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The association between chemical-induced porphyria and hepatic cancer

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The haem biosynthetic pathway is of fundamental importance for cellular metabolism both for the erythroid and nonerythroid tissues. There are several genetic variants of the pathway in the human population that cause dysfunction of one or other of the enzymes resulting in porphyrias of varying severity. Serious chronic hepatic and systemic diseases may result. Some of these can be precipitated by exposure to drugs including hormones, barbiturates and antibiotics, as well as alcohol and particular chlorinated aromatic chemicals. In experimental animals some of the steps of this pathway can also be severely disrupted by a variety of environmental chemicals, potential drugs and pesticides, especially in the liver, leading to the accumulation of uroporphyrins derived from the intermediate uroporphyrinogens or protoporphyrin IX, the immediate precursor of haem. With some of these chemicals this also leads to cholestasis and liver cell injury and eventually hepatic tumours. The review evaluates the available evidence linking hepatic porphyria with carcinogenesis in naturally occurring human genetic conditions and in chemically-induced porphyrias in laboratory animals. The existing data showing gender, strain, and species differences in sensitivity to the chemical-induced porphyrias, liver injury and liver tumours are discussed and the role that transgenically altered mouse models have played in defining the varying mechanisms. Finally, the review proposes a novel, unifying hypothesis linking the hepatotoxicity induced by the accumulation of various porphyrins, with the increased risk of developing hepatic cancer as a long term consequence.

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1. Introduction

The hepatic toxicities of a variety of drugs, pesticides and environmental chlorinated chemicals in animals include marked disturbances of the haem biosynthetic pathway.[1–4] The formation of haem in mammals is an essential pathway accounting for much of the use of iron either for transport and storage of oxygen, or for its use in cytochromes of respiration, oxygenases and signalling. Most of the haem synthesized in the body is used in the bone marrow for haemoglobin and about 10% for intracellular metabolism. However, although virtually all cells require haem, the liver accounts for the greatest amount of non-erythrocyte haem formation, not only for mitochondrial function and steroid, bile acid and prostaglandin synthesis, but particularly in the metabolism of drugs, xenobiotics and plant constituents. The hepatic pathway is very responsive to the demand for haem as in circadian rhythm and in induction of cytochrome P450, whilst turnover can be quickly stimulated by the action of inducible haem oxygenase 1 (HMOX1). In humans, dominant or recessive genetic variants of the pathway can lead to porphyrias, malfunctions and accumulation of intermediate precursors of haem some of which may be toxic (including oxidized product porphyrins), and have serious clinical outcomes.[5] The genetic penetrance can be very low and the clinical disorder may be precipitated by exogenous factors such as alcohol or by changes in physiological demand for haem controlled by hormones or nutrition.[4] Some chemicals cause porphyria in rodents similar to types of human porphyrias without the requirement of predisposing gene variants of haem synthesis.[3,4]

The incidences of liver cancer are higher than expected in surveys of patients with some types of clinical porphyria where the numbers are sufficient for valid studies. Many of the chemicals that cause porphyria in rodents cause eventually hepatocellular tumours. This review focuses on some drugs, herbicides and chlorinated aromatic chemicals that cause both porphyria and liver tumours or precancerous changes in rodents and compares with types of human porphyrias in which associations with increased incidences of liver cancer have been described.

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2. Interference of haem synthesis by chemicals

The biosynthesis of haem involves eight steps in which simple precursors from the tricarboxylic acid (TCA) cycle are built into the complex macrocyclic molecule, protoporphyrin IX, into which iron is then incorporated (Fig. 1). Both in the liver and red blood cells, the first step is the condensation between glycine and succinyl CoA to give 5-aminolaevulinate (5-ALA). Condensation differs between non-erythroid and erythroid tissue being catalyzed by different 5-aminolaevulinate synthetase enzymes (ALAS1 and ALAS2) regulated by available haem or iron pools respectively.[6] Particular enzymes of the haem pathway in the liver are affected by a variety of chemicals both directly and indirectly (Fig. 1 and Table 1). Some are susceptible to direct inhibition by metal ions, drugs, herbicides or their metabolites. Other enzymes are inhibited by xenobiotic metabolites generated endogenously from haem precursors after stimulation of xenobiotic responsive pathways, some of which still need a more complete understanding. Inhibition of a particular enzyme can lead to accumulation of intermediate porphyrinogens, and their oxidized product porphyrins, and can be compounded by feedback stimulation of the rate controlling enzyme ALAS1. When inhibition of the haem pathway occurs, it is the pathological accumulation of hepatic porphyrins in rodent liver, by some of these drugs and other chemicals, that is causal in inducing hepatic toxicity. The toxicity may not be due entirely to the porphyrins *per se*; porphyria and liver injury may also be the outcome of common mechanisms, probably of an oxidative nature. While carcinogenicity studies have been carried out for some of these porphyrogenic chemicals, many may not have been tested in cancer studies. Most of the porphyrogenic chemicals that have been shown to cause liver tumours in rodents are associated with hepatic toxicity and are thought to be carcinogenic by non-genotoxic mechanisms, perhaps as a consequence of their cellular toxicity, of which the key initiating events (KIE) may have been hypothesized but not yet elucidated.

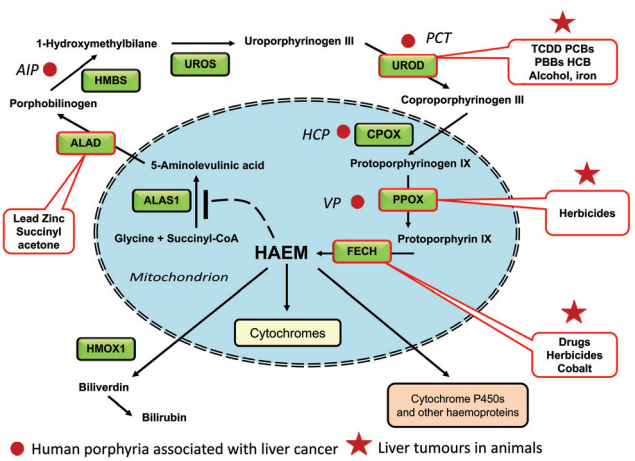

Fig. 1 Nonerythrocyte haem synthesis showing enzymic steps that are affected by chemicals and drugs *in vivo* leading to malfunctions of haem production. ALAS1, 5-aminolevulinic acid synthase; ALAD, aminolevulinic acid dehydratase; HMBS, 1-hydroxymethybilane synthase; UROS, uroporphyrinogen III synthase; UROD, uroporphyrinogen III decarboxylase; CPOX, coproporphyrinogen III oxidase; PPOX protoporphyrinogen IX oxidase; FECH, ferrochelatase; HMOX1, haem oxygenase 1. AIP, acute intermittent porphyria; HCP, hereditary coproporphyria; PCT, porphyria cutanea tarda; VP, variegate porphyria.

3. Hepatocellular cancer in porphyria patients

All but one of the enzyme steps of non-erythrocyte haem biosynthesis are associated in humans with rare, but clinically established, phenotypes of porphyria or related disorders which are caused by recessive variants, or autosomal dominant variants, triggered by endogenous or exogenous factors.[4,5] A disorder associated with genetic variants of ALAS1 is not known, possibly because either it would be lethal, or because of the potential for the gene to be upregulated to compensate for any deficiency in enzyme activity (Fig. 1). For some types of porphyria, particularly acute intermittent porphyria (AIP), and porphyria cutanea tarda (PCT), higher incidences of primary liver cancer in patients than a reference population have long been reported from different countries.[7–10]

In patients with acute hepatic porphyrias, AIP, variegate porphyria (VP) and hereditary coproporphyria (HCP) (due to variants in the *HMBS*, *PPOX*, and *CPOX*, genes respectively, see Fig. 1), acute neurovisceral attacks are triggered by the accumulation of porphyrin precursors in the circulation. Many individuals carrying these mutations will remain asymptomatic but can be triggered into disease after physiological stimuli such as changes in hormones or stress, or following the administration of porphyrogenic drugs such as sex hormones, barbiturates, and sulfonamide antibiotics. The acute attacks are probably associated with the neurotoxicity of highly elevated levels of 5-ALA produced in the liver.[5,11–13] However porphobilinogen, uroporphyrin III, coproporphyrin III and protoporphyrin IX, derived from the malfunctioning haem pathway, also accumulate in the liver depending on the type of acute porphyria. In VP and HCP these haem precursors may contribute to the liver injury, but they also provoke dermal photosensitivity due to deposition in the skin.

