Reactive oxygen species from the uncoupling of human cytochrome P450 1B1 may contribute to the carcinogenicity of dioxin-like polychlorinated biphenyls

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Polychlorinated biphenyls (PCBs) are classified by the International Agency for Research on Cancer as probable human carcinogens. A subset of PCBs are described as ‘dioxin like’ because of similarities to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Dioxin-like PCBs have been shown to tightly bind the active site of cytochrome P450 (CYP) 1A isoforms, primarily CYP1A1, resulting in inhibition of CYP activity and the generation of reactive oxygen species (ROS) as a result of uncoupling of the catalytic cycle. Human CYP1B1 (hCYP1B1) is an extrahepatic CYP closely related to hCYP1A1 and is overexpressed in the lungs of smokers. Moreover, hCYP1B1 has been found to be overexpressed in cancers derived from a number of tissue types, as well as in pre-malignant prostate tumours, implicating overexpression of hCYP1B1 as a risk factor for extrahepatic carcinogenesis. It has been demonstrated previously that hCYP1B1 is inhibited by dioxin-like PCBs, but whether or not it is uncoupled has not been investigated. In the current study, the ability of three dioxin-like PCBs 3,3',4,4'-tetrachlorobiphenyl, 3,3',4,4',5-pentachlorobiphenyl and 3,3',4,4',5,5'-hexachlorobiphenyl (PCB169) to inhibit hCYP1B1 and stimulate the formation of ROS in V79MZ cells (which lack endogenous CYPs) expressing hCYP1B1 was demonstrated. Moreover, the generation of ROS was also associated with increases in parameters of oxidative stress related to genotoxicity (DNA oxidation and lipid peroxidation). For PCB169, these effects were time and concentration dependent. These data identify a novel mechanism of genotoxicity for dioxin-like PCBs, as well as providing further evidence that overexpression of hCYP1B1 is a risk factor for extrahepatic carcinogenesis.

Introduction

Polychlorinated biphenyls (PCBs) are classified by the International Agency for Research on Cancer (IARC) as probable human carcinogens (group 2A) (1). PCBs are persistent organic contaminants found in soil, water, air and food as a result of improper disposal and bioaccumulation (2). Human exposure to PCBs can thus occur via many routes.

The term PCB describes 209 separate compounds, a subset of which have been described as ‘dioxin like’ because of their mechanism of toxicity and structural features that make them similar to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (3). TCDD is classified by the IARC as carcinogenic to humans (group 1) (4). TCDD and dioxin-like PCBs alike are potent agonists of the aryl hydrocarbon receptor (AhR), inducing the expression of members of the cytochrome P450 (CYP) 1 family (CYP1A1/1A2/1B1) of mono-oxygenases via the AhR/AhR nuclear translocator (ARNT) pathway (5).

CYP1B1 is an extrahepatic CYP, with major sites of expression and induction in humans including the breast, colon, endometrium, lungs, ovaries and prostate (6,7). In particular, human CYP1B1 (hCYP1B1) has been found to be overexpressed in the lungs of smokers due to frequent induction by AhR agonists in the form of polycyclic aromatic hydrocarbons (PAHs) (8–10). Moreover, hCYP1B1 has also been found to be overexpressed in cancers derived from all of these tissue types, as well as in pre-malignant prostate tumours, implicating overexpression of hCYP1B1 as a risk factor for extrahepatic carcinogenesis (11–17). Suggested mechanisms of hCYP1B1-dependent transformation include increased activation of PAHs and 4-hydroxylation of oestrogens to yield genotoxic metabolites (18–20).

In addition to inducing their expression, Schlezinger et al. (21–23) have reported that certain dioxin-like PCBs (3,3',4,4'-tetrachlorobiphenyl [PCB77], 3,3',4,4',5-pentachlorobiphenyl [PCB126] and 3,3',4,4',5,5'-hexachlorobiphenyl [PCB169]) can tightly bind the active site of CYP1A isoforms, primarily CYP1A1, in a number of different species. This results in inhibition of CYP1A activity and generation of reactive oxygen species (ROS) due to uncoupling of the catalytic cycle.

