oil)	Daily for 2 days, harvest after day 3	Dose used to induce tumors in NGC studies (Reddy and Rao, 1978)
Cyproterone acetate (427-51-0)	160 mg/kg po (corn oil)	Single dose, harvest after day 2 Daily for 6 days, harvest after day 7	Dose used to induce tumors in NGC studies (Kossler *et al.*, 2015)
Piperonyl butoxide (51-03-6)	1200 mg/kg ip (corn oil)	Single dose, harvest after day 2	Dose used to induce tumors in NGC studies (Kossler *et al.*, 2015)
	600 mg/kg ip (corn oil)	Daily for 7 days, harvest after day 8	Reduced dose used due to excess toxicity
Phenobarbital (57-30-7)	80 mg/kg ip (PBS)	Daily for 3 days, harvest after day 4	Conventional dosing regimen used to study CAR interactions and hyperplastic responses associated with NGCs (Ross *et al.*, 2010)
	0.05% in drinking water	For 28 or 56 days	Dose used to induce tumors in C57BL/6 mice (Braeuning *et al.*, 2014)

(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,

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 Axiocam 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 Hs03003631_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 C_T 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 $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$.

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 ($CdCl_2$) 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 $CdCl_2$ 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 immunohistochemical staining for the expressed β-Gal enzyme; expression of the endogenous HO-1 enzyme was also visualized immunohistochemically and morphological effects were visualized by H&E staining. EQ and $CdCl_2$ 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 $CdCl_2$ 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 $CdCl_2$ 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 $CdCl_2$. The $CdCl_2$-induced changes in expression occurred in the absence of any evidence of hepatotoxicity. Indeed, plasma alanine aminotransferase (ALT) levels were unchanged after $CdCl_2$ 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).