Over the last few decades it has become clear that the acute hepatic porphyrias are associated with a markedly increased risk of patients developing primary liver cancer (HCC), at least for Northern European countries and without underlying cirrhosis. This was first described for AIP from northern Sweden[9] and now confirmed in a number of other independent studies of not only AIP but also VP and HCP in countries such as Sweden, Norway, France and Switzerland. Investigations on Finnish and Swedish cohorts with AIP reported increased risk ratios for HCC compared to reference populations of up to about 100 times. It is unclear why reports are only from European countries but this may simply reflect health data organizational arrangements and clinical interest.[14–20] Study

Table 1 Summary of chemicals and human genetic conditions associated with the production of hepatic porphyria and their link to liver cancer

Chemical	Main porphyrin involved[a]	Nuclear receptor?	Molecular MOA	Hepatotoxicity	Species	Carcinogenic response (incidence)	Ref.
Rodent chemical porphyrogen							
3-[2-(2,4,6-Trimethylphenyl)-thioethyl]-4-methylsydnone (TTMS)	Protoporphyrin IX	CAR/PXR	Ferrochelatase inhibition	Wt↑; inflammation; necrosis; hepatocellular hypertrophy	Mouse	Unknown	87 and 88
Tralkoxydim	Protoporphyrin IX	CAR/PXR	Ferrochelatase inhibition	Wt↑; inflammation; necrosis; hepatocellular hypertrophy; bile duct hyperplasia; porphyrin	Mouse ≫ rat = hamster ≫ human	(–ve rat, hamster) Not conducted in mouse!	87, 94 and 100
Griseofulvin	Protoporphyrin IX	CAR/PXR	Ferrochelatase inhibition	Wt↑; inflammation; necrosis; hepatocellular hypertrophy; porphyrin	Mouse M > F ⋙ human	~90% mouse (–ve in rat, hamster)	85, 86, 98, 104 and 105
3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC)	Protoporphyrin IX	CAR/PXR	Ferrochelatase inhibition	Wt↑; inflammation; necrosis; hepatocellular hypertrophy in mouse, rat and chick, porphyrin	Rat = mouse = chick	Unknown	2, 80 and 81
Fomesafen	Protoporphyrin IX	N/A	Inhibition of PPOX	ALT↑ liver wt↑ in mouse; porphyrin	Mouse ICR	Yes; mouse (16% HCC; 100% AHF)	77
Human & rodent chemical porphyrogen							
Polychlorinated biphenyls (PCBs)	Uroporphyrin	AHR agonist	Inhibition of UROD	Wt↑; inflammation; necrosis, hypertrophy; porphyrin	Rat = mouse ≫ human	Yes; mouse > rat	36 and 56
2,3,7,8-Tetrachorodibenzo-*p*-dioxin (TCDD)	Uroporphyrin	AHR agonist	Inhibition of UROD	Wt↑; inflammation; necrosis, hypertrophy; porphyrin	Rat = mouse ≫ human	Yes; mouse > rat	3, 34, 35, 54 and 121
Hexachlorobenzene (HCB)	Uroporphyrin	AHR agonist?	Inhibition of UROD	Wt↑; inflammation, necrosis; hypertrophy; porphyrin	Rat = mouse ≫ Human	Yes; mouse > rat	37–39, 54 and 122
Human genetic disorders							
Acute intermittent porphyria (AIP)	Porphobilinogen	N/A	Hydroxymethylbilane synthase (HMBS) deficiency	Drug-induced severe hepatitis	Human	Yes	9 and 14–21
Porphyria cutanea tarda (PCT)	Uroporphyrin	N/A	Uroporphyrinogen decarboxylase (UROD) deficiency	Mild-moderate hepatitis	Human	Yes (5–12% of patients)	10 and 25–31
Variegate porphyria (VP)	Porphobilinogen	N/A	Protoporphyrinogen oxidase (PPOX) deficiency	Moderate hepatitis	Human	Yes	21
Erythropoietic porphyria (EPP)	Protoporphyrin IX	N/A	Ferrochelatase (FECH); less commonly 5-aminolevulinic acid synthase-2 (ALAS2)	Drug-induced hepatitis	Human	Yes	5, 78 and 117
Hereditary coproporphyria (HCP)	Porphobilinogen; coproporphyrin	N/A	Deficiency in coproporphyrinogen oxidase (CPOX)	Drug-induced hepatitis	Human	Yes	5, 11 and 13

[a] Does not include elevation of plasma and hepatic 5-ALA which may be substantial in some experimental and human acute porphyrias. N/A Not applicable.

of an AIP and of a VP patient has suggested that as well as the underlying *HMBS* and *PPOX* germline mutations, the hepatic tumour tissue also had second-hit somatic mutations in the respective genes.[21] Whether these second mutations occurred incidentally or were instrumental in the subsequent development of the hepatic cancer is unknown and their role in the chronic elevation of heme precursors such as 5-ALA, porphobilinogen and porphyrins, remains unclear. It is possible that 5-ALA is pro-oxidant and genotoxic in hepatocytes[22] but perhaps other mechanisms may operate such as dysfunction of the mitochondria if haem synthesis is severely disturbed. What does seem clear is that, like clinical AIP, the development of HCC is more likely to occur in women than in men. In addition, unlike most cases of HCC, the incidence of cirrhosis is low and without other major findings of underlying liver disease in these porphyric patients.[23]

Type 1 tyrosinemia is a genetic disease of tyrosine metabolism in which fumarylacetoacetate hydrolase (FAH) is inefficient and some of the neurological and other symptoms resemble those of AIP. Side reactions of accumulating fumarylacetoacetate and maleylacetoacetate form succinylacetone, a potent inhibitor of aminolevulinate dehydratase (ALAD) (Fig. 1). Patients can be treated with nitrisinone (2-(2-nitro-trifluoro-methylbenzoyl-1,3-cyclohexadione) originally developed as a pesticide) restricting precursors for FAH.[136] If untreated there is a high incidence of liver damage and HCC in these patients.[137]

PCT patients show marked inhibition of hepatic uroporphyrinogen decarboxylase activity (UROD) with mild or moderate liver damage and an increased susceptibility to developing liver cancer (Fig. 1). Under uv light the uroporphyrins and other porphyrins arising from disruption of the haem pathway fluoresce red in the liver due to their deposition. Porphyrins released into circulation are deposited elsewhere in the body and can cause marked cutaneous photosensitivity. Unlike the acute porphyrias there is no marked rise in plasma or urinary 5-ALA and no neurotoxicity. Autosomal dominant inheritance with low penetrance of a *UROD* gene variant is a contributing factor for familial form of PCT. However, most patients have a sporadic form with no apparent association with adverse variants of the gene.[5] Consumption of alcohol, oestrogenic drugs, other chemicals and hepatitis viruses have all been reported as precipitating agents for PCT, even for the familial form which may occur at a younger age.[3] Most of the PCT patients are diagnosed at middle age or older. This is the most common type of clinical porphyria in many populations. Iron metabolism is implicated in the pathogenesis of PCT and depletion of body iron stores is one treatment approach. For some patient cohorts inheritance of the haemochromatosis *HFE* variant has been a strong risk factor in the development of the porphyria.[24] The role of iron is not really understood, other than possibly promoting reactive oxygen species, but must clearly be the consequence of genetic variants in iron mobilization perhaps releasing Fe^{2+}. This has been discussed in more detail elsewhere.[3] A disorder very similar to PCT occurred in many people, particularly children, poisoned by hexachlorobenzene (HCB) and there is evidence that other highly chlorinated aromatic chemicals such as the 2,3,7,8-tetrachorodibenzo-*p*-dioxin (TCDD) and polychlorinated biphenyls (PCBs) have a similar potential.[3]

