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“PCB153-Elicited Hepatic Responses in the Immature, Ovariectomized C57BL/6 Mice: Comparative Toxicogenomic Effects of Dioxin and Non-Dioxin-Like Ligands”

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Abstract

Polychlorinated biphenyls (PCBs) are ubiquitous contaminants found as complex mixtures of coplanar and non-coplanar congeners. The hepatic temporal and dose-dependent effects of the most abundant non-dioxin-like congener, 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153), were examined in immature, ovariectomized C57BL/6 mice, and compared to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the prototypical aryl hydrocarbon receptor (AhR) ligand. Animals were gavaged once with 300 mg/kg PCB153 or sesame oil vehicle and sacrificed 4, 12, 24, 72 or 168 h post dose. In the dose-response study, mice were gavaged with 1, 3, 10, 30, 100 or 300 mg/kg PCB153 or sesame oil for 24 h. Significant increases in relative liver weights were induced with 300 mg/kg PCB153 between 24 and 168 h, accompanied by slight vacuolization and hepatocellular hypertrophy. The hepatic differential expression of 186 and 177 genes was detected using Agilent 4 x 44 K microarrays in the time course (|fold change| 1.5, P1(*t*) 0.999) and dose-response (|fold change| 1.5, P1(*t*) 0.985) studies, respectively. Comparative analysis with TCDD suggests that the differential gene expression elicited by PCB153 was not mediated by the AhR. Furthermore, constitutive androstane and pregnane X receptor (CAR/PXR) regulated genes including Cyp2b10, Cyp3a11, Ces2, Insig2 and Abcc3 were dose-dependently induced by

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PCB153. Collectively, these results suggest that the hepatocellular effects elicited by PCB153 are qualitatively and quantitatively different from TCDD and suggestive of CAR/PXR regulation.

Keywords

PCB153; TCDD; liver; mouse; microarray

INTRODUCTION

Polychlorinated biphenyls (PCBs), manufactured in the United States from 1929 to 1977, had various applications as coolants, insulating fluids for transformers and capacitors, plasticizers in paints and cements, pesticide extenders, lubricating oils and sealants (Mullin 1984; NTP 2006b). Their chemical stability and lipophilic properties facilitated their distribution, persistence and biomagnification in the food chain, particularly in fatty tissues (Kimbrough 1995).

PCBs and related compounds elicit tissue- and species-specific effects including hepatotoxicity, immune suppression, reproductive toxicity, endocrine disruption, developmental toxicity, and carcinogenicity (Poland and Knutson 1982; Knerr and Schrenk 2006). They are classified as coplanar, designated dioxin-like based on their structural similarity to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and non-coplanar (Mullin 1984). The effects of TCDD and dioxin-like PCBs, such as 3,3',4,4',5-pentachlorobiphenyl (PCB126), are mediated by the aryl hydrocarbon receptor (AhR), facilitating the toxicity assessment of dioxin-like PCB mixtures to be expressed as toxic equivalents relative to TCDD (Van den Berg *et al.* 2006). TCDD and related dioxin-like PCBs are more toxic than non-coplanar PCBs, which contain ortho chlorine substituents on the biphenyl ring, which significantly reduces their binding affinity for the AhR (Giesy and Kannan 1998). Despite their non-dioxin-like characteristics, non-coplanar PCBs account for a majority of the PCBs found in environmental and biological samples.

Non-coplanar 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153) is the congener found at the highest concentrations in human samples on a molar basis (Schecter *et al.* 1994; NTP 2006a). PCB153 bears little structural resemblance to TCDD, has little to no AhR binding affinity, and elicits a unique toxicity profile relative to TCDD and other coplanar PCBs, and therefore is not assigned a TEF value. Its mode of action has not been fully elucidated, but based on its structural similarity to phenobarbital and limited gene expression activity, it may be mediated via the constitutive androstane receptor (CAR)/ pregnane X receptor (PXR) (Craft *et al.* 2002; Honkakoski *et al.* 2003; Tabb *et al.* 2004; Strathmann *et al.* 2006; Pascussi *et al.* 2007).

Previous studies have demonstrated non-additive interactions between PCB153 and dioxin-like compounds (Biegel *et al.* 1989; Bager *et al.* 1995; Suh *et al.* 2003; Chubb *et al.* 2004). PCB153 partially antagonizes TCDD-mediated cleft palate and immunotoxicity, as well as suppresses hepatic microsomal ethoxyresorufin-O-deethylase (EROD) induction in C57BL/6 mice (Biegel *et al.* 1989). Immunohistochemistry of rat livers following co-treatment with PCB126 and PCB153 also showed differential induction of Cyp1a1

compared to PCB126 alone (Chubb *et al.* 2004). In addition, PCB126 and PCB153 co-treatment synergistically altered hepatocellular foci development and expression of γ -glutamyltranspeptidase (Bager *et al.* 1995). Consequently, a more thorough examination of PCB153 is warranted in order to further elucidate the mechanisms involved in these non-additive activities.

In this report, time course and dose-dependent gene expression studies with complementary histopathology, gas chromatography/mass spectrometry (GC/MS) lipid profiling and high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) tissue level analyses were conducted to investigate the hepatic effects elicited by PCB153 in immature, ovariectomized C57BL/6 mice. Comparisons were made to comparable TCDD and PCB126 studies, which used the same model, study design and data analysis strategies (Boverhof *et al.* 2005; Kopec *et al.* 2008).

MATERIALS & METHODS

Animal Husbandry

Immature female C57BL/6 mice, ovariectomized by the supplier on postnatal day (PND) 20, with body weights (BW) within 10% of the average, were obtained from Charles Rivers Laboratories (Portage, MI) on PND 25. Animals were housed in polycarbonate cages (n=5 per cage) containing cellulose fiber chips (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensburg, NY) with 30-40% humidity and a 12 h light/dark cycle (07:00 h – 19:00 h). Mice had free access to deionized water and were fed *ad libitum* with Harlan Teklad 22/5 Rodent Diet 8640 (Madison, WI). Animals were acclimatized for 3 days prior to dosing on PND 28. The immature ovariectomized mouse was used to facilitate comparisons with other data sets obtained using the same model, study design and analysis methods (Boverhof *et al.* 2005; Kopec *et al.* 2008). Immature animals were used, since they are more responsive to AhR ligands. The animals were ovariectomized to negate potential interactions with estrogens produced by the developing ovaries, since some animals in our studies are approaching reproductive maturity. All procedures were carried out with the approval of the Michigan State University All-University Committee on Animal Use and Care.