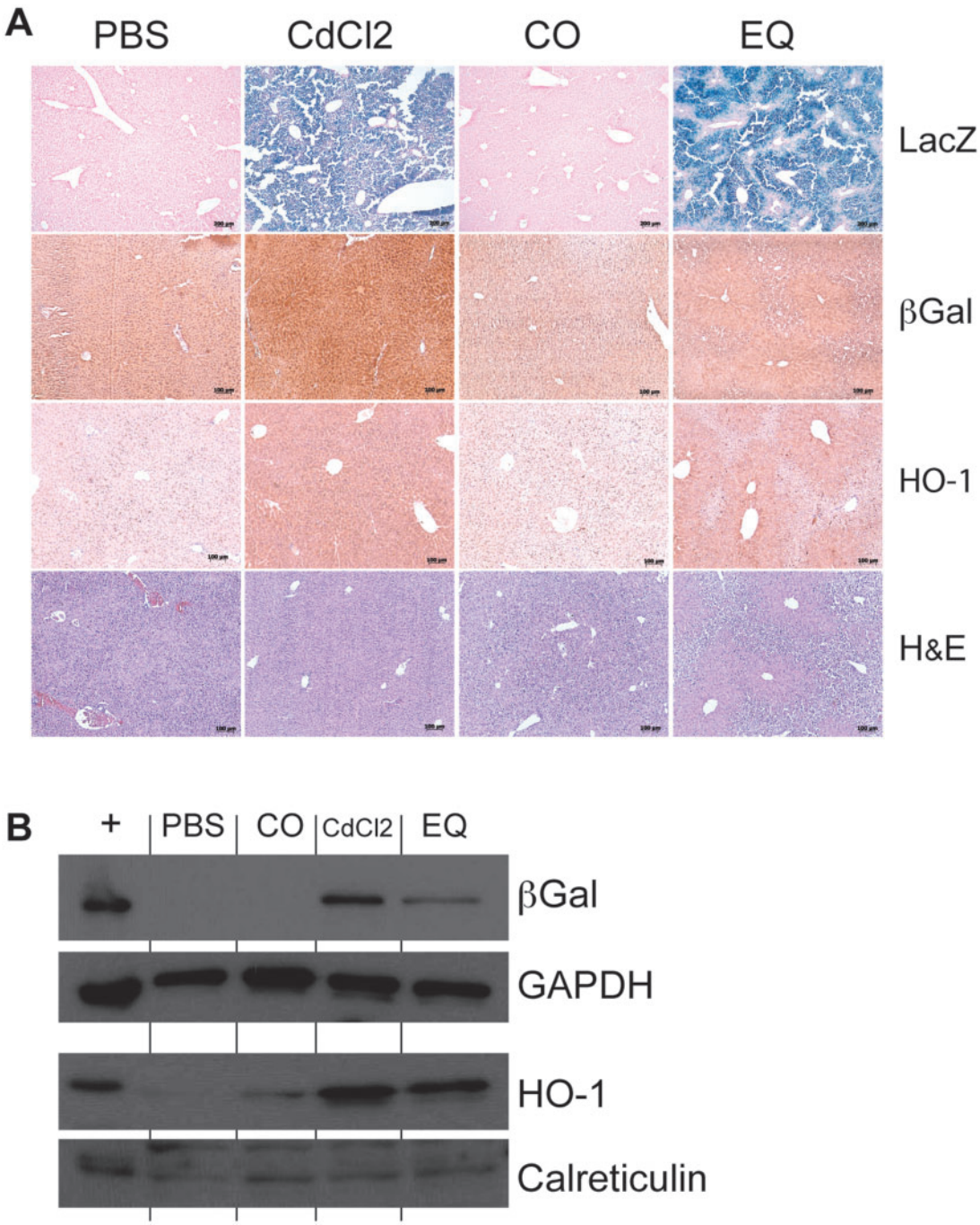

FIG. 2. Validation of heme oxygenase-1 (HO-1) reporter using compounds which induce oxidative stress. Adult male heme oxygenase-1 dual mice ($n = 3$) were treated with PBS (ip), corn oil (po), cadmium chloride (4 mg/kg ip in PBS), or ethoxyquin (350 mg/kg po in corn oil) as described in Materials and 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).

Structurally Diverse NGCs Have Compound-specific Effects on HO-1 Reporter Expression

Having demonstrated induction of the HO-1 reporter by $CdCl_2$ 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. 3G and H). The common features of the response to NGC treatment were that hepatocytes appeared to