The roles of ROS, including superoxide (O2·−), hydrogen peroxide (H2O2), the hydroxyl radical (·HO) and singlet molecular oxygen (¹O2) in genotoxicity and carcinogenesis have been reviewed extensively (24–26). Major mechanisms include DNA oxidation, such as formation of 8-oxo-2′-deoxyguanosine (8-oxo-dG) (27), and the formation of the genotoxic aldehydes malondialdehyde and 4-hydroxynonenal as end products of membrane phospholipid oxidation (lipid peroxidation) (28). Thus uncoupling of CYP activity leading to generation of ROS may represent a risk factor for chemical carcinogenesis.

hCYP1A1 and hCYP1B1 share extensive sequence homology and overlapping substrate specificity, and the expression of both is regulated via the AhR/ARNT pathway. Based on these similarities and previous evidence that dioxin-like PCBs inhibit hCYP1B1 (29,30) and uncouple CYP1A isoforms (21–23), we hypothesized that dioxin-like PCBs can uncouple the catalytic cycle of CYP1B1, resulting in the generation of ROS and that this mechanism contributes to carcinogenesis mediated by dioxin-like PCBs.

In order to test this hypothesis, the ability of PCB77, 126 and 169 to inhibit hCYP1B1 and stimulate the formation of
ROS in V79MZ cells (which lack endogenous CYP expression/activity) engineered to constitutively express high levels of hCYP1B1 was investigated. Inhibition of hCYP1B1, together with a concomitant increase in intracellular ROS was observed for all three dioxin-like PCBs tested, providing strong evidence that these compounds uncouple hCYP1B1. In addition to intracellular ROS, increases in parameters of oxidative stress related to genotoxicity (DNA oxidation and lipid peroxidation) were also observed. These data identify a novel mechanism of genotoxicity for dioxin-like PCBs as well as providing further evidence that overexpression of hCYP1B1 should be considered a risk factor for extrahepatic carcinogenesis.

Materials and methods

Materials

PCB77, 126 and 169 (purity ≥99.7%) were purchased from AccuStandard Europe (Niederbipp, Switzerland). Arylsulphatase/β-glucuronidase from Helix pomatia was purchased from Roche (Basel, Switzerland). Cis-parinaric acid (CPA), 2′,7′-dichlorodihydrofluorescein diacetate (DCF) and SYBR gold were purchased from Invitrogen (Paisley, UK). Escherichia coli formamidopropionymidine DNA glycosylase (FPG) was purchased from AMS Biotechnology (Abingdon, Oxfordshire, UK). All other reagents were purchased from Sigma Aldrich Chemical Company (Poole, Dorset, UK). All cell culture plastics were purchased from Falcon (Becton Dickinson Labware, Le Pont De Claix, France).

Cell culture

Parental V79MZ cells and V79MZ cells expressing hCYP1B1 were a kind gift from Professor Johannes Doehmer (GenPharmTox, BioTech AG, Germany) (31). Parental V79MZ cells do not express CYPs (32), thus all CYP activity observed in V79MZ + hCYP1B1 cells can be attributed to the product of the hcyP1B1 transgene. Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 0.4 mg/ml streptomycin. Cells were routinely cultured in 25 cm² flasks and passaged twice weekly using a standard trypsin–ethylenediaminetetraacetic acid (EDTA) protocol. Prior to commencement of experiments, cells were subcultured into either 96-well plates [for the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay] or 6-well plates (for all other experiments).

Treatment of cells with dioxin-like PCBs

PCB77, 126 and 169 were prepared as 2.5 mM stock solutions in sterile dimethyl sulfoxide (DMSO). Stock solutions of PCBs were diluted to the required final concentrations in supplemented DMEM, with the final DMSO concentration maintained at 0.1% (v/v), before being added to the culture vessel.