Dose-Response and Time Course Studies

A stock solution of PCB153 (99.9% purity, AccuStandard, New Haven, CT) was first dissolved in acetone (J.T. Baker), followed by a dilution with sesame oil (Sigma, St. Louis, MO), and evaporation of the acetone using nitrogen gas. The PCB153 stock solution was further diluted using sesame oil to achieve the desired dose. Animals were orally gavaged using 1.5 inch feeding needle with a 2.25 mm ball end (Cadence Science: formerly Popper and Sons, Lake Success, NY). For the dose-response study, mice (n=5 per group) were administered 0.1 ml of a single dose of 1, 3, 10, 30, 100 or 300 mg/kg of PCB153 or 0.1 ml pure sesame oil vehicle (Sigma) and sacrificed 24 h post-treatment. The 300 mg/kg PCB153 dose was used in a complementary time course study, as it was the highest dose examined that did not alter body weight gain. Animals were gavaged with either 300 mg/kg PCB153 or sesame oil vehicle and sacrificed at 4, 12, 24, 72 or 168 h. For direct comparisons

between dioxin-like and non-dioxin-like responses, additional animals were treated with 30 µg/kg TCDD and sacrificed at the same time points. Furthermore, additional data sets generated using the same animal model, study design and analysis methods for 30 µg/kg TCDD and 300 µg/kg PCB126 (Boverhof *et al.* 2005; Kopec *et al.* 2008) served as comparators for this study. Mice were sacrificed by cervical dislocation and tissue samples were removed, weighed, flash frozen in liquid nitrogen, and stored at -80°C. For both the time course and dose-response studies, the section of the right liver lobe was fixed in 10% neutral buffered formalin (Sigma) for histological analysis.

Histological Analyses

Fixed liver tissues were sectioned and processed in ethanol, xylene, and paraffin using a Thermo Electron Excelsior tissue processor (Waltham, MA). Tissues were embedded in paraffin with Miles Tissue Tek II embedding center, after which paraffin blocks were sectioned at 5 µm with a rotary microtome. Liver sections were placed on glass microscope slides, washed twice in xylene for 5 min, followed by four quick washes in ethanol and rinsing in water. Slides were placed in Gill 2 hematoxylin (Thermo Fisher Scientific, Waltham, MA) for 1.5 min followed by 2-3 quick dips in 1% glacial acetic acid water and rinsed with running water for 2-3 min. Slides were then rinsed in ethanol and counterstained with 1% eosin Y-phloxine B solution (Sigma) followed by multiple rinses in ethanol and xylene. For lipid staining, liver sections were frozen in Tissue-Tek O.C.T. compound (Sakura, Torrance, CA) before further processing. Liver samples were sectioned at 6 µm, fixed in 10% neutral buffered formalin for 5 min, rinsed with water and immersed in 100% propylene glycol for 5 min. The slides were stained with Oil Red O solution (Sigma) for 8 min at 60°C. Following staining, slides were placed in 80% propylene glycol for 5 min and rinsed in water for 15 min. Slides were counterstained with Gill 2 hematoxylin for 30 sec and washed with water for 30 min. Coverslips were attached using aqueous mounting media. All the histological processing was performed at Michigan State University Investigative HistoPathology Laboratory, Division of Human Pathology using a modified version of previously published procedures (Sheehan and Hrapchak 1980).

Lipid Analysis by Gas Chromatography-Mass Spectrometry (GC-MS)

Liver samples (~100 mg) from the 24, 72, 168 h PCB153, TCDD and vehicle control groups were homogenized (Polytron PT2100, Kinematica AG, Luzern, CH) in 40% methanol and acidified with concentrated HCl. Lipids were extracted with chloroform: methanol (2:1) containing 1 mM 2,6-di-tert-butyl-4-methylphenol (BHT; Sigma) and extraction efficiency controls (19:1n9 FFA [free fatty acid] and 19:0 TAG [triacylglycerol]) were added (Nu-Chek Prep, Elysian, MN). Protein and aqueous phases were re-extracted with chloroform and the organic phases were pooled. A derivatization standard (19:2n6 FFA; Nu-Chek Prep) was added and samples were dried under nitrogen, resuspended in 2% non-aqueous methanolic HCl and incubated at 60°C overnight. Samples were cooled to room temperature (RT) and 0.9% (w/v) NaCl and hexane were added. The organic phase was collected and a loading control (17:1n9 fatty acid methyl ester [FAME]; Nu-Chek Prep) was added. Samples were dried under nitrogen, resuspended in equal volumes of hexane and separated on Agilent 6890N gas chromatograph interfaced to Agilent 5973 mass spectrometer with DB23 column (30 m length, 0.25 mm internal diameter, 0.25 µm film thickness). Samples

were run on the following temperature program: 50°C to 150°C at 40°C/ min, to 185°C at 5°C/ min, to 235°C at 3°C/ min, to 250°C at 10°C/ min. Principal component analysis (PCA) of lipid profiles was performed in R 2.6.0, data were extracted and used to generate PCA plots in GraphPad Prism 4.0.

Hepatic Triglyceride Measurement

Frozen liver samples (~100 mg) were homogenized (Polytron PT2100, Kinematica) in 1 ml of 1.15% KCl. Triglycerides were extracted from 200 µl of hepatic homogenate with 800 µl of isopropyl alcohol by vortex-mixing for 10 min. The samples were centrifuged for 5 min at 800 x g at RT and supernatant was collected into separate vials. The concentration of hepatic triglycerides was determined using a commercial L-Type Triglyceride M kit (Wako Diagnostics, Richmond, VA) with Multi-Calibrator Lipids as a standard (Wako Diagnostics). The measurements were performed according to manufacturer's protocol with 20 µl of the triglyceride extract incubated with 150 µl of Reagent 1 followed by incubation with 50 µl of Reagent 2. Final results were normalized to the starting amount of liver (Supplementary Figure 1).

Quantification of PCB153 and TCDD Tissue Levels

Liver samples were processed in parallel with laboratory blanks and a reference or background sample at Wellington Laboratories Inc. (Guelph, ON, Canada). The samples (100 to 500 mg) were transferred to a tared screw cap culture tube and weight recorded. The sample was spiked with $^{13}\text{C}_{12}$ -PCB153 and $^{13}\text{C}_{12}$ -2,3,7,8-TCDD surrogates and then digested with hydrochloric acid. Each digested sample was then split between two screw cap tubes and hexane (3-4 ml) was added to each tube followed by vigorous mixing. The tubes were centrifuged, and the organic layer removed. The hexane extraction was performed three times per screw cap tube and the six hexane fractions were combined. The hexane fraction was then split evenly prior to clean-up and one fraction was archived. The other fraction was cleaned-up using a small multilayer (acid/base/neutral) silica gel column eluted with 20-25 ml of hexane. The eluate was concentrated on a rotary evaporator and then transferred to a conical micro-vial with pentane and dichloromethane rinses and allowed to dry. Immediately prior to injection on the high-resolution gas chromatograph/high-resolution mass spectrometer (HRGC/HRMS) system $^{13}\text{C}_{12}$ -PCB111 and $^{13}\text{C}_{12}$ -1,2,3,4-TCDD injection standards were added to the conical micro-vial.