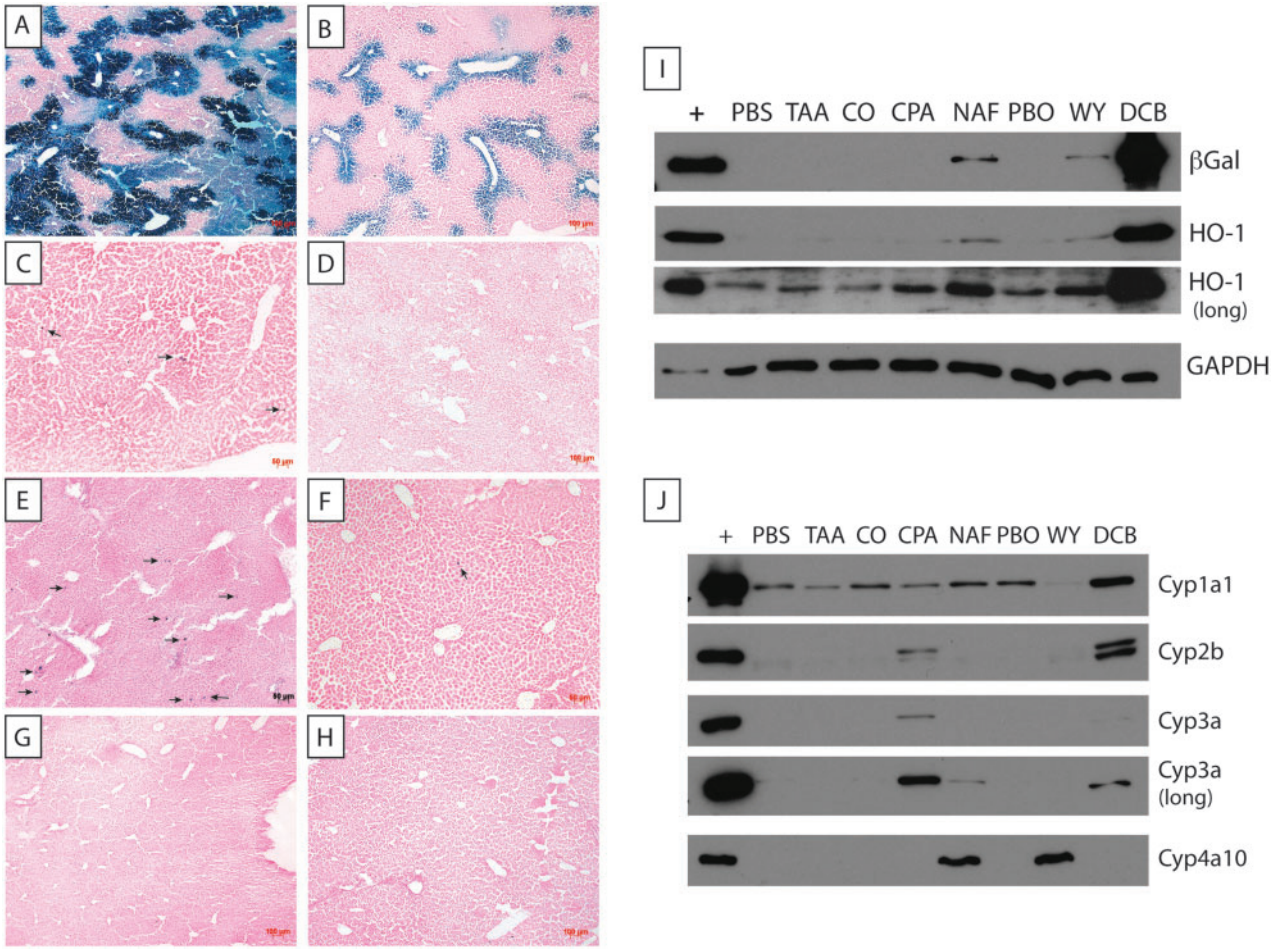

FIG. 3. Nongenotoxic carcinogens have compound-specific effects on hepatic heme oxygenase-1 (HO-1) reporter induction. Adult male heme oxygenase-1 dual mice ($n = 3$) were treated as described in Materials and Methods and Table 1. A-H, Detection of LacZ in liver sections by histochemical staining. A, 1,4-dichlorobenzene; B, nafenopin; C, WY-14,643; D, cyproterone acetate; E, piperonyl butoxide; F, thioacetamide (TAA), 20 mg/kg; G, corn oil; H. PBS. Arrows indicate individual LacZ-positive cells; photomicrograph shown in D is also shown in Figure 5 for TAA. I, Immunoblotting of pooled liver cell lysates or microsomal samples for β-galactosidase and HO-1, respectively, with GAPDH as loading control. J, Immunoblotting of pooled microsomal samples for Cyp1a1, Cyp2b10, Cyp3a, and Cyp4a10; loading control GAPDH. In I and J, "Long" refers to a longer exposure of the immunoblot above.

be the only cell population affected and the induction of oxidative stress where 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). Little is known about the molecular mode of action of DCB; however, we observed strong induction of Cyp2b10 after DCB treatment (Fig. 3J) suggesting that it is a CAR ligand. 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/