As with the acute porphyrias, there is clear evidence of an association between PCT and the risk of developing HCC.[10,25–30] In one of these studies, 342 patients with PCT were observed over a 15-year period, 5% of them died from primary liver cancer.[31] In another study, 6 out of 78 patients with HCC had PCT. Most studies have confirmed the early findings while others have been less positive, probably because of the complex nature of PCT development, and the possible confounding role of concurrent cirrhosis. Recognition of PCT and subsequent treatment may be resulting in a decrease in the likelihood of patients progressing to HCC. However, the subject is of interest in consideration of the risk assessment of cancer associated with exposure to environmental levels of polychlorinated aromatic chemicals.

4. Hepatic tumours in rodents caused by porphyria-inducing chemicals

Although metals such as lead and mercury are known to interfere in haem synthesis, both in liver and in erythrocytes, the resulting clinical chemical signs from human and experimental studies are usually porphyrinurias, frequently increased coproporphyrin I or III, or excretion in stool of protoporphyrin rather than overt porphyrias.[1] Sometimes these reflect relatively mild disturbances of haem synthesis. What appears to be especially important in determining the onset, or otherwise, of hepatic porphyrias is the particular enzyme in the pathway that is affected and permits the accumulation of toxic haem precursors. In lead poisoning there is inhibition of haem biosynthesis but the metal has been shown to inhibit ALA dehydratase resulting in increased urinary 5-ALA without affecting porphobilinogen or resulting in the hepatic build-up of toxic porphyrins. Hence while lead is known to cause porphyrinuria with an increased excretion of coproporphyrins, its inability to inhibit markedly later enzymes prevents the onset of hepatic porphyrias and their dire consequences.[135] Drugs and chemicals that trigger AIP in humans do not do so in animals without genetic models particularly the disrupted *HMBDS* gene.[32,33]

Some of the chemicals and drugs known to cause disturbance of hepatic haem synthesis in animals, and sometimes in humans, induce not only overt porphyrias in rodents but also induce hepatocellular cancer, or forms of chronic liver injury which might predispose to cancer.

4a. Chemicals causing hepatic uroporphyria

HCB, dioxins and PCBs have been known for many years to cause hepatic symptoms in rodents similar to human PCT with inhibition of UROD (probably through an oxidative

sequence involving oxidation of uroporphyrinogen),[34,35] porphyria and liver injury.[3] As with human patients there is strong evidence for a synergistic role of iron metabolism, and genetic predispositions. One key mechanistic aspect to these chemicals for porphyria seems to be through their binding to, and activation of, the aryl hydrocarbon receptor (AHR) with associated induction of hepatic CYP1A enzymes.[3] For chemicals, such as chlorinated dioxins and dioxin-like PCB constituents of commercial PCB mixtures such as Aroclor 1254 and 1260, porphyria potency can be ascribed partly to the toxicity equivalence compared to the prototype AHR ligand, TCDD.[36] However, for HCB the evidence is less clear.[37] Hahn *et al.* concluded that porphyria induced by HCB is caused by weak AHR binding activity of the chemical[38,39] and a genetic locus for susceptibility in mice corresponds to variants of the *Ahr* gene.[40] World Health Organization views did not regard HCB as an AHR ligand and noted that the chemical could be contaminated by dioxins.[36] This has been a four decade debate. In fact, the particular commercial HCB source used by many research laboratories for porphyria studies was chosen originally for its known high purity as a chemical standard[41,42] and shown to have CYP1A inducing properties in rodents.[43,44] Up to now the levels of contaminants present in this source have not been fully reported. The levels of TCDD equivalents due to PCDDs and PCDFs present in this HCB are now shown in Table 2. Comparison with previous porphyria experiments suggest the levels are too low to account significantly for *in vivo* hepatic porphyrogenic activity of HCB in rodents and that probably it can act as a weak AHR ligand.

In subchronic studies in rats HCB, TCDD, PCB and PBB mixtures have all shown a marked preponderance of females compared to males in inhibition of hepatic UROD activity and developing porphyria.[45,46] Interestingly, this marked sexual dimorphism in porphyria also occurs in chronic studies of the development of liver tumours with these chemicals (Fig. 2).[47–53] This is in contrast to a range of other genotoxic and nongenotoxic hepatocarcinogens. With HCB, the hepatotoxicity gives rise to peliosis and necrosis with haemosiderosis which may increase tumourgenic potential.[54] The development of hepatocellular cancer in rats has been of some interest for the risk assessment of polyhalogenated aromatic chemicals such as TCDD and PCBs. The promotion of rodent liver hepatic carcinogenesis by these chemicals has been investigated and discussed in detail[55–57] but the reason for initiation of tumourgenesis has not been explored fully although a number of earlier hypotheses sought to explain the predominance of tumours in females. Initiation with diethylnitrosamine (DEN) followed by TCDD or other polyhalogenated chemicals has often been used to model the complete neoplastic process.[57] Comparison of hepatic tumours in the rat, caused by HCB, with hepatic tumours from rats first given an initiating dose of DEN followed by a promoting regimen of HCB, showed markedly different results. There was no consistent sex difference in timing or severity with the initiation-promotion regimen.[58] When tissue from the two regimens was compared under uv light for uroporphyrin fluorescence as a consequence of hepatic porphyria the distribution of porphyrins between tumour and non tumour areas were very different (Fig. 3).

Table 2 Concentrations of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in Organic Standard grade hexachlorobenzene (HCB) a source used in many published experimental studies[a]

PCDDs or PCDFs in HCB	Concentration ng g^{-1} of solid	WHO TEF (2005)	TEQ concentration ng g^{-1} of solid
2,3,7,8-TCDD	88.00	1	88.0
1,2,3,7,8-PeCDD	<0.59	1	0.6
1,2,3,4,7,8-HxCDD	<0.59	0.1	0.6
1,2,3,6,7,8-HxCDD	<0.94	0.1	0.1
1,2,3,7,8,9-HxCDD	<0.71	0.1	0.1
1,2,3,4,6,7,8-HpCDD	18.94	0.01	0.2
OCDD	16 011	0.0003	4.8
2,3,7,8-TCDF	<1.03	0.1	0.1
1,2,3,7,8-PeCDF	<0.47	0.03	0.01
2,3,4,7,8-PeCDF	<4.35	0.3	0.1
1,2,3,4,7,8-HxCDF	2.82	0.1	0.3
1,2,3,6,7,8-HxCDF	1.76	0.1	0.2
1,2,3,7,8,9-HxCDF	<1.06	0.1	0.1
2,3,4,6,7,8-HxCDF	<1.41	0.1	0.1
1,2,3,4,6,7,8-HpCDF	1060	0.01	10.6
1,2,3,4,7,8,9-HpCDF	30.6	0.01	0.3
OCDF	5978	0.0003	1.8
Total			108

[a] Organic Standard grade HCB for elemental analyses was from B.D.H. Ltd, UK. and analysed for A.G.S. (2005) at the Central Science Laboratory, DEFRA, York, UK by high resolution gas chromatography with high resolution mass spectrometry for PCDDs and PCDFs using ^{13}C-labelled analogues for standardisation. Results were UKAS accredited. WHO Toxic Equivalent Factors (TEFs)[36] were used to calculate Toxic equivalents to TCDD (TEQs) with upper values of ranges. Comparison of TEQs present in HCB with doses of TCDD that cause porphyria in rats and mice showed levels were insufficient to account for the responses.[46,59,133,134]

Given the evidence for a role of iron metabolism in PCT it was of interest that HCB porphyria in female rats seemed to be associated with iron status,[47,59] and also CYP1A2 inducibility.[46] Iron overload moderately enhanced hepatic carcinogenesis induced by HCB alone.[60] In other studies with aflatoxin B1 and DEN, iron overload had no, or a reducing, effect on the incidence of liver carcinogenesis in rats (A. G. Smith, unpublished data). Thus rat studies might suggest a link between porphyria, perhaps inducing cytotoxicity, and liver cancer caused by polyhalogenated aromatic chemicals, and which may have a parallel in human PCT.