Sesame oil (control) samples (100 to 500 mg) were weighed into tared screw cap tubes, spiked with $^{13}\text{C}_{12}$ -PCB153 and $^{13}\text{C}_{12}$ -2,3,7,8-TCDD surrogates and diluted in 5 ml of hexane. The controls were then mixed and digested with 5 ml of sulfuric acid. The tube was centrifuged and the hexane layer was removed, split evenly with one half subjected to the same clean-up as the liver samples.

Harlan Teklad 22/5 Rodent Diet 8640 feed samples were crushed and weighed (~8 g) into a pre-extracted cellulose thimble. The samples were then spiked with $^{13}\text{C}_{12}$ -PCB153 and $^{13}\text{C}_{12}$ -2,3,7,8-TCDD surrogates prior to soxhlet extraction overnight with dichloromethane. The samples were concentrated on a rotary evaporator and, transferred to a screw cap tube with hexane. The samples were then mixed and digested with 5 ml of

sulfuric acid. Following centrifugation, hexane layer was removed, split evenly and processed as described above.

The identification and quantification of PCB153 and TCDD was performed using an Agilent (Santa Clara, CA, USA) 6890 series HRGC with direct capillary interface to a Waters (Milford, MA, USA) Autospec Ultima HRMS. Chromatographic separations were carried out on a 60 m DB5 (0.25 mm ID, 0.25 μ m film thickness) column in constant flow mode (Helium, 1 ml /min). All injections were 1 μ l and a splitless injection was used. The mass spectrometer was operated in EI+ selective ion recording mode (SIR) at a mass resolving power of 10,000 or greater.

RNA Isolation

Frozen liver samples (~100 mg, stored at -80°C) were immediately transferred to 1 ml TRIzol (Invitrogen, Carlsbad, CA) and homogenized using a Mixer Mill 300 tissue homogenizer (Retsch, Germany). Total RNA was isolated according to the manufacturer's protocol with an additional acid phenol:chloroform extraction. Isolated RNA was resuspended in RNA storage solution (Ambion Inc., Austin, TX), quantified (A_{260}), and quality was assessed by determining the A_{260}/A_{280} ratio and by visual inspection of 2 μ g on a denaturing gel.

Microarray Experimental Design

PCB153-, TCDD-treated and vehicle samples were individually hybridized to 4 x 44K whole mouse genome oligo microarrays (Agilent Technologies, Inc., Santa Clara, CA). Hybridizations were performed with three biological replicates using one-color labeling (Cy3) for each time point and dose, according to the manufacturer's protocol (Agilent Manual: G4140-90040 v. 5.7). Published reports suggest that one- and two-color microarrays provide comparable data, with no significant differences detected when compared to studies utilizing Cy3 and Cy5 labeling (Patterson *et al.* 2006). Microarray slides were scanned at 532 nm (Cy3) on a GenePix 4000B scanner (Molecular Devices, Union City, CA). Images were analyzed for feature and background intensities using GenePix Pro 6.0 (Molecular Devices). All data were managed in TIMS dbZach data management system (Burgoon and Zacharewski 2007).

Microarray Analysis

All microarray data in this study passed our laboratory quality assurance protocol (Burgoon *et al.* 2005). Microarray data were normalized using a semiparametric approach (Eckel *et al.* 2005) and the posterior probabilities were calculated using an empirical Bayes method based on a per gene and time point or dose basis using model-based t -values (Eckel *et al.* 2004). Gene expression data were ranked and prioritized using a $|\text{fold change}| \geq 1.5$ and $P1(t)$ values ≥ 0.999 for the temporal PCB153 and TCDD data sets. However, relaxed $P1(t)$ values (≥ 0.985) were also used for the PCB153 dose-response data to identify differentially expressed genes that approached the selection cut-offs to ensure the identification of ligand-specific regulation. In addition, the different statistical cut-offs were used to obtain a comparable number of differentially expressed genes between the temporal and dose-

dependent microarray data sets and to account for studies being performed at two different times.

Dose-Response Modeling

Dose-response modeling was performed using a grid-enabled version of the ToxResponse Modeler (Burgoon and Zacharewski 2008). ToxResponse Modeler performs automated dose-response modeling by identifying the best fit model amongst five different mathematical model families (linear, exponential, Gaussian, sigmoidal, quadratic). The algorithm then chooses the best-fit of the five best in-class models. The overall best-fit model is then used to calculate the ED₅₀ values. The microarray dose-response PCB153 data set was first filtered using a P1(*t*)>0.90 cut-off and the filtered data were analyzed to identify genes exhibiting a sigmoidal dose-response profile.

DNA Response Element Analysis Modeling

Dioxin response elements (DREs) (Sun *et al.* 2004), constitutive androstane receptor response elements (CAREs) (Phillips *et al.* 2009) and pregnane X receptor response elements (PXREs) were identified computationally using position weight matrices (PWMs) specific to each site (Supplementary Figure 2). The PXRE PWM was generated using seven published response elements that were aligned to the genome (Wang *et al.* 2003; Echchgadda *et al.* 2004; Vyhldal *et al.* 2004; Pascussi *et al.* 2005; Zhou *et al.* 2006; Igarashi *et al.* 2007). Gene regulatory regions (–10,000 relative to the transcription start site [TSS] together with 5′-untranslated region [UTR]) were obtained from the University of California, Santa Cruz, Genome Browser for mouse (build 37), computationally searched, and each DRE, CARE and PXRE was scored. Matrix similarity scores (MSS) >0.80 are considered to be putative functional response elements.