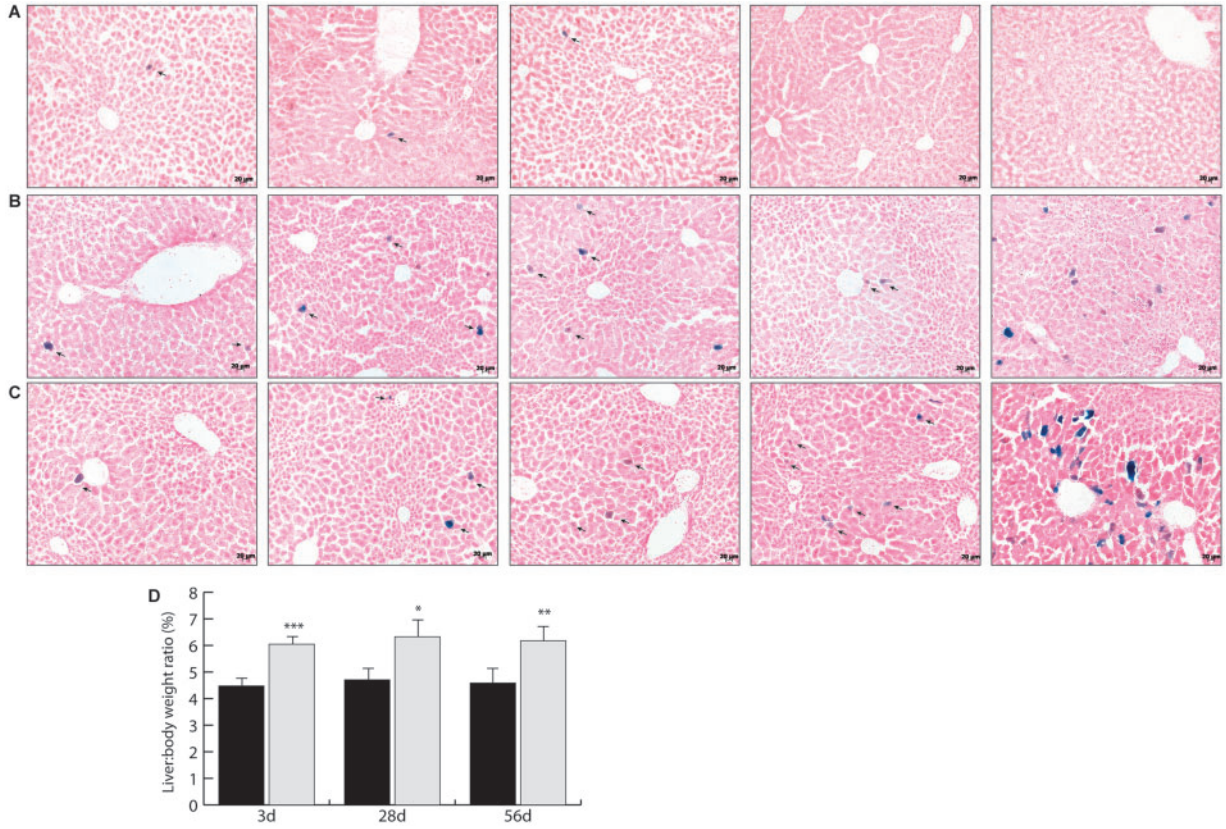

FIG. 4. Hepatic heme oxygenase-1 (HO-1) reporter expression in response to phenobarbital depends on the duration of exposure. Adult male heme oxygenase-1 dual mice ($n = 5$) were treated with phenobarbital (PB) ip (80 mg/kg/day) for 4 days or via drinking water (0.05%) for 28 or 56 days as described in the Materials and Methods and Table 1. A-C, Detection of LacZ in liver sections by histochemical staining. A, 4 days; B, 28 days; C, 56 days. Arrows indicate individual LacZ-positive cells. Photomicrographs at ×20 magnification, bright field, scale bar = 20μM. D, Liver:body weight ratios for mice treated with PB as described above (mean ± standard deviation). Black bars = vehicle control; gray bars = PB. Statistical comparison between PB and vehicle groups at each time point: $^{*}p < .5$, $^{**}p < .05$, $^{***}p < .001$.

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. 3J). 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).

For chronic dosing of CPA and PBO, 6 doses of 160 mg/kg/day po and 7 doses of 600 mg/kg/day ip were administered, respectively. A lower dose of PBO was used than in the single dose experiment because of toxicity (data not shown). These dosing regimens produced increases in liver:body weight ratios for both compounds (1.85-fold for CPA [not statistically significant] and 1.75-fold for PBO [$p \leq .05$], see Supplementary Table S1). However, although there was a slight increase in the number of cells staining for LacZ (Fig. 6A), they were still few in number. No induction of HO-1 or β-Gal was observed by immunoblotting (Fig. 6B), but both CPA and PBO induced Cyp2b10 protein expression, the former to a greater extent (Fig. 6B). Cyp3a protein expression was observed after CPA treatment, but not following PBO, while the converse was true for Cyp4a (Fig. 6B). Through the application of CAR and PXR null mice, we have subsequently shown that this effect is due to the ability of CPA to induce both CAR and PXR (unpublished).