In mice, the development of porphyria by TCDD, PCBs and HCB was found to be influenced profoundly by hepatic iron status during experiments designed to explore mechanistic models of human PCT.[3] Administration of iron not only enhanced porphyria, and hepatic toxicity, in mice with an AHR showing high affinity for TCDD, but overcoming resistance in some mouse strains with low affinity AHR.[61,62] Subsequent studies demonstrated that there are genetic loci in mice independent of the *AHR* gene that contribute to the development

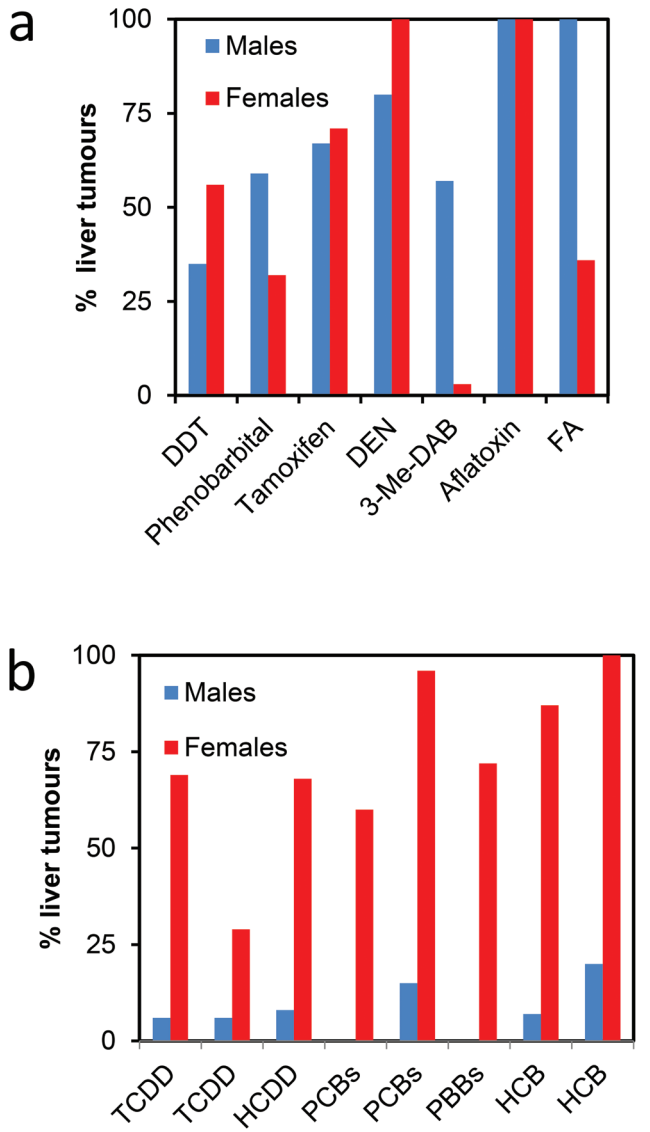

Fig. 2 Incidences of liver tumours in toxicological studies of male and female rats. (a). Selection of some genotoxic and nongenotoxic carcinogens.[126–131] (b). Polyhalogenated aromatic chemicals that also cause hepatic porphyria.[47–53] Additional references of hepatocarcinogens can be found in Smith (2003).[139] DEN, diethylnitrosamine, FA or AAF, *N*-2-fluorenylacetamide or 2 acetylaminofluorene; 3-Me-DAB, 3′-methyl-4-dimethylaminoazobenzene, tamoxifen and aflatoxin may be regarded as genotoxic carcinogens; DDT and phenobarbital may be regarded as nongenotoxic.

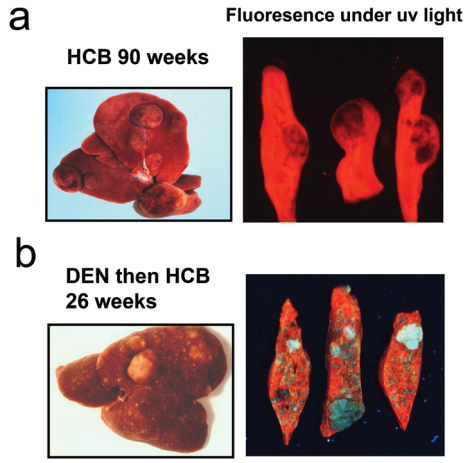

Fig. 3 Difference between liver tumours in female rats caused by HCB alone and in those initiated with diethylnitrosamine (DEN) and promoted with HCB. (a) Female rats were fed HCB in diet (0.02%) for 90 weeks.[47] (b) Female rats received DEN in the drinking water (0.015%) for 3 weeks then after 2 weeks' rest were administered HCB in the diet (0.02%) for 30 weeks.[58] Sections of liver were examined under uv light to demonstrate red fluorescence of uroporphyrin. Studies were conducted originally under the authorizations of UK Home Office regulations in the MRC toxicology unit.

of porphyria and hepatic toxicity.[63] The genes responsible have not yet been identified but we would suggest some of them are associated with iron mobilization and use. Importantly, in chronic experiments with the AHR responsive strain C57BL/10ScSn, not only were the porphyria and hepatic toxicity inducing properties of HCB and PCBs markedly potentiated by prior iron treatment but so too was the subsequent incidence of liver adenomas and cancer to a marked degree.[64,65] In contrast, the low affinity AHR DBA/2 strain also possessing other resistant loci was highly refractory to both porphyria and hepatocarcinogenesis following iron and PCBs. *CYP1A2* gene null C57BL/6J mice with no CYP1A2 expression (mostly under control of the AHR) are highly resistant to porphyria caused by TCDD and HCB, even after iron loading pretreatment.[66,67] The exact role of CYP1A2 in the porphyria mechanisms is still unclear although postulated to be in uroporphyrinogen oxidation. *CYP1A2* knockout mice did not develop hepatocellular foci or adenomas following treatment with PCBs[68] Octachlorostyrene, which only causes mild porphyria in iron-loaded C57BL10ScSn mice, was only weakly tumourgenic in contrast to HCB in the same mouse strain.[69] Other nongenotoxic carcinogens in mice such as phenobarbital and nafenopin, were unaffected by iron pretreatment. Hepatocellular tumors resected from mice following treatment with HCB and PCBs did not show increased incidences of *Ha-ras* mutations but did show the emergence of a mononucleated diploid population consistent with precancerous changes.[70,71] Tumour explants derived from the induced hepatocellular cancers could be passaged subcutaneously many times in syngeneic mice and showed many similarities whether of HCB or PCB origins. Homozygous knockout of the *P53* gene in mice had little influence on the liver tumour incidence. Uroporphyria in rodents and PCT are likely due to an oxidative mechanism. Increased oxidative DNA damage was detected in C57BL/10ScSn mice after treatment with PCBs.[3,72] Point mutations could not be detected using an *in vivo* mutation system suggesting that more complex chromosomal deletions or rearrangement are involved in the oxidative damage or in the mutagenicity of the porphyrins.[73] However, other mechanisms associated with disturbed production of haem could be involved but are yet to be understood.