Cytotoxicity assay

Cytotoxicity was determined using the MTT assay. Cells (2 × 10⁵ per well in 96-well plates) were incubated with vehicle (0.1% DMSO) or PCB (0.5 or 2.5 μM) for 1, 3, 12 or 24 h as required. The final volume of medium was 200 μl/ well. Subsequently, the medium was removed and replaced with fresh medium (200 μl/well) containing 0.5 mg/ml MTT and the cells incubated for a further 2 h. Precipitated formazan was dissolved in 200 μl DMSO/well and the absorbance measured at 540 nm against a DMSO blank.

hCYP1B1 activity assay

hCYP1B1 activity was measured using the 7-ethoxyresorufin O-deethylase (EROD) assay. Members of the CYP1 family catalyse the O-deethylation of 7-ethoxyresorufin (7-ER) to yield the fluorescent product resorufin, which can be readily detected and quantified. Cells (3 × 10⁵ per well in 6-well plates) were incubated with vehicle (0.1% DMSO) or PCB (0.5 or 2.5 μM) for 1, 3, 12 or 24 h as required. The final volume of medium was 2 ml/well. Following incubation, 2 μl of a dicumaron solution (10 mM in DMSO) was added directly into the incubation medium of the wells to be assayed, followed by 2 μl of a 7-ER solution (8 mM in DMEM) 5 min later. The cells were incubated for a further 30 min before 750 μl of the incubation medium was removed from each well and transferred to 3 ml polystyrene fluorescence cuvettes containing 250 μl of a freshly prepared arylsulphatase/β-glucuronidase solution (12000 units arylsulphatase, 150 Fishman units β-glucuronidase, dissolved in 100 mM sodium acetate, pH 4.5). The cuvettes were covered and incubated at 37°C for 2 h before the addition of 1 ml of ethanol. Fluorescence was measured at λexitation 530 nm and λemission 590 nm. Resorufin yields (pmol) were determined from a resorufin standard curve (0–1000 pmol). Subsequently, the remaining medium was removed from the cells and, following washing with 1 ml of phosphate-buffered saline (PBS)/well, replaced with 1 ml of 0.1% (v/v) Triton X-100/well, into which the cells were scraped. Aliquots (5 μl) of the resulting lysates were used for protein quantification as described. The picomol resorufin yielded (pmol) 7-ER metabolized was normalized to protein mass (mg) and divided by the incubation time (30 min) to obtain rates of 7-ER turnover (pmol/min/mg protein).

ROS and lipid peroxidation assays

DCF and CPA were used for the detection of intracellular ROS and lipid peroxidation (33). Briefly, non-fluorescent DCF is taken up by cells, where it can be oxidized by ROS to yield a fluorescent product; therefore, an increase in DCF-derived fluorescence acts as a surrogate marker of intracellular ROS. Conversely, CPA is a fluorescent compound that incorporates into cellular membranes, where it can be oxidized by ROS to yield a non-fluorescent product; therefore, a decrease in CPA-derived fluorescence acts as a surrogate marker of lipid peroxidation. Cells (3 × 10⁵ per well in six-well plates) were incubated in 3 ml of supplemented DMEM/well containing 0.1% (v/v) ethanol and either 10 μM DCF or 10 μM CPA for 30 min. Following incubation, the medium was removed and the cells washed with 3 × 1 ml of PBS/well. Following washing, the cells were incubated with vehicle (0.1% DMSO) or PCB (0.5 or 2.5 μM) for 1, 3, 12 or 24 h as required. The final volume of medium was 3 ml/well. Following incubation, the medium was removed and the cells washed with 1 ml of PBS/well. Next, the cells were scraped into 2 ml of PBS/well and the resulting suspensions transferred to 3 ml polystyrene fluorescence cuvettes. Fluorescence (units) were measured at 505 nm and λemission 520 nm (for DCF) or λexitation 312 nm and λemission 455 nm (for CPA) against a PBS blank. In addition, the background fluorescence of a population of cells not labelled with DCF/CPA was also determined. Next, 0.5 ml of the suspensions were transferred to 1.5 ml microcentrifuge tubes containing 0.5 ml of 0.2% (v/v) Triton X-100 and 10 μl of the resulting lysates used for protein quantification as described. Fluorescence units (F) at 520/455 nm were blank adjusted, normalized to protein mass (mg) and corrected for endogenous fluorescence.