Quantitative Real-Time PCR (QRT-PCR)

QRT-PCR verification of selected microarray data was performed as described previously (Boverhof *et al.* 2005). Briefly, 1 µg of total RNA was reverse transcribed by SuperScript II (Invitrogen) using an anchored oligo-dT primer as described by the manufacturer. The cDNA (1.0 µl) was used as a template in a 30 µl PCR reaction containing 0.1 µM of forward and reverse gene-specific primers, 3 mM MgCl₂, 1 mM dNTPs, 0.025 IU AmpliTaq Gold, and 1X SYBR Green PCR buffer (Applied Biosystems, Foster City, CA). PCR amplification was conducted on an Applied Biosystems PRISM 7500 Sequence Detection System. cDNAs were quantified using a standard curve approach and the copy number of each sample was standardized to 3 housekeeping genes (ActB, Gapdh, Hprt) to control for the differences in RNA loading, quality, and cDNA synthesis (Vandesompele *et al.* 2002). For graphing purposes, the relative expression levels were scaled such that the expression level of the time-matched control group was equal to one.

Functional Gene Annotation and Statistical Analysis

Annotation and functional categorization of differentially regulated genes was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis *et al.* 2003). All statistical analyses were performed with SAS 9.1 (SAS Institute, Cary, NC).

All data were analyzed by analysis of variance (ANOVA) followed by Tukey's or Dunnett's *post hoc* tests. Differences between treatment groups were considered significant when $p < 0.05$.

RESULTS

Organ and Body Weights

In the time course study, 300 mg/kg PCB153 increased ($p < 0.05$) relative liver weight (RLW) at 72 and 168 h (Table 1), comparable to other reports using lower doses (Craft *et al.* 2002; NTP 2006a). In the dose-response study, 300 mg/kg significantly increased RLW at 24 h (Table 2), similarly to 30 µg/kg TCDD and 300 µg/kg PCB126, which also increased RLW at later time points (Table 1) (Boverhof *et al.* 2005; Kopec *et al.* 2008). No significant decreases in body weight gain were observed at any of the PCB153 doses or time points, in agreement with other reports with doses as high as 360 mg/kg (Biegel *et al.* 1989; Craft *et al.* 2002).

Hepatic Tissue Level Quantification

HRGC/HRMS analysis of liver samples at 24 and 168 h time points indicate that PCB153 levels (in pg/g) exhibited different hepatic accumulation kinetics relative to TCDD (Fig. 1A). PCB153 levels dramatically decreased (~3.3 fold) after 7 days, compared to TCDD which exhibited modest reductions (~1.4 fold), and in contrast to PCB126 which continued to increase throughout the study, reaching the highest concentration at 7 days (Kopec *et al.* 2008). These differences may be partially due to the induction of Cyp1a2 (~30 fold), which sequesters TCDD and dioxin-like compounds in the liver (DeVito *et al.* 1998) (Fig. 6A). PCB153 only marginally induced Cyp1a2 (~1.8 fold) (Fig. 6B), which may account for the time dependent decrease of hepatic PCB153 levels.

PCB153 exhibited a dose-dependent increase in hepatic levels that was significantly different from controls (Fig. 1B). Absolute hepatic quantification results (in pg/g) from vehicle and treated mice from Fig. 1A-B have been included in Supplementary Table 1. Interestingly, hepatic PCB153 levels for sesame oil treated controls (Fig. 1B) were ~500 times higher than the corresponding PCB126 or TCDD levels (Kopec *et al.* 2008). High levels in vehicle animals have been attributed to the ingestion of PCB153 found in rodent chow (Luotamo *et al.* 1991; Jordan and Feeley 1999). However, only 62.1 pg/g of PCB153 was detected in Harlan Teklad 22/5 Rodent Diet 8640 using HRGC/HRMS, while levels in sesame oil were below the limits of detection (data not shown). Others suggest that non-dioxin-like PCBs may also accumulate in animals via lactational transfer (Vodicnik and Lech 1980).

Histopathology

PCB153 induced minimal hepatocellular vacuolization (Fig. 2C), comparable to the levels observed in vehicle controls (Fig. 2A). In contrast, TCDD-elicited vacuolization was more severe and localized to the periportal regions and extended to the midzonal and centrilobular regions in more severely affected mice. TCDD treatment also resulted in moderate multifocal inflammation (Fig. 2B, arrow), which was almost absent in the PCB153-treated

mice. However, PCB153 elicited increasing hypertrophic responses between 24 and 168 h. Furthermore, Oil Red O staining (ORO) identified significant lipid accumulation only in the TCDD-treated animals (Supplementary Figure 3E), while PCB153 livers showed no fatty accumulation (Supplementary Figure 3F).

In the dose-response study, 300 mg/kg PCB153 induced the highest levels of hepatocellular vacuolization, however incidences of hypertrophy were not observed. Compared to our previous studies (Boverhof *et al.* 2005; Boverhof *et al.* 2006), 30 µg/kg TCDD elicited more dramatic vacuolization, and necrosis, as well as mixed cell infiltration that was absent in PCB153 treatment.

Lipid Profiling

Total lipids were extracted from control, TCDD and PCB153-treated livers at 24, 72 and 168 h, derivatized to fatty acid methyl esters (FAMES) and analyzed by GC-MS. The temporal and treatment dependent separation of FAME profiles (as fold changes) is summarized in Fig. 3. The cumulative proportion of variance for principal component (PC) 1 and PC2 is 99%, indicating that the PCA plot accurately represents the separation of the data. Comparison of PCB153- and TCDD-elicited FAME profiles identified separation around PC1 and PC2 indicating differences due to treatment and time, respectively (Fig. 3). In agreement with ORO staining, TCDD exhibited a time-dependent induction in the level of several individual FAMES that was the highest at 168 h (Fig. 4 and Supplementary Table 2B) and led to hepatic lipid accumulation (Boverhof *et al.* 2006; Kopec *et al.* 2008) that was not detected in PCB153-treated animals (Fig. 4 and Supplementary Table 2A).

Hepatic triglyceride measurement identified a time-dependent increase in triglycerides in the TCDD group. In contrast, there was no difference in triglyceride levels between vehicle and PCB153-exposed mice (Supplementary Figure 1), consistent with ORO and GC-MS FAME analysis.

Temporal and Dose-Dependent PCB153 Gene Expression Changes

Hepatic gene expression was assessed using 4 x 44K Agilent oligonucleotide microarrays, containing ~21,000 unique annotated genes. PCB153 elicited the differential expression of 186 unique, annotated genes at one or more time points ($P1(t) < 0.001$ and $|\text{fold change}| > 1.5$) relative to vehicle controls with 72 h exhibiting the most changes (127 unique genes). In the 24 h dose-response study, PCB153 differentially regulated 177 unique genes at one or more doses ($P1(t) < 0.001$ and $|\text{fold change}| > 1.5$). Complete time course and dose-response data sets are available as Supplementary Tables 3 and 4, respectively.