4b. Protoporphyrinogen oxidase inhibitors

Some chemicals that are inhibitors of protoporphyrinogen IX oxidase (PPOX) in green plants have been formulated as herbicides for post emergence weeds.[74] Early products were diphenyl ethers, although later generation herbicides are often based on non-oxygen bridge compounds. The protoporphyrinogen that accumulates in an unregulated way is light sensitive and is oxidized chemically, rather than enzymically, to protoporphyrin IX which may catalyse oxygen free radical-induced damage in the photosynthetic plant cell. These herbicides can have similar inhibitory properties in mammals and although the protoporphyrin IX formed in the liver is not exposed to light, it still has hepatotoxic potential. The toxicity of these PPOX inhibitors in animals might be considered as having some similarities with variegate porphyria (Fig. 1) but the risk to humans from herbicide use has been considered to be very low due to their low exposure, rapid metabolism and excretion. However, chronic administration to rodents of a diphenyl ether PPOX inhibitor, fomesafen, has been studied particularly with respect to its possible mammalian porphyria inducing properties and toxicity.[75,76] Interestingly, in iron treated C57BL/6 mice fomesafen enhanced preneoplastic changes in the liver such as frequency of altered foci and nodules.[77]

4c. Drugs and pesticides that inhibit ferrochelatase

In humans with erythropoietic porphyria (EPP) the enzyme ferrochelatase that catalyzes the incorporation of iron into protoporphyrin IX is compromised and some patients have severe hepatic disease probably reflecting mostly the deposition of toxic erythrocytic protoporphyrin from the blood into the liver (Table 1).[5,78] There are several chemicals that have been shown to induce a similar porphyria in rodents and other animals through inhibition of hepatic ferrochelatase (Fig. 1). These chemicals are of a variety of types but are common in their ability to convert the haem in some liver cytochrome P450s (CYPs) into *N*-alkylated porphyrins which can be strong inhibitors of ferrochelatase.[2,79] As well as porphyria these drugs and pesticides often cause cholestasis and hepatic toxicity in animals, which may lead to neoplastic lesions, but this can be species specific and has been the subject of risk assessment concerns.

The generalized molecular mechanism of induced protoporphyria with these types of chemicals involves suicidal inactivation of cytochrome P450 with loss of associated enzyme activities (Fig. 4).[2,80,81,94] Reactive metabolites from the parent molecules bind covalently to the prosthetic haem of the cytochrome through *N*-alkylation of one of the pyrrole moieties. The alkylated haem then dissociates from the apoprotein and the iron atom is liberated to yield *N*-alkylprotoporphyrin IX compounds (Fig. 5). The subsequent loss of haem results in a feedback upregulation of hepatic ALAS1.[82,83] Stimulation of this enzyme drives increased haem synthesis compounding the inhibition of ferrochelatase and adding to the subsequent hepatic accumulation of porphyrin. In porphyria induced by 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC),

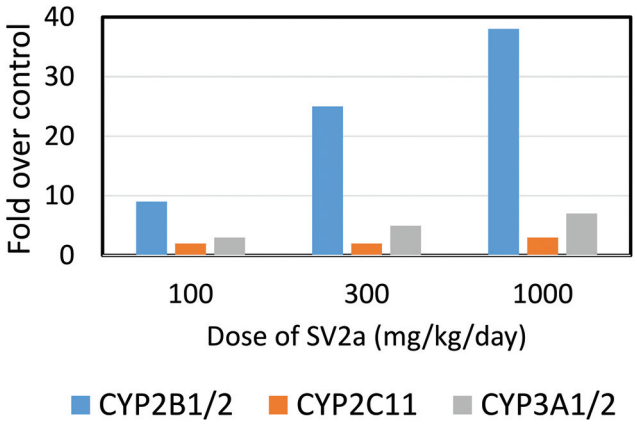

Fig. 4 Effect of a porphyrinogenic agent, an anti-epileptic synaptic vesicle 2a (SV2a) ligand, 2-[4-(2-chloro-2,2-difluoroethyl)-2-oxopyrrolidin-1-yl]butanamide, in causing depressed activity of hepatic phenobarbital-type CYP2B family enzyme in the dog. Data are means from 4 male and 4 female dogs following 4 weeks' administration. Redrawn from Nicolas *et al.* (2014).[94] CYP2B11 was induced by the prodrug but at the highest dose given reduced enzyme activity was observed (but not the apoprotein by immunoblot). At the highest dose ferrochelatase was inhibited, hepatic protoporphyrin IX levels were highly elevated, *N*-alkylated porphyrins were detected, and hepatic transaminases in the plasma were markedly elevated indicating liver injury. Rats dosed with even higher amounts of the ligand (up to 1000 mg kg^{-1} day^{-1}) showed much higher induction of CYP2B enzyme activities but no decrease at the highest dose and only 2-fold increase in porphyrins and very small elevations of plasma transaminases.

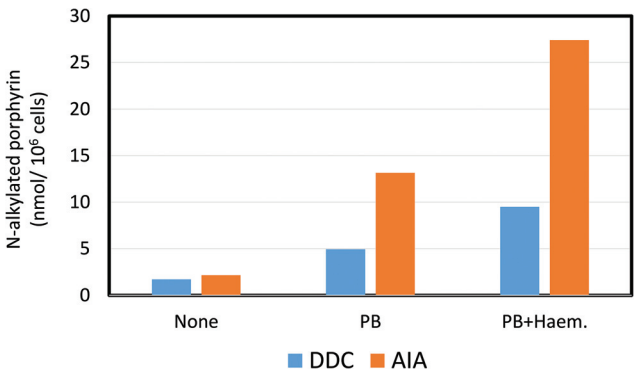

Fig. 5 Effect of the ferrochelatase inhibiting porphyrinogenic agents, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) and allylisopropyl acetamide (AIA) on the *N*-alkylated porphyrin content of isolated rat hepatocytes. PB = the effect of pre-dosing *in vivo* with phenobarbital on the production of *N*-alkylated porphyrins by increasing cytochrome P4502B content. PB + Haem = the effect of pre-exposure with phenobarbital and haematin (to provide additional source of haem) on the production of *N*-alkylated porphyrins. Drawn from data in De Matteis *et al.* (1982).[132]

cytochrome P450 mediated metabolism generates a metabolite from which a 4-methyl substituent is transferred onto the pyrrole nitrogen atom of the haem molecule and the alkylated product, *N*-methyl protoporphyrin, is a powerful inhibitor of ferrochelatase.[79,84] In mice fed (1% of diet) the antifungal

agent griseofulvin, hepatic ferrochelatase activity was decreased to 24% after 3 days. Concomitantly, the ALAS1 activity was increased by 6.6 fold.[85,86]

Other chemicals inducing protoporphyria or causing *N*-alkylated porphyrins in rodents include the anti-arthritic drug, 3-[2-(2,4,6-trimethylphenyl)-thioethyl]-4-methylsydnone (TTMS), ethylene, diethylnitrosamine, norethindrone, and the herbicides, tralkoxydim, ATMP, and ETC.[87–92]