Protein quantification

Protein quantification was performed according the method of Bradford (34), with the standard curve constructed using bovine serum albumin (BSA; 0–10 μg).

Alkaline comet assay

The alkaline comet assay, originally described by Singh et al. (35), was performed with FPG digestion for the detection of 8-oxo-7,8-dihydro-ÿ--terminal desoxynucleotides as described by the European Standards Committee on oxidative DNA damage (36). Cells (3 × 10⁵ per well in six-well plates) were incubated with vehicle (0.1% DMSO) or PCB (0.5 or 2.5 μM) for 1, 3, 12 or 24 h as required. The final volume of medium was 3 ml/well. Following incubation, the cells were harvested by gentle scraping into 1 ml of cold phenol red-free DMEM/well and cell pellets collected by centrifugation at 300 × g for 5 min at 4°C. Cell pellets were then resuspended in 150 μl of phenol red-free DMEM and aliquots of 300 μl of each suspension were layered onto two glass slides [pre-coated with a thin layer of 0.5% (w/v) PBS normal melting point agarose] and covered with a glass coverslip. Slides were maintained at 4°C for 30 min before the coverslips were removed and the slides immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 1% sodium N-lauryl sarcosinate, 10% DMSO, 1% Triton X-100, 10 mM Tris, pH 10.0) for 1 h. Following lysis, slides were washed (3 × 5 min) with 1 ml of FPG buffer (100 mM KCl, 500 mM EDTA, 0.2 mg/ml BSA, 40 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, pH 8.0). Parallel slides were then treated with either 50 μl of FPG buffer containing 1 unit of FPG or 50 μl of FPG buffer alone and covered with a coverslip before incubation at 37°C for 1 h. Following incubation, coverslips were removed and the slides placed in a 2 l horizontal electrophoresis tank containing electrophoresis buffer (75 mM Tris, pH 10.0) for 30 min. Following electrophoresis, slides were washed (3 × 5 min) with 1 ml of neutralization buffer (400 mM Tris, pH 7.5) before staining with 50 μl of SYBR gold (diluted 1/1000 in neutralization buffer). Comet images were examined using a fluorescence microscope and analysed with Comet Assay IV analysis software (Perceptive Instruments, Steeple Bumpstead, Suffolk, UK). Measurements of percentage tail DNA of 100 comets per slide were taken and the median value used as the unit for statistical analysis as recommended by Duez et al. (37). FPG-sensitive
sites (FSS), which are mostly 8-oxo-dG lesions (36), were calculated as the difference in median percentage tail DNA between paired slides ± FPG.

Statistical analyses

All statistical analyses were performed using MINITAB® software (version 14; Minitab Inc., State College, Pennsylvania, USA).

Results

Cytotoxicity of dioxin-like PCBs

Treatment with either PCB77, 126 or 169 did not result in statistically significant levels of cytotoxicity relative to time-matched vehicle (0.1% DMSO) controls at any of the times and concentrations tested in either parental V79MZ or V79MZ + hCYP1B1 cells as assessed using the MTT assay (Figure 1A and B). This is critical as DNA fragmentation resulting from cell death may otherwise interfere with the alkaline comet assay.