Functional annotation of PCB153-elicited differential gene expression was associated with xenobiotic metabolism and oxidoreductase activity, lipid metabolism, cell cycle and cell death, and transport (Table 3). Position weight matrices (PWMs) were used to computationally identify putative dioxin, PXR/CAR response elements (Wang *et al.* 2003; Echchgadda *et al.* 2004; Sun *et al.* 2004; Vyhldal *et al.* 2004; Pascucci *et al.* 2005; Zhou *et al.* 2006; Igarashi *et al.* 2007; Phillips *et al.* 2009) in the promoter region (–10,000 bp

relative to the [TSS] together with the 5' UTR) of responsive genes identified from the microarray analysis (Table 3).

Xenobiotic and oxidoreductase activity functions were the most highly induced among all functional clusters and almost exclusively included cytochrome P450s (e.g. Cyp2b9, Cyp2b10, Cyp2c54, Cyp3a25) and glutathione S-transferases (e.g. Gsta2, Gstt3, Gstm4), with Cyp2c55 showing the highest (48-fold) induction in both the time course and dose-response study. PCB153 modestly induced the typical “AhR-battery” genes such as Cyp1a1, Cyp1a2 and Tiparp compared to TCDD (Fig. 6A-B), while TCDD induction of PCB153-responsive genes was reciprocally modest (Table 3 and Fig. 5C).

PCB153 down-regulated the lipid metabolism acyl-CoA synthetase long-chain family member 3, Acs13, and sterol regulatory element binding factors (Srebf1 and Srebf2) genes, -2.1 to -3.1-fold, respectively. Moreover, PCB153 repressed Elov15 -1.9-fold, while it was induced 2.2-fold by TCDD, suggesting divergent regulation (Fig. 6A-B). The down-regulation of many lipid biosynthesis and metabolism genes by PCB153 is consistent with GS-MS FAMES and triglyceride analysis, all indicating a lack of lipid accumulation compared to TCDD.

Genes involved in cell cycle and DNA replication, including Mad111 and Zwint, were up-regulated 2.4- and 2.0-fold, respectively, by PCB153. However, in contrast to TCDD, PCB153 did not induce necrosis or immune cell infiltration, even though genes involved in cell death and immune response were differentially regulated. A number of transport genes were also differentially expressed by PCB153, including ATP-binding cassette and solute carrier family members, such as Abcc3, Slc1a4, and Slc23a1.

Computational Dose-Response Modeling

Dose-response microarray data were filtered using $P(t) > 0.90$ cut-off and identified 846 unique annotated genes exhibiting a sigmoidal dose-response profile with reasonable ED_{50} values (i.e. between 1 and 300 mg/kg) (Fig. 7). 315 genes exhibited ED_{50} s between 1 and 10 mg/kg, 124 genes were between 10 and 30 mg/kg, 140 genes between 30 and 100 mg/kg and 267 genes had ED_{50} s between 100 and 300 mg/kg. The CAR/PXR regulated genes, Cyp2b10, Cyp2c55, Nl3, Entpd5 and Abcc3 exhibited ED_{50} s of 38.1, 31.7, 33.9, 34.2 and 2.9 mg/kg, respectively. Supplementary Table 5 contains the list of 846 genes with their corresponding ED_{50} values.

Verification of Microarray Responses

QRT-PCR was used to verify the temporal and dose-dependent changes in expression for a subset of genes. Supplementary Table 6 provides the gene names, gene abbreviations, accession numbers, forward and reverse primer sequences, and amplicon sizes. In total, 20 differentially regulated genes were confirmed, including Cyp3a11, which did not satisfy the Agilent microarray data selection criteria. The differential expression of CAR/PXR-regulated genes Cyp2b10, Cyp3a11 and Gadd45b (Maglich *et al.* 2002; Honkakoski *et al.* 2003; Rosenfeld *et al.* 2003), as well as Cyp2c55 (48-fold) by PCB153 and TCDD was also confirmed (Fig. 5A-C). In addition, AhR-responsive genes (Cyp1a1, Cyp1a2 and Tiparp)

were included as a positive control for TCDD, and a negative control for PCB153 (Fig. 6A-B). Elov15 was included as an example of divergent regulation (Fig. 6A-B). Overall, there was a good agreement in temporal and dose-dependent expression patterns between QRT-PCR and microarray analysis.

Comparison of TCDD and PCB153 elicited differential gene expression

Overall, when compared to TCDD and PCB126, PCB153 elicited a significantly different gene expression profile that did not include “AhR battery genes”, but CAR/PXR regulated genes, such as *Ces2*, *Cyp2b10*, *Fmo5*, *Fasn*, *Insig2*, *Abcc3* and *Gsta2* (Table 3). To further investigate these differences, a comprehensive comparison of the Agilent 300 mg/kg PCB153 and 30 µg/kg TCDD temporal microarray data between 4 and 168 h was performed (Fig. 8A). Using the same filtering criteria ($P(t) < 0.999$ and $|\text{fold change}| > 1.5$), 170 and 186 differentially expressed genes elicited by TCDD and PCB153, respectively, were identified, of which only 14 were regulated by both compounds. Relaxing the statistical cut-off to $P(t) < 0.985$ increased the overlap to 74 genes (Fig. 8A). A correlation plot of expression ratios compared to significance was then used to identify conserved and divergent responses between TCDD and PCB153 (Fig. 8B). Overall, only 54% of the commonly regulated genes were positively correlated in terms of fold change and significance, suggesting that TCDD and PCB153 elicit different expression patterns via different mechanisms of regulation. All 74 genes regulated by PCB153 and TCDD are listed in Supplementary Table 7.

DISCUSSION

Hepatic gene expression, histopathology and lipid profiling analysis quantitatively and qualitatively indicate that the temporal and dose-dependent effects elicited by PCB153 are mechanistically different when compared to TCDD and PCB126. These data are consistent with the effects elicited by PCB153 being regulated by CAR/PXR.

PCB153 elicited a gene expression profile that included the induction of xenobiotic metabolism genes such as *Cyp2c55*, *Cyp2b10*, *Cyp3a11*, *Ces2*, *Fmo5* and *Gsta2* (Maglich *et al.* 2002; Honkakoski *et al.* 2003; Rosenfeld *et al.* 2003) in the absence of “AhR gene battery” induction. PWM analysis computationally identified putative CAREs and PXREs within the regulatory region of numerous genes that exhibited differential expression following PCB153 treatment, further suggesting CAR/PXR regulation. This is also consistent with the *in vivo* induction of *Cyp3a23* by highly chlorinated non co-planar PCBs (Schuetz *et al.* 1998), and more specifically, the induction of Gal4-PXR-regulated reporter gene activity (Tabb *et al.* 2004). In addition, Sprague-Dawley rat studies concluded PCB153 did not activate AhR and may be associated with the induction of phenobarbital-responsive genes (Connor *et al.* 1995; Vezina *et al.* 2004; NTP 2006a).