The chemical-induced protoporphyria is associated with significant liver weight increases in susceptible animal species and although the actual cause of the weight increases has not been definitively ascribed, it is most probably multifactorial involving a combination of microsomal CYP induction, hepatic inflammation and necrosis, and hepatocellular hypertrophy.[93,94] Dependent upon the degree of hepatopathy (liver pathology), the weight increases are frequently accompanied by increases in plasma alanine aminotransferase (ALT), alkaline phosphatase, aspartate aminotransferase and, mostly dependent upon the animal species involved, γ-glutamyl transferase.[87,94] Although none of these enzyme biomarkers are diagnostic of the porphyria they reflect the degree of liver damage that may be associated with accumulation of protoporphyrin. In some studies, elevations in bilirubin can also be seen indicating a degree of cholestasis associated with the porphyrin-induced liver damage.[94,95] In mice fed griseofulvin characteristic gene expression profiles were associated with disruption of lipid metabolism, and the pathologic states of liver toxicity, inflammation, early fibrosis, and cholestasis.[86]

Histopathologically the initial accumulation of the protoporphyrin IX occurs as red "Maltese cross" accumulations within the hepatocytes and Kupffer cells. Prolonged dosing of the porphyrogenic chemicals leads to progressive accumulation within the bile canaliculi between hepatocytes, the appearance of crystalline forms of the protoporphyrin IX within distended and hypertrophic bile ducts (Fig. 6a),[87,94,96,97] and hepatocyte degeneration and inflammation, all of which are most severe in the periportal regions of the liver lobules (Fig. 6c).[87,98] The protoporphyrin IX deposits appear birefringent under polarized light and display a red fluorescence under ultraviolet light (Fig. 6b and d). Of particular importance, with regard to the subsequent development of neoplasia by some of these porphyrogenic chemicals, is that induction of mitotic activity in the hepatocytes occurs between 2 to 5 days following administration of griseofulvin in the food[97] and, while not recorded with other porphyrogenic drugs, would be expected to have occurred from the increased liver weight that almost invariably occurs following exposure to high doses of these porphyrogenic chemicals. The increase of mitotic activity in the mouse liver correlates with the increase in liver weight over time reaching 4.2-fold concurrent control liver weight after 4 to 6 months of feeding of griseofulvin at a concentration of 2.5% in the diet. Although few in number, where histological investigations are available of humans with porphyria, in PCT the pattern of pathology is similar to that described in the livers of mice given griseofulvin with the prominent presence of needle-like porphyrin crystals (although uroporphyrin not protoporphyrin), hepatocellular necrosis and regenerative hyperplasia.[25]

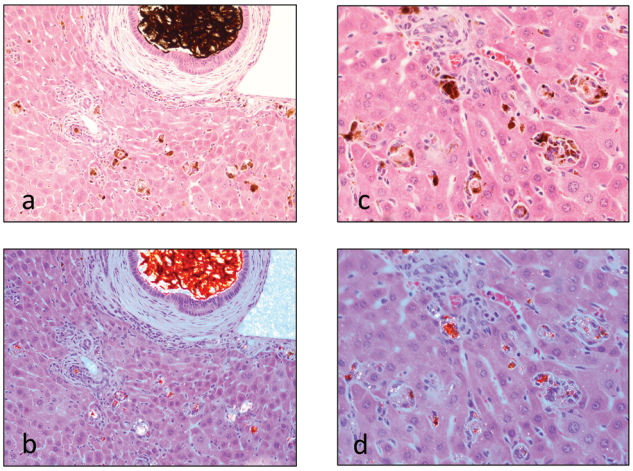

Fig. 6 Periportal area in liver from a mouse given tralkoxydim for 28 days showing distended bile duct with hypertrophic lining mucosa (×20 microscope magnification) (a and b) and bile duct and phagocytic cells with brown pigment accumulation in hepatocytes and phagocytic cells (×40 magnification) (c and d). Viewed by conventional light (a and c) and polarized light demonstrating birefringent red porphyrin crystals (b and d). Liver sections were from studies conducted under the authorizations of UK Home Office regulations.

While some of these chemicals appear to induce porphyria irrespective of animal species, others show distinct strain, species and sex differences in sensitivity, with griseofulvin being more toxic to male than female mouse liver, and tralkoxidim being a potent porphyrogen in the mouse but not in the rat or hamster.[83,95,99,100] Similarly an antiepileptic, synaptic vesicle 2a (SV2a) ligand, drug candidate was found to form a CYP mediated *N*-alkylprotoporphyrin derivative that inhibited ferrochelatase in the dog but not in the rat nor in human hepatocytes *in vitro* (Fig. 4).[94] The species difference operating in this instance is the metabolic ability to generate the reactive metabolite needed to alkylate protoporphyrin IX in the dog as opposed to the insensitive species. In contrast, the porphyrinogenic drug, DDC, induces porphyria as a result of ferrochelatase inhibition in rats, mice and chick livers,[2] while TTMS induces porphyria in rats, mice and dogs.[87] For those chemicals that induce protoporphyria in one or more animal species the outcome appears to be largely, if not solely, determined by the rate of generation of the appropriate CYP dependent hepatic metabolite.[101] Those species that fail to generate appropriate amounts of the alkylated protoporphyrin will not inhibit ferrochelatase leading to protoporphyria. This has been adequately demonstrated for tralkoxydim which induces protoporphria in the mouse *in vivo*, and in mouse hepatocytes *in vitro*, but not in rat *in vivo* nor *in vitro*, nor human hepatocytes.[102] Clearly these species differences are important in consideration of extrapolation to human risks.

In a survey of drug-induced liver injury in humans in Taiwan,[103] of a total of 90 847 patients taking one or more fungicides, including griseofulvin, eight were reported to have suffered hepatic injury, necessitating termination, following treatment with high doses of griseofulvin. Increased porphyrin has been reported in humans given high doses of griseofulvin but it is normally mild in comparison with that seen in the mouse model.[93] In rare cases where the drug was taken at high doses over prolonged periods of time liver damage and serious skin reactions were reported accompanying the protoporphyria.[93] Evidence supporting the drug-induced production of porphyria in man includes a survey of patients receiving griseofulvin and compared to a further group who had been off treatment for between 2–80 weeks.[104] Hence griseofulvin is not recommended for patients suffering pre-existing porphyrias.[105]

Porphyrins are reported to be generators of reactive oxygen species, they affect hepatic redox balance and lead to induction of the antioxidant defense enzyme systems such that mice treated with griseofulvin show increased activity of glutathione reductase, superoxide dismutase, glutathione-*S*-transferase, and increased levels of reduced glutathione and malondialdehyde. In contrast the hepatic activities of glutathione peroxidase and catalase were reduced following griseofulvin administration.[106] Griseofulvin has also been shown to reduce bile flow as a result of direct liver damage and effects on the biliary system, leading to the hepatocytes accumulating toxic endogenous bile acids such as taurocholic and taurodeoxycholic acid.[107]

Hepatocellular carcinomas, at incidences up to 90%, have been observed in mice fed griseofulvin at maximum concentrations of 3% in the diet for periods of up to 120 weeks.[95,108,109] As well as causing accumulation of porphyrin within the liver, griseofulvin also induces necrosis and inflammation in a dose dependent fashion.[106,110] Lifetime studies with griseofulvin, of similar duration to those in mice, in the rat and hamster have not shown the development of liver cancer and neither do these two species show the protoporphyria following shorter duration exposure.[93] It is of note that the NOEL (approximately 0.1% in the diet) for griseofulvin induced liver enlargement and porphyria in mice[111,112] is similar to the NOEL for tumor induction in mice (0.3% in the diet).[109] To complicate factors further, hepatocellular carcinomas have been reported to develop in male mice offspring suggesting that irreversible nuclear changes had occurred after as little as 21 days of exposure.