Inhibition of hCYP1B1 by dioxin-like PCBs

Treatment (3 h) of V79MZ + hCYP1B1 cells with 2.5 µM of either PCB77, 126 or 169 resulted in highly significant inhibition of hCYP1B1, with the rate of 7-ER turnover being reduced to 14, 8 and 13% of vehicle control, respectively (Figure 2A). Additionally, these data show that the parental V79MZ cells have no appreciable EROD activity (Figure 2A), confirming the lack of CYP1A/1B expression in this cell line (31).

Treatment of V79MZ + hCYP1B1 cells with PCB169 for 1, 3, 12 or 24 h resulted in time- and concentration-dependent inhibition of hCYP1B1 (Figure 2B). Treatment with 2.5 µM resulted in rapid inhibition of hCYP1B1, with 27 ± 3% of hCYP1B1 activity remaining relative to time-matched vehicle control after 1 h, decreasing in a time-dependent manner to <1% after 24 h (Figure 2B). Treatment with 0.5 µM achieved a maximum of 92% inhibition of hCYP1B1 activity relative to time-matched vehicle control after 3 h, with inhibition decreasing to 84 and 81% after 12 and 24 h, respectively (Figure 2B).

Induction of ROS by dioxin-like PCBs in V79MZ + hCYP1B1 cells

Treatment (3 h) with 2.5 µM of either PCB77, 126 or 169 resulted in statistically significant increases in ROS, measured...
as the increase in DCF-derived fluorescence per milligram protein relative to vehicle control, to 149 ± 8, 177 ± 18 and 196 ± 23% of vehicle control in V79MZ + hCYP1B1 cells, respectively, while having no detectable effect in parental V79MZ cells (Figure 3A).

Treatment of V79MZ + hCYP1B1 cells with PCB169 for 1, 3, 12 or 24 h resulted in cumulative increases in DCF-derived fluorescence per milligram protein, indicating continued induction of ROS throughout the 24 h treatment period (Figure 3B). Treatment with 2.5 μM resulted in a statistically significant increase in DCF-derived fluorescence per milligram protein to 166 ± 8% of time-matched vehicle control after 1 h, increasing in a time-dependent manner to 231 ± 23% after 24 h (Figure 3B). Treatment with 0.5 μM, however, did not result in a statistically significant increase in DCF-derived fluorescence per milligram protein until 3 h after treatment, at which point a statistically significant increase to 159 ± 15% of time-matched vehicle control was observed, increasing in a time-dependent manner to 201 ± 27% after 24 h (Figure 3B).

**Induction of lipid peroxidation by dioxin-like PCBs in V79MZ + hCYP1B1 cells**

Treatment (3 h) with 2.5 μM of either PCB77, 126 or 169 resulted in statistically significant increases in lipid peroxidation, measured as the decrease in CPA-derived fluorescence per milligram protein relative to vehicle control, to 75 ± 1, 71 ± 3 and 82 ± 4% of vehicle control in V79MZ + hCYP1B1 cells, respectively, while having no detectable effect in parental V79MZ cells (Figure 4A).

Treatment of V79MZ + hCYP1B1 cells with 2.5 μM PCB169 did not result in statistically significant increases in lipid peroxidation until 3 h after treatment, when CPA-derived fluorescence per milligram protein decreased to 84 ± 22% of time-matched vehicle control (Figure 4B). CPA-derived fluorescence per milligram protein continued to decrease with time, dropping to 53 ± 23% of time-matched vehicle control.

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**Fig. 3.** DCF-derived fluorescence per milligram protein of V79MZ and/or V79MZ + hCYP1B1 cells following treatment with (A) 2.5 μM PCB77, 126 or 169 (3 h) [graph represents mean ± standard error of the mean (SEM) of five independent experiments] and (B) (V79MZ + hCYP1B1 cells only) 0.5 or 2.5 μM PCB169 (1, 3, 12 or 24 h) (graph represents mean ± SEM of three independent experiments). ○, ●: significantly different from vehicle control with \( P < 0.05 \) and \( P < 0.01 \), respectively. +, ++: data for 0.5 μM PCB169 significantly different from time-matched vehicle control with \( P < 0.05 \) and \( P < 0.01 \), respectively. ●, ●: data for 2.5 μM PCB169 significantly different from time-matched vehicle control with \( P < 0.05 \) and \( P < 0.01 \), respectively. \( P \)-values determined by two-tailed Student’s t-test.