Hepatic responses induced by PCB153 are also in agreement with reported CAR/PXR-mediated hepatic hypertrophy (Wei *et al.* 2000; Staudinger *et al.* 2003; Blanco-Bose *et al.* 2008). In contrast, hepatocyte cell size and number are not affected by TCDD and PCB126 (Boverhof *et al.* 2005; Kopec *et al.* 2008). PCB153 induced hepatocellular hypertrophy between 24 and 168 h compared to controls, in agreement with the NTP Technical Report that identified pronounced and persistent increase in cell size elicited by PCB153 (NTP

2006a). In addition, liver hypertrophic responses to known CAR inducers (TCPOBOP or phenobarbital) are abolished in mice lacking the CAR gene (Wei *et al.* 2002; Honkakoski *et al.* 2003).

TCDD, PCB126 and PCB153 all induced RLW in the time course and dose-response studies (Boverhof *et al.* 2005; Kopec *et al.* 2008). However, PCB153 elicited minimal vacuolization which completely subsided by 168 h. Histopathology and lipid profiling, also suggest that PCB153 did not induce hepatic steatosis (Boverhof *et al.* 2005; Kopec *et al.* 2008), and microarray analysis indicated that PCB153 down-regulated many lipid metabolism genes. For example, *Elovl5* was repressed by PCB153, but induced by TCDD, suggesting divergent regulation that may partially explain the histopathology differences. Interestingly, *Elovl5* null mice (*Elovl5*^{-/-}) have higher SREBP1 protein levels that increase fatty acid synthesis and the development of steatosis (Moon *et al.* 2008). However, PCB153 did not affect *Srebf1* expression and did not promote lipid synthesis. Other studies have shown that the CAR/PXR-mediated insulin induced genes (*Insig1* and *Insig2*) also repress *Srebf1*, thereby lowering hepatic fatty acids (Rosenfeld *et al.* 2003; Roth *et al.* 2008). The PCB153 induction of *Insig2* by 5.9-fold and repression of *Srebf1* by -2.4-fold suggests that *Elovl5* repression does fully explain the observed lipid changes. In contrast, *Insig2* was down-regulated -1.7-fold and *Srebf1* was repressed -2.3-fold by TCDD suggesting that this down-regulation was not sufficient to affect lipid biosynthesis.

Histopathology revealed a lack of PCB153-elicited hepatocellular necrosis/apoptosis, when compared to TCDD (Boverhof *et al.* 2005), consistent with other studies suggesting PCB153 reduces apoptosis in mouse hepatocytes (Tharappel *et al.* 2002; Lu *et al.* 2004). PCB153 induced *Gadd45b* ~11 fold, a known inhibitor of c-Jun N-terminal kinase (JNK)-mediated apoptosis (Papa *et al.* 2004). In addition, *Gadd45b* null mice have attenuated *Cyp2b10* expression, linking *Gadd45b* with direct co-activation of CAR-mediated transcription (Yamamoto and Negishi 2008). PCB153 also regulated the expression of genes involved in cell death, although there was no evidence of necrosis. For example, *Myc* induction following chronic PCB153 exposure has been associated with increased apoptosis in HepG2 cells (Ghosh *et al.* 2007). However, the single dose of PCB153 used in the current study may not be sufficient to induce cell death within 168 h.

Furthermore, there was a lack of significant immune cell accumulation associated with PCB153 treatment, unlike TCDD, which increased mixed cell infiltration, primarily in the centrilobular regions (Boverhof *et al.* 2005). Nevertheless, PCB153 induced expression of chemokine (C-C motif) ligand 9 (*Ccl9*), in agreement with the increased incidence of liver inflammation in chronically treated rats (NTP 2006a). This suggests that a single dose of PCB153 may not be sufficient to increase mixed cell infiltration within a week.

In summary, PCB153 and possibly other non-coplanar congeners elicit responses that are qualitatively and quantitatively different than TCDD and other planar PCBs. PCB153 elicited no instances of inflammation, necrosis/apoptosis and did not lead to hepatic lipid accumulation. These effects are consistent with the differential gene expression responses and suggestive of CAR/PXR-mediated regulation. However, the relevance of these effects in risk assessment warrants further investigation due to significant species-specific differences

in ligand preference, binding, and receptor activation when comparing human and rodent CAR/PXR orthologs (Blumberg and Evans 1998; Jones *et al.* 2000; Maglich *et al.* 2002; Tabb *et al.* 2004).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Hepatic PCB153 and TCDD levels per g liver wet weight measured using HRGC/HRMS. **(A)** Mice were gavaged with 300 mg/kg PCB153 or 30 µg/kg TCDD. Hepatic levels of PCB153 showed a dramatic decrease (~3.3 fold) after 7 days, compared to TCDD, which exhibited more modest decreases (~1.4 fold). **(B)** PCB153 levels attained in the 24 h dose-response study. The results are displayed as the mean ± standard error (SE) of at least three independent samples. Dose-response data are displayed on a log scale to visualize tissue levels at all doses. An asterisk (*) indicates a significant ($p < 0.05$) difference between the treated samples and vehicle (VEH) controls.

Figure 2.

Representative histopathology results from vehicle, 30 µg/kg TCDD and 300 mg/kg PCB153-treated mice at 168 h. Liver sections from (A) vehicle showed an overall lack of vacuolization, (B) 30 µg/kg TCDD-treated animal exhibited slight to moderate vacuolization and instances of multifocal inflammation (arrow), and (C) an animal treated with 300 mg/kg PCB153 exhibited minimal vacuolization and hepatocellular hypertrophy. PCB153 did not elicit immune cell infiltration or necrosis. PV – Portal Vein; CV – Central Vein.

Figure 3. GC-MS lipid profile analysis at 24, 72 and 168 h. Principal component analysis indicated temporal and treatment dependent separation of PCB153 and TCDD fatty acid methyl ester profiles (fold change ratios) relative to their respective time-matched controls.

Figure 4.

Fatty acid methyl ester (FAME) GC-MS analysis of PCB153- and TCDD-treated mouse livers at 168 h. Treatment with 30 µg/kg TCDD induced a variety of individual FAMEs compared to 300 mg/kg PCB153 which did not affect FAME composition relative to control animals.

Figure 5.