Lifetime carcinogenicity studies on tralkoxydim have not been carried out in mice, which show protoporphyria after subchronic studies. It is not known if mice would have developed liver cancer after chronic exposure. Carcinogenicity studies have been conducted in rats and hamsters even though porphyria is not seen in sub-chronic studies in either the rat or the hamster. In neither species were hepatocellular neoplasms observed following lifetime exposure to tralkoxydim. The prediction from these studies is that tralkoxydim would be a liver carcinogen in mice if lifetime studies had been carried out, and that the absence of hepatocarcinogenicity in the non-porphyrinogenic rat and hamster is a consequence of the lack of porphyria induced by tralkoxydim in these species.[100]

5. Evidence for pathogenesis from mutated mice

Direct evidence that disruption of haem biosynthesis and the resulting porphyrin-induced hepatopathy can cause hepatic tumours in rodents, has come from genetically modified mice. BALB/c *Fech(m1/Pas1)* mice that have a radiation-induced missense mutation in the ferrochelatase gene (resulting in ~6% activity of wild type), have markedly increased hepatic and serum protoporphyrin IX levels. They also have severe liver toxicity as evidenced by elevated plasma transaminases and bilirubin, cholestasis and pathological evidence of hepatic degeneration and inflammation (Fig. 7).[113,114] In less than a year, these mice develop hepatocellular tumors in the absence of chemical treatment, demonstrating that porphyria alone can be carcinogenic to the liver.[114,115] These mice also show depressed expression of total cytochrome P450 and many of its isoforms in comparison to their expression in wild-type mice. A related mouse model, more pertinent of human EPP, and carrying a targeted mutated ferrochelatase gene, showed significantly enlarged livers and spleens, and a high grade hepatobiliary pathology with a prominent accumulation of protoporphyrin IX in the biliary canaliculi and portal macrophages[116] very similar to that shown in human porphyric patients with a pronounced liver pathology.[117]

Mitoferrin 1 protein is a mitochondrial iron transporter critical for haem synthesis. In a mouse model with a deleted mitoferrin 1 gene, animals fed 5-ALA to stimulate haem synthesis showed increased liver weight in comparison to wild type mice, with homozygotes having appreciably higher liver weights than the heterozygotes.[118] Livers from heterozygous mice showed bile duct proliferation and chronic inflammation in the periportal area with a marked accumulation of protoporphyrin IX pigment in the bile ducts of the 5-ALA-administered mutated mice that was not present in wildtype mice treated similarly. The heterozygous mice had a cholestatic pathology and early fibrosis which was more severe in the homozygous mice. Fibrosis was bridged between the portal and centrilobular areas with the development of hyperplastic liver nodules. Hence in this model, in the presence of increased porphyrin synthesis, deletion of the mitoferrin gene in mouse liver resulted in a decreased ability to convert protoporphyrin IX into haem, leading to protoporphyria, chronic hepatic damage, cholestasis and bridging fibrosis showing similar characteristics to that seen in human EPP.

It is important to consider that mechanisms other than porphyrin-induced liver injury may occur associated with disruption of enzymes of the haem and related pathway, perhaps as a consequence of mitochondria malfunction. Although mitochondrial failure is observed in mice with partial knockout of the hydroxymethylbilane synthase (HMBS) gene, no chronic studies appear to have been conducted that might

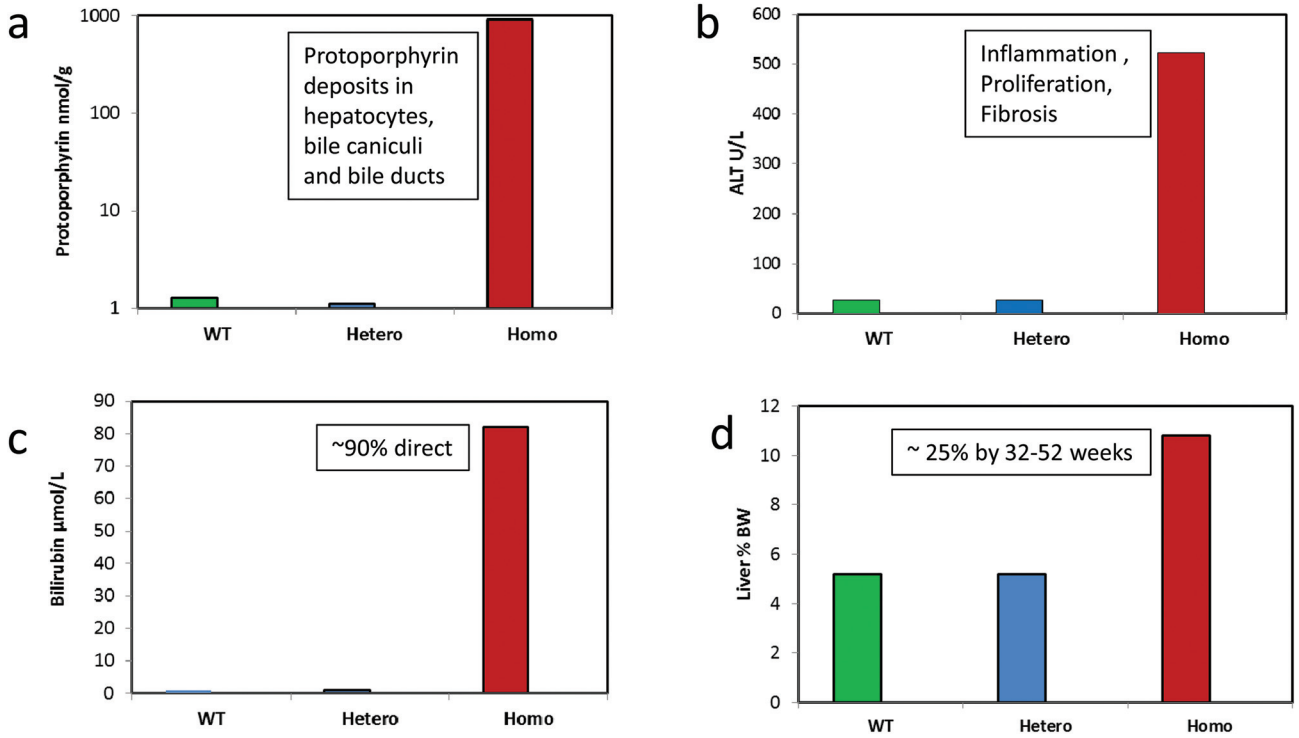

Fig. 7 Effect of severely depressed hepatic ferrochelatase activity causing protoporphyria and hepatic toxicity in homozygote BALB/c *Fech(m1/Pas1)* mice carrying a mutated ferrochelatase gene. (a) Hepatic protoporphyrin levels, (b) plasma transaminase (ALT) levels, (c) plasma total bilirubin levels, (d) liver weight relative to body weight. Data are 8 weeks old male mice from Davies *et al.* (2005) descended from those described by Tutois *et al.* (1991) and produced as an F2 cross from wild type BALB/C and mutant mice (6 WT, 14 heterozygotes, 5 homozygotes).[113,114] Studies were conducted originally in the MRC toxicology Unit under the authorization of UK Home office regulations. Homozygous mice eventually developed eosinophilic and clear cell foci, adenomas and adenocarcinomas.

explain the incidence of liver cancer in AIP patients in which high levels of non-porphyrin precursors of haem are implicated in toxicity.[119] That oxidative processes alone can be tumorigenic is illustrated by the mitochondrial disruption, oxidative stress and subsequent liver tumors that are observed in mice with mutation of the frataxin gene which is involved in hepatic iron and heme metabolism.[120] In a fumarylcetoacetate hydrolase (FAH) knockout mouse model of type 1 tyrosinemia (section 3) producing succinylacetone that inhibits ALAD, sustained activation of stress pathways were associated with hepatocellular damage, steatosis, oval cell proliferation and development of liver adenomas when the protective effect of nitrisinone was withdrawn.[138] One possibility is that this is due to alkylating activity of tyrosine metabolites and related products.