**Fig. 4.** CPA-derived fluorescence per milligram protein of V79MZ and/or V79MZ + hCYP1B1 cells following treatment with (A) 2.5 μM PCB77, 126 or 169 (3 h) [graph represents mean ± standard error of the mean (SEM) of five independent experiments] and (B) (V79MZ + hCYP1B1 cells only) 0.5 or 2.5 μM PCB169 (1, 3, 12 or 24 h) (graph represents mean ± SEM of three independent experiments). ○: significantly different from vehicle control with \( P < 0.05 \). +, ++: data for 0.5 μM PCB169 significantly different from time-matched vehicle control with \( P < 0.05 \) and \( P < 0.01 \), respectively. ●, ●: data for 2.5 μM PCB169 significantly different from time-matched vehicle control with \( P < 0.05 \) and \( P < 0.01 \), respectively. \( P \)-values determined by two-tailed Student’s t-test.
24 h after treatment (Figure 4B). Treatment with 0.5 μM PCB169 resulted in a decrease in CPA-derived fluorescence per milligram protein of 25% between 3 and 12 h after treatment (Figure 4B), with no change observed between 1 and 3 h treatment, and only a minor decrease observed between 12 and 24 h after treatment (Figure 4B).

**Induction of DNA damage by dioxin-like PCBs in V79MZ + hCYP1B1 cells**

Treatment (3 h) with 2.5 μM of either PCB77, 126 or 169 did not result in statistically significant increases in frank DNA strand breaks (FSB; DNA strand breaks detectable in the alkaline comet assay without requiring FPG digestion) relative to vehicle control in either parental V79MZ or V79MZ + hCYP1B1 cells (Figure 5A). However, statistically significant increases in FSS were observed, with percentage tail DNA for FSS increasing by 5.9, 8.2 and 14.3 fold (to 2.6 ± 0.47, 3.61 ± 0.39 and 6.28 ± 0.84% tail DNA; vehicle control was 0.44 ± 0.35% tail DNA) following treatment with PCB77, 126 and 169, respectively (Figure 5B).

Treatment of V79MZ + hCYP1B1 cells with 2.5 μM PCB169 resulted in statistically significant increases in FSS as early as 3 h after treatment, when percentage tail DNA for FSS increased 7.6-fold to 6.94 ± 0.52% tail DNA (time-matched vehicle control was 0.81 ± 0.73% tail DNA) (Figure 5D). However, 12 h after treatment, percentage tail DNA for FSS was increased by only 4.4-fold to 3.45 ± 0.73% tail DNA (time-matched vehicle control was 0.79 ± 0.73% tail DNA) (Figure 5D). Associated with the decrease in FSS 12 h after treatment was a statistically significant increase in percentage tail DNA for FSB, rising 1.8-fold to 3.37 ± 0.58% tail DNA (time-matched vehicle control was 1.99 ± 0.23% tail DNA) (Figure 5C).