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

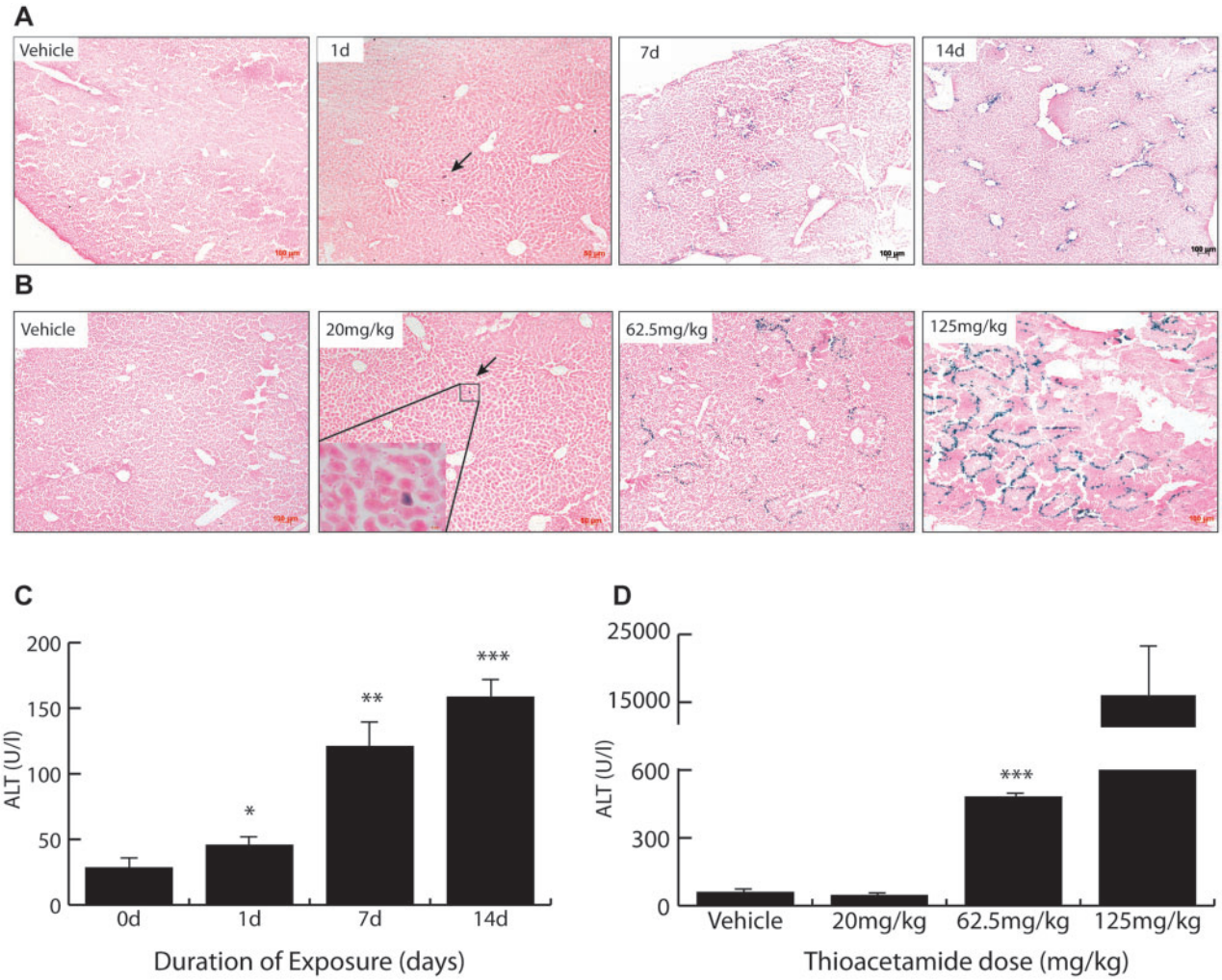

FIG. 5. Time response and dose response of hepatic heme oxygenase-1 reporter expression in response to thioacetamide. Adult male heme oxygenase-1 dual (HOD) mice ($n = 3$) were treated with thioacetamide (TAA) at a fixed dose (20 mg/kg) for 1, 7, or 14 days, or at differing doses (20, 62.5, or 125 mg/kg) for a fixed time (24 h), as described in the Materials and Methods. Vehicle controls received PBS. A, Hepatic LacZ reporter expression in HOD mice treated with TAA (20 mg/kg, ip) daily for 1, 7, or 14 days. B, Hepatic LacZ reporter expression 24 hours after a single administration of TAA at the doses indicated. C, Serum alanine aminotransferase (ALT) levels (mean ± standard deviation [SD]) in HOD mice treated with TAA (20 mg/kg, ip) daily for 1, 7, or 14 days. D, Serum ALT levels (mean ± SD) in HOD mice 24 h after a single administration of TAA at the doses indicated. $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$.

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

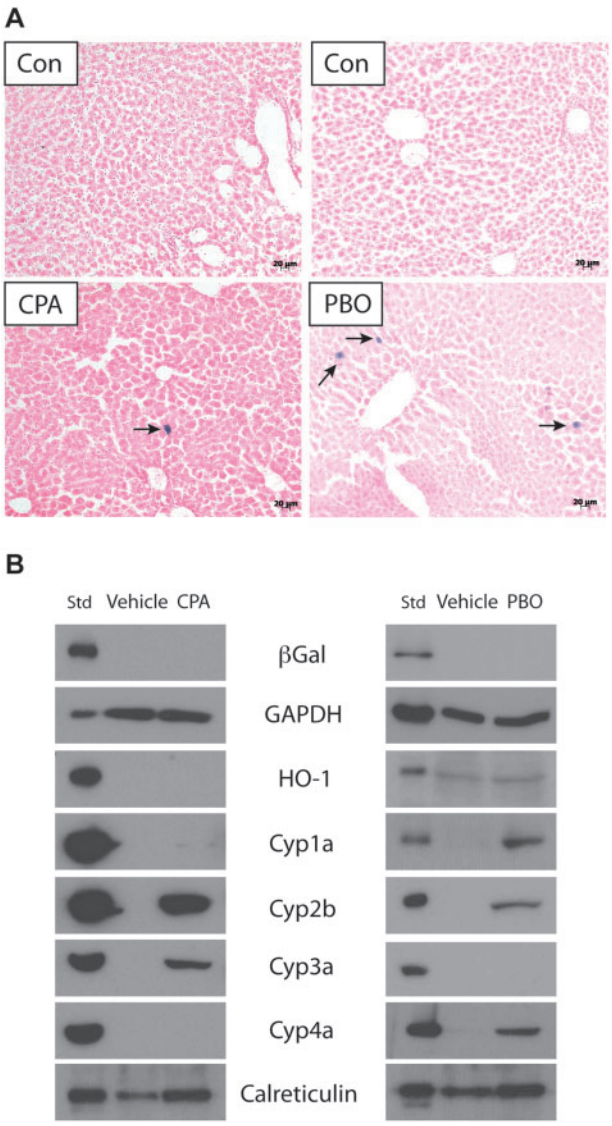

FIG. 6. Effects on hepatic heme oxygenase-1 (HO-1) reporter expression for extending the duration of exposure to cyproterone acetate or piperonyl butoxide. Adult male heme oxygenase-1 dual mice were treated with cyproterone acetate (160 mg/kg po daily for 6 days), piperonyl butoxide (600 mg/kg daily 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).

