TOXICOGENOMIC ANALYSIS REVEALS THAT THE RELATIVE POTENCY OF PCB 126 IN HUMANS IS FAR LESS THAN THE WHO TEF OF 0.1

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Introduction

The use of toxic equivalency factors (TEFs) for dioxin-like compounds in human health-related risk assessment is increasing. The World Health Organization (WHO) has recently updated the TEF values for several so-called "dioxin-like" polychlorinated biphenyls (PCBs)[1]. In this WHO report, the expert panel decided to keep the PCB 126 TEF at 0.1 based upon relative potency (REP) values derived from various *in vivo* rat studies in the Haws *et al.*[2] database. Although the database included REP_{PCB126} estimates from several *in vitro* human studies[3,4,5,6], human data was not given any weight in the WHO analysis. Data from these human studies, along with several more recent *in vitro* human studies[7,8,9], indicate that the REP_{PCB126} in humans is far less than the TEF of 0.1 would suggest. Van den Berg *et al.*[1] recognized this fact, but deemed the information "too limited to make a decision other than to retain 0.1 as the WHO 2005 TEF." The current study was designed to address this perceived dearth of information regarding human sensitivity to PCB 126 by examining the dose-response changes in gene expression elicited in 2,3,7,8-tetrachlorodibezo-*p*-dioxin (TCDD)- and PCB 126-exposed primary hepatocytes from humans and Sprague-Dawley rats using microarrays.

Materials & Methods

Chemicals

TCDD was obtained from Accustandard (New Haven, CT; catalog no. D404N; CAS no. 1746-01-6; lot no. 970401R-AC; 99.1% pure). The single contaminant was a pentachlorohydroxydiphenyl ether by GC/MS. PCB 126 was obtained from Accustandard (Catalog no. C-126N;CAS no. 57465-28-8; Lot no. 081699MT-AC; 99.2% pure). The single contaminant was identified as a tetrachlorobiphenyl by GC/MS.

Hepatocyte sources

Human hepatocytes were prepared from non-transplantable human tissue acquired after informed consent for use in research by In Vitro Technologies, Inc. (IVT). An external FDA-certified Institutional Review Board approved the use of human tissue for ADME-Tox research at IVT. Human donors included 1 male and 1 female Caucasian and 1 male of African descent. Rat hepatocytes (2 pools from 3 rats/pool) were isolated by IVT from female Sprague-Dawley rats (Crl:CD®(SD)IGS BR, Charles River Laboratories, Wilmington, MA). Rats were treated in accordance with the Animal Welfare Act.

Hepatocyte culture, chemical treatments

Isolated primary hepatocytes were cultured as previously described by Silkworth *et al.*[7] Established 48h cell cultures were exposed to TCDD (concentrations ranging from 10^{-14} to $10^{-6.5}$ M), PCB 126 (10^{-12} to 10^{-5} M), or vehicle control (DMSO) in serum-free media for an additional 48h. Exposure media was changed once at 24h post-exposure initiation. Culture viability was assessed in replicate sets of cultures for each exposure group via the 3-[4,5-dimathylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. TCDD and PCB 126 did not affect culture viability at any concentration tested.

RNA extraction & microarray analysis

Total RNA was isolated from each culture and quality-control (QC) tested as previously described[10]. One RNA sample from each exposure group was analyzed using Affymetrix Genechip® technology according to the standard protocol. Human samples were analyzed using HG-U133A arrays (22,283 probe sets) and rat samples with RG-U34A arrays (8,799 probe sets). The final data set utilized 40 human and 26 rat arrays.

Dose-response modeling

Raw microarray data was background-corrected and quantile-normalized using default setting of the *gcrma*[11] package version 2.8.0 in R. Pre-processed data was then back-transformed and fold change expression estimates (i.e., exposed divided by vehicle control) were obtained for each probe set and within each human subject /rat

pool. Gene expression data was filtered to remove probe sets that did not change greater than 2-fold (induced or repressed) in at least 2 out of 3 human subjects for at least one dose. The same fold change filter was applied to the rat data where both pools required at least a 2-fold response. Filtered probe sets were then modeled as nonlinear sigmoid curves using a modified Hill equation (Equation 1),