QRT-PCR verification of selected PCB153-induced genes. The same RNA samples used in the time course and dose-response microarray studies were also used for QRT-PCR analysis. PCB153 induced CAR/PXR regulated genes, Cyp2b10, Cyp3a11, Cyp2c55 and Gadd45b, in (A) time course and (B) dose-response studies. (C) TCDD elicited minimal differential expression of CAR/PXR responsive genes. All fold changes were calculated relative to vehicle controls. Bars (left y-axis) and lines (right y-axis) represent QRT-PCR and microarray data, respectively. The genes are represented by their official gene symbols. Bars represent the mean $\pm$ SE of at least four independent samples. Data were analyzed by ANOVA followed by Tukey's and Dunnett's *post hoc* test. The asterisk (*) indicates $p < 0.05$.

Figure 6.

QRT-PCR verification of selected TCDD-regulated genes. The same RNA samples used in the time course microarray studies were also used for QRT-PCR analysis. **(A)** TCDD induced AhR-responsive genes, Cyp1a1, Cyp1a2, Tiparp, as well as Elov15. **(B)** PCB153 elicited modest regulation of AhR-responsive genes, but down-regulation of Elov15. All fold changes were calculated relative to vehicle controls. Bars (left y-axis) and lines (right y-axis) represent QRT-PCR and microarray data, respectively. The genes are represented by their official gene symbols. Bars represent the mean $\pm$ SE of at least four independent samples. Data were analyzed by ANOVA followed by Tukey's *post hoc* test. The asterisk (*) indicates $p < 0.05$.

Figure 7.

Dose-response modeling. 846 genes exhibited a dose-response profile and were categorized according to their ED_{50} value.

Figure 8.

(A) Microarray data sets for 30 $\mu\text{g/kg}$ TCDD and 300 mg/kg PCB153 were compared at 4, 12, 24, 72 and 168 h with stringent ($|\text{fold change}| \geq 1.5$, $P1(t) \geq 0.999$) and relaxed ($|\text{fold change}| \geq 1.5$, $P1(t) \geq 0.985$) selection criteria. Numbers in the Venn Diagram represent unique genes. (B) The correlation plot illustrates that the 74 genes regulated by TCDD and PCB153 are poorly correlated, indicating that many of the genes exhibited divergent regulation. The x-axis represents the correlation of gene expression, while the y-axis represents the correlation between significance values. Genes (dots) located within the upper right quadrant exhibit good correlation across their gene expression profiles and significance values indicating that their expression patterns are similar. Genes in the lower left quadrant are poorly correlated with respect to fold induction and significant values indicating that the TCDD and PCB153-elicited expression patterns are different, suggesting different mechanisms of regulation. Approximately 54% of the genes (40/74) were located within the upper right hand quadrant, indicating that 34 of the 74 common genes were poorly correlated. In contrast, when PCB126 and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDF) were compared to TCDD in the same model, 68% and 82% of the genes were located in the upper right hand quadrant (Kopec *et al.* 2008; N'Jai *et al.* 2008), further illustrating the differences between PCB153 and TCDD, PCB126 and TCDF.

Temporal Effects of 300 mg/kg PCB153 and 30 µg/kg TCDD on Terminal Body, Body Weight Gain, Absolute Liver Weight and Relative Liver Weight.

Table 1

Sacrifice time (h)	Treatment	Terminal body weight (g)	Body weight gain	Liver weight (g)	Relative liver weight
4	Vehicle	12.5 ± 0.5	1.0 ± 0.0	0.666 ± 0.028	0.053 ± 0.002
	PCB153	12.5 ± 0.8	1.0 ± 0.0	0.712 ± 0.048	0.057 ± 0.001
	TCDD	13.6 ± 0.8	1.0 ± 0.0	0.733 ± 0.044	0.054 ± 0.003
12	Vehicle	14.0 ± 0.7	1.0 ± 0.0	0.766 ± 0.048	0.055 ± 0.001
	PCB153	13.0 ± 0.6	1.0 ± 0.0	0.730 ± 0.075	0.056 ± 0.003
	TCDD	12.8 ± 0.7	1.1 ± 0.0	0.724 ± 0.025	0.056 ± 0.001
24	Vehicle	14.4 ± 0.9	1.1 ± 0.0	0.814 ± 0.070	0.056 ± 0.003
	PCB153	13.3 ± 0.9	1.1 ± 0.0	0.813 ± 0.137	0.061 ± 0.009
	TCDD	14.2 ± 0.9	1.1 ± 0.0	0.973 ± 0.088	0.068 ± 0.003 [*]
72	Vehicle	15.6 ± 1.5	1.2 ± 0.0	0.918 ± 0.055	0.059 ± 0.005
	PCB153	15.7 ± 0.7	1.1 ± 0.0	1.086 ± 0.135	0.069 ± 0.007 [*]
	TCDD	15.1 ± 0.7	1.2 ± 0.1	1.068 ± 0.060	0.071 ± 0.003 [*]
168	Vehicle	17.6 ± 0.4	1.4 ± 0.1	1.017 ± 0.082	0.058 ± 0.004
	PCB153	17.9 ± 1.2	1.4 ± 0.1	1.280 ± 0.074	0.072 ± 0.002 [*]
	TCDD	17.5 ± 0.4	1.3 ± 0.0	1.263 ± 0.080	0.072 ± 0.003 [*]

Values represent mean ± standard deviation (SD) of five independent replicates. Body weight gain is represented as terminal body weight divided by body weight prior to dosing.

^(*) Asterisk indicates p<0.05 vs. vehicle.

Table 2

Dose-Dependent Effects of PCB153 on Terminal Body, Body Weight Gain, Absolute Liver Weight and Relative Liver Weight at 24 h.

Dose (mg/kg)	Treatment	Terminal body weight (g)	Body weight gain	Liver weight (g)	Relative liver weight
0	Vehicle	11.8 ± 1.9	1.1 ± 0.0	0.694 ± 0.085	0.059 ± 0.006
1	PCB153	14.3 ± 1.8	1.1 ± 0.0	0.850 ± 0.121	0.059 ± 0.002
3	PCB153	14.4 ± 1.7	1.1 ± 0.0	0.865 ± 0.119	0.060 ± 0.002
10	PCB153	12.7 ± 1.1	1.1 ± 0.1	0.721 ± 0.073	0.056 ± 0.004
30	PCB153	13.4 ± 1.0	1.1 ± 0.0	0.836 ± 0.066	0.062 ± 0.001
100	PCB153	13.8 ± 1.1	1.1 ± 0.0	0.829 ± 0.071	0.060 ± 0.002
300	PCB153	12.7 ± 1.9	1.1 ± 0.0	0.855 ± 0.152	0.067 ± 0.003*

Values represent mean ± SD of five independent replicates. Body weight gain is represented as terminal body weight divided by body weight prior to dosing.