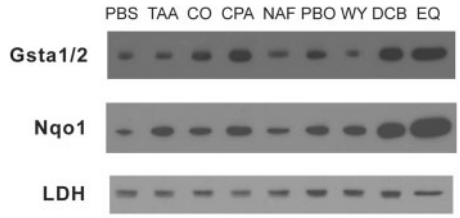

FIG. 7. Nongenotoxic carcinogens have compound-specific effects on the expression of Nrf2 target genes. Adult male heme oxygenase-1 dual mice ($n = 3$) were treated with nongenotoxic carcinogen thioacetamide (TAA, 20 mg/kg), cyproterone acetate, nafenopin, piperonyl butoxide, WY-14,643, 1,4-dichlorobenzene, and ethoxyquin as described in Materials and Methods and Table 1. PBS was used as a vehicle control for TAA, with corn oil for the remaining compounds. Liver cell lysates were prepared, pooled for each treatment, and immunoblotted for Gsta1/2 and Nqo1 as described in Materials and Methods. Lactate dehydrogenase was used as a loading control.

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 PPARα 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 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 difference 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 produced 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 (Klepeisz *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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REFERENCES

Abu-Bakar, A., Moore, M. R., and Lang, M. A. (2005). Evidence for induced microsomal bilirubin degradation by cytochrome P450 2A5. *Biochem. Pharmacol.* **70**, 1527–1535.

Alam, J. (1994). Multiple elements within the 5′ distal enhancer of the mouse heme oxygenase-1 gene mediate induction by heavy metals. *J. Biol. Chem.* **269**, 25049–25056.

Block, K., and Gorin, Y. (2012). Aiding and abetting roles of NOX oxidases in cellular transformation. *Nat. Rev. Cancer* **12**, 627–637.

Braeuning, A., Gavrilov, A., Brown, S., Wolf, C. R., Henderson, C. J., and Schwarz, M. (2014). Phenobarbital-mediated tumor promotion in transgenic mice with humanized CAR and PXR. *Toxicol. Sci.* **140**, 259–270.

Buckley, D. B., and Klaassen, C. D. (2009). Induction of mouse UDP-glucuronosyltransferase mRNA expression in liver and intestine by activators of aryl-hydrocarbon receptor, constitutive androstane receptor, pregnane X receptor, peroxisome proliferator-activated receptor alpha, and nuclear factor erythroid 2-related factor 2. *Drug Metab. Dispos.* **37**, 847–856.

Burka, L. T., Sanders, J. M., and Matthews, H. B. (1996). Comparative metabolism and disposition of ethoxyquin in rat and mouse. II. Metabolism. *Xenobiotica* **26**, 597–611.

Cascales, M., Martin-Sanz, P., Craciunescu, D. G., Mayo, I., Aguilar, A., Robles-Chillida, E. M., and Cascales, C. (1991). Alterations in hepatic peroxidation mechanisms in thioacetamide-induced tumors in rats. Effect of a rhodium(III) complex. *Carcinogenesis* **12**, 233–240.

Eichner, J., Kossler, N., Wrzodek, C., Kalkuhl, A., Bach Toft, D., Ostenfeldt, N., Richard, V., and Zell, A. (2013). A toxicogenomic approach for the prediction of murine hepatocarcinogenesis using ensemble feature selection. *PLoS One* **8**, e73938.

Gold, L. S., Manley, N. B., Slone, T. H., Rohrbach, L., and Garfinkel, G. B. (2005). Supplement to the Carcinogenic Potency Database (CPDB): Results of animal bioassays published in the general literature through 1997 and by the National Toxicology Program in 1997–1998. *Toxicol. Sci.* **85**, 747–808.

Hayes, J. D., Chanas, S. A., Henderson, C. J., McMahon, M., Sun, C., Moffat, G. J., Wolf, C. R., and Yamamoto, M. (2000). The Nrf2 transcription factor contributes both to the basal expression of glutathione S-transferases in mouse liver and to their induction by the chemopreventive synthetic antioxidants, butylated hydroxyanisole and ethoxyquin. *Biochem. Soc. Trans.* **28**, 33–41.

Irie, H., Asano-Hoshino, A., Sekino, Y., Nogami, M., Kitagawa, T., and Kanda, H. (2010). Striking LD50 variation associated with fluctuations of CYP2E1-positive cells in hepatic lobule during chronic CCl4 exposure in mice. *Virchows Arch.* **456**, 423–431.

Jonker, M. J., Bruning, O., van Iterson, M., Schaap, M. M., van der Hoeven, T. V., Vrieling, H., Beems, R. B., de Vries, A., van Steeg, H., Breit, T. M., *et al.* (2009). Finding transcriptomics biomarkers for in vivo identification of (non-)genotoxic carcinogens using wild-type and Xpa/p53 mutant mouse models. *Carcinogenesis* **30**, 1805–1812.