$$R = \frac{A}{1 + 10^{HS(EC50 - d)}} \quad \textbf{(Equation 1)}$$

where ***R*** is the average response (fold change) of an individual from a larger population for a single probe set; ***A*** is the upper right asymptote (i.e., maximum response); ***HS*** is the Hillslope at 50% maximal response; ***EC50*** is the effective concentration ($\log_{10}$) at 50% maximal response for the base chemical (i.e., TCDD); and, ***d*** is the $\log_{10}$ molar dose. Data was modeled for each species separately, but for both chemicals (i.e., TCDD and PCB 126) simultaneously with TCDD as the base chemical and ***dEC50*** equating to $EC50_{PCB126} - EC50_{TCDD}$. Thus, the REP_{PCB126} was obtained by calculating 10^{-dEC50}. Each chemical was assumed to generate parallel dose-response curves of equal efficacy (i.e., ***A*** and ***HS*** do not vary with chemical type). Upon visual inspection of the dose-response relationships for aryl hydrocarbon receptor (AHR) battery genes (i.e., *CYP1A1, CYP1B1, CYP1A2, ALDH3A1*), a mixed-effects model, which allowed for ***A*** to vary among subjects/pools (i.e., the random effect), was deemed adequate to fit these responsive probe sets. However, for all filtered probe sets, models that contained either (1) both ***A*** and the ***EC50*** as random effects varying among subjects or (2) no random effects at all (i.e., a generalized nonlinear least squares model) were also attempted and the simplest model of the three was chosen using nested log-likelihood ratio tests (LRT; $p \leq 0.05$) and, if significantly different, the lowest Akaike Information Criterion (AIC). Since initial analyses revealed significant heteroskedasticity in residual variance of some probe sets (e.g., *CYP1A1*), the adequacy of models containing a weighted variance term was also tested using LRTs ($p \leq 0.05$) and the Breusch-Pagan test against heteroskedasticity ($p \geq 0.05$). The maximum likelihood estimate (MLE) and 95% lower and upper confidence bounds of the MLE were reported for model terms ***A, HS, EC50*** and ***dEC50***. For the human subjects, it was also anticipated that 1 out of the 3 humans might not have responded at all to the chemical treatment (i.e., a non-responder). Thus, if any human did not respond at least 1.5 fold to TCDD for at least one dose for any filtered probe set, this human was removed from further analyses and the data was modeled for only the two responding humans. All nonlinear modeling was performed with the *nlme* and *gnls* functions of the *nlme*[12] package version 3.1-86 in *R*.

Results & Discussion

The current study sought to determine the species-specific relative potencies of PCB 126 at the transcriptomic level in primary hepatocytes from both humans and Sprague-Dawley rats. The dose-response models utilized rely upon a set of assumptions that form the criteria of the TEF concept[1]. These major assumptions include (1) responsive genes are potentially regulated by the AHR pathway, (2) PCB126 and TCDD are equally efficacious AHR agonists, and (3) dose-response curves for both compounds are parallel. Although not presented here, these hypotheses were tested in successfully modeled probe sets and, overall, were likely valid.

Dose-response models were generated for 97 rat probe sets (48 induced and 49 repressed) and 57 human probe sets (45 induced and 12 repressed). Expectedly, probe sets representing well-known AHR battery genes (i.e., *CYP1A1, CYP1A2,* and *CYP1B1*) were among the most highly responsive for both species. Chemical-specific dose-response models were also attempted, resulting in 14 PCB- and 7 TCDD-specific models for human probe sets and 17 PCB- and 5 TCDD-specific models for rat probe sets. The observance of few chemical-specific changes in gene expression in the current study using both TCDD and PCB 126 further substantiates the very similar higher-level phenotypic changes induced by both of these compounds in chronic *in vivo* rodent studies[13,14].

The human dose-response data generated in the current study was particularly challenging to model. This was mainly due to measurable differences in maximal fold change response seen among the human subjects for ~50% of the probe sets modeled (e.g., *CYP1A1;* Figure 1). Large inter-individual heterogeneity in chemical efficacy is to be expected in such a diverse sampling (i.e., both male and female Caucasian subjects and a male subject of African descent). This problem, for the most part, was successfully addressed by the use of a mixed-effects nonlinear modeling approach. Furthermore, 47% of the 57 modeled probe sets possessed 1 non-responsive human. Interestingly, a term to describe the variability of the $EC50_{TCDD}$ among subjects was not

necessary for the vast majority of human probe sets modeled (only ~ 16%); suggesting that TCDD and PCB 126 might express fairly uniform chemical-specific potencies for responsive individuals in the human population.