6. An adverse outcome pathway for porphyria-induced liver cancer in rodents

An adverse outcome pathway (AOP) has been proposed for liver cancer induced by dioxin-like compounds through promotion following sustained activation of the AHR.[56] Fig. 8a summarizes this AOP developed by Becker *et al.*[56] In this case the molecular initiating event (MIE) is binding and activation of the AHR but while the MIE is a necessary effect, it is not, on its own sufficient to produce the adverse outcome (AO). The downstream biological effects, termed key events (KE), within the proposed mode of action (MOA) for the development of liver cancers are similarly necessary but not in isolation, sufficient to provoke the final AO.[121,122] This proposal suggests that following activation of the AHR, downstream KEs involve changes in the dynamics of cellular growth as a response to altered gene expression following AHR activation, observed as the development of hepatic foci, and decreases in the apoptotic rate both generally and especially within the foci, extensive liver toxicity described in the publication as "toxic hepatopathy", and subsequent cellular proliferation and hyperplasia in several hepatic cell types. This progression of KEs then leads to the development of hepatocellular and cholangiocellular neoplasms. There is experimental evidence to support much of the proposed AOP but a critical review of the dose response and time relationships between the various key events and their subsequent KE would suggest that the sequence, although not necessarily the roles, of several of the KE may need to be evolved. Even with potent AHR ligands hepatic neoplasia still appears to only occur at relatively high dose levels when saturation of the AHR binding and activation would be expected to occur. Indeed in experimental situations in

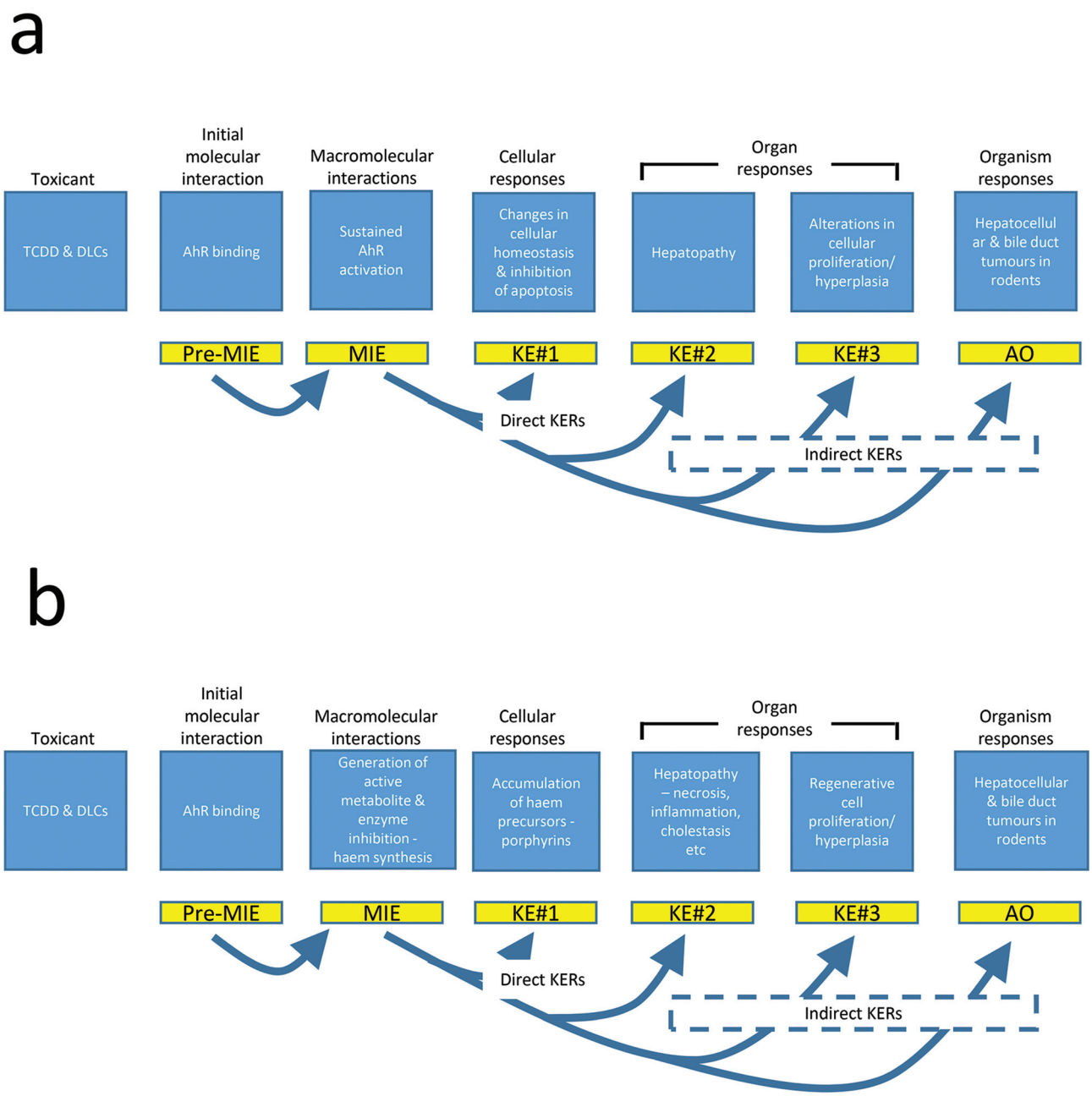

Fig. 8 (a) An adverse outcome pathway analysis for the production of liver cancer with AHR ligands (redrawn from Becker *et al.* 2015).[56] (b) An alternative AOP for the production of liver cancer in rodents *via* the hepatic accumulation of porphyrins.

rodents, the dose–response for neoplasia correlates best with elevations in liver weight above a threshold.[109,111,112] The causes of the liver weight induction with these AHR agonists are also unlikely to be a simple product of sustained AHR activation since the "toxic hepatopathy" is far more likely to be the main driver for the chronic regenerative hepatic hyperplasia that is implicit in the AOP. For those AHR agonists that induce porphyria, a main initiator of the "toxic hepatopathy" could be the porphyria that develops following dosing at high concentrations and the hepatotoxicity induced by the build-up of porphyrin. Its interference with biliary excretion are most likely major KEs that determine the evolution/promote the development of neoplasia in the rodent livers through chronic liver injury and the regenerative cell replication that ensues.[123,124] Support for a potential role for hepatic porphyrin accumulation in the induction of neoplasia comes from the induction of rodent hepatic neoplasia by compounds that don't activate the AHR but that do induce porphyria, and the subsequent "toxic hepatopathy", through inhibition of ferrochelatase. These compounds also induce CYPs, although there is no evidence of their activation of the AHR, but their commonality with the AHR agonists is the production of porphyria and the

development of toxic hepatopathy. Therefore a revision of the Becker *et al.* AOP[56] is presented below (Fig. 8b) which changes the order of some of the KEs but also introduces the key role of porphyrin in the subsequent induction of the hepatopathy and consequentially of all of the downstream events leading to hepatic neoplasia.

7. Summary

Some chemicals, drugs and pesticides are known to interfere in experimental animals with hepatic haem synthesis causing porphyria. With some clinical human porphyrias there is a known association with increased incidences of hepatocellular cancer. Hepatic porphyrin accumulation caused in rodents may occur either by mechanisms that produce oxidized haem precursors which act as inhibitors of enzymes in the pathway or by metabolism of the chemical that by a side reaction produces an inhibitor of ferrochelatase, from the haem of cytochrome P450. In both mechanisms there is evidence that under chronic conditions the accumulation of porphyrins, mostly either uroporphyrins or protoporphyrin IX may cause liver injury and aid subsequent liver tumour formation.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Preliminary presentation on this topic took place to a HESI Workshop U.S. EPA, 2004.[125]

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