**Discussion**

Because of the extensive sequence homology and overlapping substrate specificity of hCYP1A1 and hCYP1B1 and previous evidence that dioxin-like PCBs inhibit CYP1B1 (29,30) and (time-matched vehicle control was 3.38 ± 0.62% tail DNA) (Figure 5C). After 24 h treatment, percentage tail DNA for both FSB and FSS were still significantly elevated from time-matched vehicle controls (Figure 5C and D). V79MZ + hCYP1B1 cells treated with 0.5 μM PCB169 also showed a peak increase in FSS 3 h after treatment, with percentage tail DNA for FSS increased 4.9-fold to 3.97 ± 0.19% tail DNA (time-matched vehicle control was 0.81 ± 0.1% tail DNA) (Figure 5D). After 12 h treatment, percentage tail DNA for FSS of treated cells was still significantly higher than that of vehicle control-treated cells, being increased 3.8-fold to 3.00 ± 0.56% tail DNA (time-matched vehicle control was 0.79 ± 0.24% tail DNA), but to a lesser extent than was observed 3 h after treatment (Figure 5D). Associated with this decrease in FSS at 12 h was a small, statistically insignificant, increase in percentage tail DNA for FSB of 1.2-fold to 3.96 ± 1.1% tail DNA (time-matched vehicle control was 3.38 ± 0.62% tail DNA) (Figure 5C). After 24 h treatment, percentage tail DNA for FSS of treated cells had decreased further and was no longer significantly elevated from that of vehicle control-treated cells (Figure 5D). Associated with this decrease in FSS was a statistically significant increase in percentage tail DNA for FSB of 1.7-fold to 3.37 ± 0.58% tail DNA (from (time-matched vehicle control was 1.99 ± 0.23% tail DNA)) (Figure 5C).

**Fig. 5.** Effects of dioxin-like PCBs on FSB and FSS in V79MZ and/or V79MZ + hCYP1B1 cells. (A, B) Effects of 2.5 μM PCB77, 126 or 169 (3 h) on percentage tail DNA for FSB and FSS, respectively [graphs represent mean ± standard error of the mean (SEM) of five independent experiments]. (C, D) Effects of 0.5 or 2.5 μM PCB169 (1, 3, 12 or 24 h) on percentage tail DNA for FSB and FSS of V79MZ + hCYP1B1 cells, respectively (graphs represent mean ± SEM of three independent experiments). oo, ooo significantly different from vehicle control with P < 0.01 and P < 0.001, respectively. +, ++: data for 0.5 μM PCB169 significantly different from time-matched vehicle control with P < 0.05 and P < 0.01, respectively. ●, ●●: data for 2.5 μM PCB169 significantly different from time-matched vehicle control with P < 0.05 and P < 0.01, respectively. P-values determined by two-tailed Student’s t-test.
Uncouple CYP1A isoforms (21–23), we hypothesized that dioxin-like PCBs can uncouple the catalytic cycle of \( h\)CYP1B1, resulting in the generation of ROS and that this mechanism contributes to carcinogenesis mediated by dioxin-like PCBs.

The data presented in the current study are strongly supportive of this hypothesis and we have shown that not only are all three dioxin-like PCBs tested potent inhibitors of \( h\)CYP1B1 [consistent with the observations of Pang et al. (29)] but also result in \( h\)CYP1B1-dependent elevation of ROS and parameters associated with intracellular oxidative stress. In the current study, inhibition of \( h\)CYP1B1 activity by 2.5 \( \mu M \) PCB169 was rapid, resulting in >70% inhibition after 1 h, and time dependent, with almost complete inhibition after 24 h. However, 0.5 \( \mu M \) PCB169 did not result in increased inhibition of \( h\)CYP1B1 activity with time, instead maximum inhibition (92%) was achieved after 3 h, with apparent recovery of \( h\)CYP1B1 activity 12 and 24 h after treatment. This recovery of \( h\)CYP1B1 activity may reflect oxidation of PCB169 to dihydroxy and quinone metabolites by \( h\)CYP1B1 that do not bind CYP1 active sites as tightly as the parent compound (38).

The ability of dioxin-like PCBs to stimulate ROS production in V79MZ + \( h\)CYP1B1 was related to the degree of chlorination, with PCB169 (hexachloro) > PCB126 (pentachloro) > PCB77 (tetrachloro). This may reflect the greater ability of more heavily chlorinated dioxin-like PCBs to uncouple \( h\)CYP1B1. This is in agreement with Hennig et al. (39), who observed that treatment of porcine pulmonary arterial endothelial cells with PCB77, 126 or 169 resulted in increases in intracellular ROS related to the degree of dioxin-like PCB chlorination.