(*) Asterisk indicates $p < 0.05$ (PCB153 vs. vehicle).

Table 3

Functional Categorization and Regulation of Select Hepatic Genes Identified as Differentially Regulated in Response to 300 mg/kg PCB153 and 30 µg/kg TCDD (Boverhof *et al.* 2005).

Functional category	Gene name	Gene symbol	Entrez Gene ID	Regulation	PCB153 fold change ^a	TCDD fold change [*]	TCDD time course Boverhof <i>et al.</i> ^(*)	DREs ^b	PXRES ^c	CAREs ^d
Metabolizing enzymes/ Oxidoreductase activity	aldehyde oxidase 1	Aox1	11761	▲	2.9	2.2	N/A	4	5	2
	aldo-keto reductase family 1, member B7	Akr1b7	11997	▲	22.1	no change	no change	4	6	2
	aldo-keto reductase family 1, member C20	Akr1c20	116852	▼	-2.3	-1.7	N/A	-	1	-
	carboxylesterase	Ces2	234671	▲	5.8	2.5	1.6	1	4	1
	cytochrome P450, family 2, subfamily b, polypeptide 10	Cyp2b10	13088	▲	27.5	2.3	no change	5	5	1
	cytochrome P450, family 2, subfamily b, polypeptide 23	Cyp2b23	243881	▲	19.7	no change	N/A	2	5	-
	cytochrome P450, family 2, subfamily b, polypeptide 9	Cyp2b9	13094	▲	7.6	no change	N/A	-	4	1
	cytochrome P450, family 2, subfamily c, polypeptide 54	Cyp2c54	404195	▲	2.4	no change	N/A	-	5	1
	cytochrome P450, family 2, subfamily c, polypeptide 55	Cyp2c55	72082	▲	48.4	1.8	N/A	4	3	1
	cytochrome P450, family 3, subfamily a, polypeptide 25	Cyp3a25	56388	▲	3.8	no change	N/A	-	5	-
	dihydrofolate reductase	Dhfr	13361	▼	-2.1	no change	-1.5	5	5	1
	flavin containing monooxygenase 5	Fmo5	14263	▲	3.0	no change	no change	2	5	1
	glutathione peroxidase 2	Gpx2	14776	▲	1.7	no change	no change	8	9	3
	glutathione S-transferase, alpha 2 (Yc2)	Gsta2	14858	▲	4.1	5.9	7.2	4	10	2
	glutathione S-transferase, mu 4	Gstm4	14865	▲	4.0	2.4	N/A	2	5	1
Lipid metabolism	glutathione S-transferase, theta 3	Gstt3	103140	▲	1.7	1.5	no change	3	10	3
	UDP-glucose dehydrogenase	Ugdh	22235	▲	2.5	2.7	3.1	6	2	2
	acyl-CoA synthetase long-chain family member 3	Acsl3	74205	▼	-3.1	-2.3	N/A	2	4	-
	1-acylglycerol-3-phosphate O-acyltransferase 4 (lysophosphatidic acid acyltransferase, delta)	Agpat4	68262	▼	-1.9	no change	no change	7	4	1
	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	Hmgcr	15357	▼	-5.3	-2.6	N/A	4	5	1

Functional category	Gene name	Gene symbol	Entrez Gene ID	Regulation	PCB153 fold change ^a	TCDD fold change [*]	TCDD time course Boverhof <i>et al.</i> ^(*)	DREs ^b	PXRES ^c	CAREs ^d
Cell cycle/ Cell death	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2	Cds2	110911	▲	1.7	no change	no change	5	4	3
	ELOVL family member 6, elongation of long chain fatty acids (yeast)	Elov16	170439	▼	-3.8	-4.3	-1.8	3	5	1
	fatty acid synthase	Fasn	14104	▼	-3.9	-4.3	-1.9	4	7	1
	hydroxysteroid (17-beta) dehydrogenase 2	Hsd17b2	15486	▼	-2.0	2.2	N/A	6	6	-
	insulin induced gene 2	Insig2	72999	▲	5.9	-1.7	no change	1	4	1
	phospholipase A2, group XIIA	Pla2g12a	66350	▲	1.7	3.1	N/A	14	9	1
	sterol regulatory element binding factor 2	Srebf2	20788	▼	-2.1	-1.7	1.5	8	6	4
	sterol regulatory element binding transcription factor 1	Srebf1	20787	▼	-2.4	-2.3	-2.1	4	16	1
	mitotic arrest deficient 1-like 1	Mad11l	17120	▲	2.4	no change	N/A	8	8	1
	cell division cycle associated 2	Cdc42	108912	▲	2.8	no change	N/A	2	3	1
	growth arrest and DNA-damage-inducible 45 alpha	Gadd45a	13197	▲	2.0	-3.6	N/A	3	-	2
	growth arrest and DNA-damage-inducible 45 beta	Gadd45b	17873	▲	10.7	3.2	4.6	4	14	4
	myelocytomatosis oncogene	Myc	17869	▲	2.9	3.0	3.7	9	2	1
	nucleolar protein 3 (apoptosis repressor with CARD domain)	Nol3	78688	▲	4.9	no change	no change	12	6	-
Transport	sphingomyelin phosphodiesterase 3, neutral	Smpd3	58994	▲	10.8	-2.6	N/A	4	6	1
	ZW10 interactor	Zwint	52696	▲	1.8	no change	N/A	2	5	-
	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	Abcc3	76408	▲	1.9	1.7	1.5	2	9	5
	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	Slc1a4	55963	▼	-2.5	no change	no change	6	10	-
	solute carrier family 23 (nucleobase transporters), member 1	Slc23a1	20522	▲	2.3	-1.7	N/A	3	2	1

^aMaximum fold change, |fold change| 1.5, P1(*t*) 0.985

^{*}Maximum fold change, |fold change| 1.5, P1(*t*) 0.90

^bPutative DREs, PXREs and CAREs identified by computational searches (See *Materials & Methods* and Supplementary Figure 2) N/A – not available on cDNA microarray

^c Putative DREs, PXRREs and CAREs identified by computational searches (See *Materials & Methods* and Supplementary Figure 2) N/A – not available on cDNA microarray

^d Putative DREs, PXRREs and CAREs identified by computational searches (See *Materials & Methods* and Supplementary Figure 2) N/A – not available on cDNA microarray