Kawai, M., Saegusa, Y., Dewa, Y., Nishimura, J., Kemmochi, S., Harada, T., Ishii, Y., Umemura, T., Shibutani, M., and Mitsumori, K. (2010). Elevation of cell proliferation via generation of reactive oxygen species by piperonyl butoxide contributes to its liver tumor-promoting effects in mice. *Arch. Toxicol.* **84**, 155–164.

Kelly, V. P., Ellis, E. M., Manson, M. M., Chanas, S. A., Moffat, G. J., McLeod, R., Judah, D. J., Neal, G. E., and Hayes, J. D. (2000). Chemoprevention of aflatoxin B1 hepatocarcinogenesis by coumarin, a natural benzopyrone that is a potent inducer of aflatoxin B1-aldehyde reductase, the glutathione S-transferase A5 and P1 subunits, and NAD(P)H:quinone oxidoreductase in rat liver. *Cancer Res.* **60**, 957–969.

Klaunig, J. E., and Kamendulis, L. M. (2004). The role of oxidative stress in carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* **44**, 239–267.

Klepeisz, P., Sagmeister, S., Haudek-Prinz, V., Pichlbauer, M., Grasl-Kraupp, B., and Gerner, C. (2013). Phenobarbital induces alterations in the proteome of hepatocytes and mesenchymal cells of rat livers. *PLoS One* **8**, e76137.

Kobliakov, V. A. (2010). Mechanisms of tumor promotion by reactive oxygen species. *Biochemistry (Mosc)* **75**, 675–685.

Kossler, N., Matheis, K. A., Ostenfeldt, N., Bach Toft, D., Dhalluin, S., Deschl, U., and Kalkuhl, A. (2015). Identification of specific mRNA signatures as fingerprints for carcinogenesis in mice induced by genotoxic and non-genotoxic hepatocarcinogens. *Toxicol. Sci.* **143**, 277–295.

Lempiainen, H., Couttet, P., Bolognani, F., Muller, A., Dubost, V., Luisier, R., Del Rio Espinola, A., Vitry, V., Unterberger, E. B., Thomson, J. P., *et al.* (2013). Identification of Dlk1-Dio3 imprinted gene cluster noncoding RNAs as novel candidate biomarkers for liver tumor promotion. *Toxicol. Sci.* **131**, 375–386.

Lempiainen, H., Muller, A., Brasa, S., Teo, S. S., Roloff, T. C., Morawiec, L., Zamurovic, N., Vicart, A., Funhoff, E., Couttet, P., *et al.* (2011). Phenobarbital mediates an epigenetic switch at the constitutive androstane receptor (CAR) target gene Cyp2b10 in the liver of B6C3F1 mice. *PLoS One* **6**, e18216.

Luisier, R., Lempiainen, H., Scherbichler, N., Braeuning, A., Geissler, M., Dubost, V., Muller, A., Scheer, N., Chibout, S. D., Hara, H., *et al.* (2014). Phenobarbital induces cell cycle transcriptional responses in mouse liver humanized for constitutive androstane and pregnane X receptors. *Toxicol. Sci.* **139**, 501–511.

Mena, S., Ortega, A., and Estrela, J. M. (2009). Oxidative stress in environmental-induced carcinogenesis. *Mutat. Res.* **674**, 36–44.

Muller, M. (2002). 1,4-Dichlorobenzene-induced liver tumors in the mouse: Evaluation of the role of chlorohydroquinones. *Rev. Environ. Health* **17**, 279–290.

Natarajan, S. K., Thomas, S., Ramamoorthy, P., Basivireddy, J., Pulimood, A. B., Ramachandran, A., and Balasubramanian, K. A. (2006). Oxidative stress in the development of liver cirrhosis: A comparison of two different experimental models. *J. Gastroenterol. Hepatol.* **21**, 947–957.

O'Connor, T., Ireland, L. S., Harrison, D. J., and Hayes, J. D. (1999). Major differences exist in the function and tissue-specific expression of human aflatoxin B1 aldehyde reductase and the principal human aldo-keto reductase AKR1 family members. *Biochem. J.* **343**(Pt 2), 487–504.

Reddy, J. K., and Rao, M. S. (1978). Enhancement by Wy-14,643, a hepatic peroxisome proliferator, of diethylnitrosamine-initiated hepatic tumorigenesis in the rat. *Br. J. Cancer* **38**, 537–543.

Reddy, J. K., Rao, S., and Moody, D. E. (1976). Hepatocellular carcinomas in acatalasemic mice treated with nafenopin, a hypolipidemic peroxisome proliferator. *Cancer Res.* **36**, 1211–1217.