Importantly, treatment of V79MZ + \( h\)CYP1B1 cells with all three dioxin-like PCBs resulted in significant induction of lipid peroxidation. Similar observations have been made both in vivo in rats (40,41) and chicken and duck embryos (42,43) and in vitro in cultured rat leydig cells (44). However, in none of these studies were the mechanisms of ROS production or lipid peroxidation clearly identified.

In the current study, treatment with all three dioxin-like PCBs resulted in DNA oxidation, inducing the formation of FSS, but not FSB. When investigated in more detail in the case of PCB169, this was found to be time- and concentration dependent. The degree of FSS induced by dioxin-like PCBs appeared to be related to the degree of ROS generation (and thus the degree of dioxin-like PCB chlorination also), strongly suggesting a causative relationship between the degree of \( h\)CYP1B1 uncoupling, the generation of ROS and DNA oxidation in the form of 8-oxo-dG lesions (which comprise the majority of FSS). The 8-oxo-dG lesions are highly mutagenic, causing G:C to T:A transversion mutations if not repaired prior to DNA replication. Such mutations are often found in a range of tumour suppressor genes (e.g. p53) in human cancers (27). In contrast, no significant FSB induction was seen until 12 h after treatment. The decline in FSS together with a concomitant appearance to be related to the degree of ROS generation (and thus the degree of ROS generation also), strongly suggesting a causative relationship between the degree of \( h\)CYP1B1 uncoupling, the generation of ROS and DNA oxidation in the form of 8-oxo-dG lesions (which comprise the majority of FSS). The 8-oxo-dG lesions are highly mutagenic, causing G:C to T:A transversion mutations if not repaired prior to DNA replication. Such mutations are often found in a range of tumour suppressor genes (e.g. p53) in human cancers (27). In contrast, no significant FSB induction was seen until 12 h after treatment. The decline in FSS together with a concomitant increase in FSB observed at the later time points is likely to reflect the removal of 8-oxo-dG lesions via base excision repair, which generates FSB as part of its mechanism (45,46).

Overall, these data provide strong evidence to support our hypothesis that dioxin-like PCBs can uncouple \( h\)CYP1B1 in vitro in an analogous way to CYP1A, resulting in the generation of intracellular ROS and increases in levels of parameters of oxidative stress related to genotoxicity including DNA oxidation and lipid peroxidation. Furthermore, we suggest that such uncoupling of \( h\)CYP1B1 represents a novel mechanism of genotoxicity for dioxin-like PCBs and contributes to their carcinogenicity in vivo. In addition, low levels of ROS are also known to promote cellular proliferation (47) and as such PCBs may also promote carcinogenesis by a non-genotoxic mechanism. We believe these mechanisms are likely to be most biologically relevant in extrahepatic tissues where \( h\)CYP1B1 expression is highly inducible by dioxin-like PCBs acting as AhR agonists, providing substantial amounts of protein which can potentially be uncoupled, thus increasing the capacity for ROS generation. Therefore, these data provide further evidence that overexpression of \( h\)CYP1B1 is a risk factor for extrahepatic carcinogenesis. However, we are aware of the need for in vivo studies in order to validate our hypothesis to the in vivo situation.

We believe the findings of this study may also provide a mechanistic interpretation for epidemiological studies that have identified associations between PCBs and cancers of the breast, endometrium, lungs and prostate (48–52), all of which are derived from tissues in which \( h\)CYP1B1 is highly inducible and for all of which previous studies have reported \( h\)CYP1B1 overexpression relative to adjacent normal tissues. Finally, we suggest that smokers may be at elevated risk due to overexpression of \( h\)CYP1B1 in the lungs where, as well as resulting in the formation of directly genotoxic metabolites such as those of PAHs, \( h\)CYP1B1 may contribute to tumour promotion as the result of dioxin-like PCB-mediated uncoupling and the formation of ROS.

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