Ross, J., Plummer, S. M., Rode, A., Scheer, N., Bower, C. C., Vogel, O., Henderson, C. J., Wolf, C. R., and Elcombe, C. R. (2010). Human constitutive androstane receptor (CAR) and pregnane X receptor (PXR) support the hypertrophic but not the hyperplastic response to the murine nongenotoxic hepatocarcinogens phenobarbital and chlordane in vivo. *Toxicol. Sci.* **116**, 452–466.

Rusyn, I., Asakura, S., Pachkowski, B., Bradford, B. U., Denissenko, M. F., Peters, J. M., Holland, S. M., Reddy, J. K., Cunningham, M. L., and Swenberg, J. A. (2004). Expression of base excision DNA repair genes is a sensitive biomarker for in vivo detection of chemical-induced chronic oxidative stress: Identification of the molecular source of radicals responsible for DNA damage by peroxisome proliferators. *Cancer Res.* **64**, 1050–1057.

Sandalio, L. M. (2013). Peroxisomes and their key role in cellular signaling and metabolism. In *Subcellular Biochemistry* (L. A. del Rio, ed.), **69**, 231–255. Springer, The Netherlands.

Schaap, M. M., Zwart, E. P., Wackers, P. F., Huijskens, I., van de Water, B., Breit, T. M., van Steeg, H., Jonker, M. J., and Luijten, M. (2012). Dissecting modes of action of non-genotoxic carcinogens in primary mouse hepatocytes. *Arch. Toxicol.* **86**, 1717–1727.

Shirai, M., Arakawa, S., Miida, H., Matsuyama, T., Kinoshita, J., Makino, T., Kai, K., and Teranishi, M. (2013). Thioacetamide-induced hepatocellular necrosis is attenuated in diet-induced obese mice. *J. Toxicol. Pathol.* **26**, 175–186.

Skolimowski, J. J., Cieslinska, B., Zak, M., Osiecka, R., and Blaszczyk, A. (2010). Modulation of ethoxyquin genotoxicity by free radical scavengers and DNA damage repair in human lymphocytes. *Toxicol. Lett.* **193**, 194–199.

Son, Y., Lee, J. H., Chung, H. T., and Pae, H. O. (2013). Therapeutic roles of heme oxygenase-1 in metabolic diseases: Curcumin and resveratrol analogues as possible inducers of heme oxygenase-1. *Oxid. Med. Cell. Longev.* **2013**, 639541.

Suhua, W., Rongzhu, L., Changqing, Y., Guangwei, X., Fangan, H., Junjie, J., Wenrong, X., and Aschner, M. (2010). Lipid peroxidation and changes of trace elements in mice treated with paradichlorobenzene. *Biol. Trace Elem. Res.* **136**, 320–336.

Tasaki, M., Kuroiwa, Y., Inoue, T., Hibi, D., Matsushita, K., Ishii, Y., Maruyama, S., Nohmi, T., Nishikawa, A., and Umemura, T. (2013). Oxidative DNA damage and in vivo mutagenicity caused by reactive oxygen species generated in the livers of p53-proficient or -deficient gpt delta mice treated with non-genotoxic hepatocarcinogens. *J. Appl. Toxicol.* **33**, 1433–1441.

Thomson, J. P., Hunter, J. M., Lempiainen, H., Muller, A., Terranova, R., Moggs, J. G., and Meehan, R. R. (2013). Dynamic changes in 5-hydroxymethylation signatures underpin early and late events in drug exposed liver. *Nucleic Acids Res.* **41**, 5639–5654.

Thomson, J. P., Moggs, J. G., Wolf, C. R., and Meehan, R. R. (2014). Epigenetic profiles as defined signatures of xenobiotic exposure. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* **764–765**, 3–9.

Thomson, J. P., Lempiaeinen, H., Hackett, J. A., Nestor, C. E., Mueller, A., Bolognani, F., Oakeley, E. J., Schuebeler, D., Terranova, R., Reinhardt, D., *et al.* (2012). Non-genotoxic carcinogen exposure induces defined changes in the 5-hydroxymethylome. *Genome Biol.* **13**, R93.

Wang, P. R., Xu, M., Toffanin, S., Li, Y., Llovet, J. M., and Russell, D. W. (2012). Induction of hepatocellular carcinoma by in vivo gene targeting. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 11264–11269.

Wijsman, J. A., Obert, L. A., Paulissen, J., Garrido, R., Toy, K. A., and Dunstan, R. W. (2007). A practical method to determine the amount of tissue to analyze using laser scanning cytometry. *Cytometry A* **71**, 501–508.

Young, R., Wolf, C. R., Brown, K., Hayes, J. D., and Whitelaw, C. B. A. (2010). Spatial monitoring of toxicity in HMOX-LacZ transgenic mice. *Transgenic Res.* **19**, 897–902.