To summarize the transcriptomic responses observed in the current study, the distributions around each species-specific geometric mean REP_{PCB126} estimate were simulated using a Monte Carlo procedure which accounted for the inherent uncertainty in the estimation process (i.e., the 95% confidence interval on parameter estimates). Ten thousand Monte Carlo trials were used. Probe sets representing redundant genes were conservatively eliminated by removing the redundant probe set(s) with the lowest REP_{PCB126}, leaving 74 and 48 non-redundant probe sets for rat and humans, respectively. The Monte Carlo procedure made several assumptions including (1) every probe set represented an independent response to the chemicals, (2) each dose-response model was of equal quality and importance, and (3) each probe set-specific REP_{PCB126} was log-normally distributed around the MLE. Following this procedure the geometric mean REP_{PCB126} for rat and human were 0.057 and 0.0022, respectively, and the simulated full distributions are depicted in Figure 2 (dotted red line indicates the TEF_{PCB126} of 0.1). Clearly, the species-specific geomean REP_{PCB126} distributions did not overlap and the human geomean REP_{PCB126} distribution was far lower than the current TEF of 0.1. Although the distribution of the rat geomean REP_{PCB126} obtained in the current study was slightly lower than 0.1, it is certainly within the "order of magnitude" uncertainty prescribed to WHO TEFs[1]. Other caveats revealed in the current study that further substantiate a lower PCB 126 sensitivity for humans include: (1) overall greater $EC50_{TCDD}$ estimates in humans compared to rats; (2) probe sets with higher human REP_{PCB126} displayed much greater $EC50_{TCDD}$ estimates and not lower $EC50_{PCB126}$ estimates; and, (3) significant induction of gene ontology and KEGG pathway categories in humans required at least 2 orders of magnitude more PCB 126 than TCDD.

Thus, data from the current study certainly helps fill in data gaps present in the interspecies and *in vitro-in vivo* extrapolation parallelogram as modified from that of Sobels *et al.*[15] (Figure 3). Since the speculated potency of PCB 126 drives the estimated dioxin toxic equivalency (TEQ) load in exposed humans, recent data on human sensitivity to this PCB congener reported here and elswhere[3-9] may help explain the lack of excess hepatic tumors reported in worker populations most heavily exposed to PCBs[16]. Furthermore, this new data for humans demonstrates the necessity for lowering the PCB 126 TEF by greater than an order of magnitude if it is to be accurately applied in human health risk assessment.

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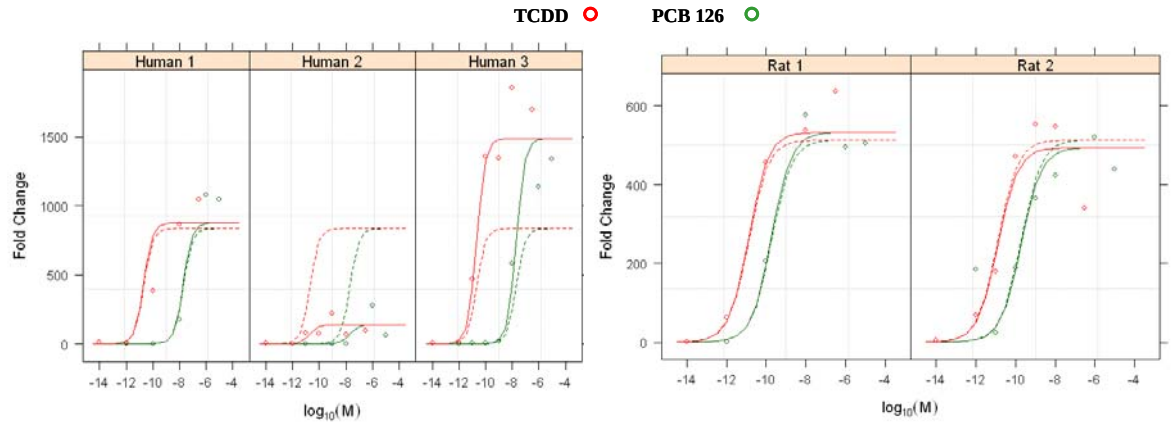
Figure 1. Dose response model output for probe sets representing *CYP1A1* and *Cyp1a1* genes in human (left) and rat (right), respectively*



* Dotted lines denote population-level model estimates, solid lines indicate subject-level estimates, and circles are the actual gene expression values

Figure 2. Species-specific REP_{PCB126} distributions for human and rat gene expression data

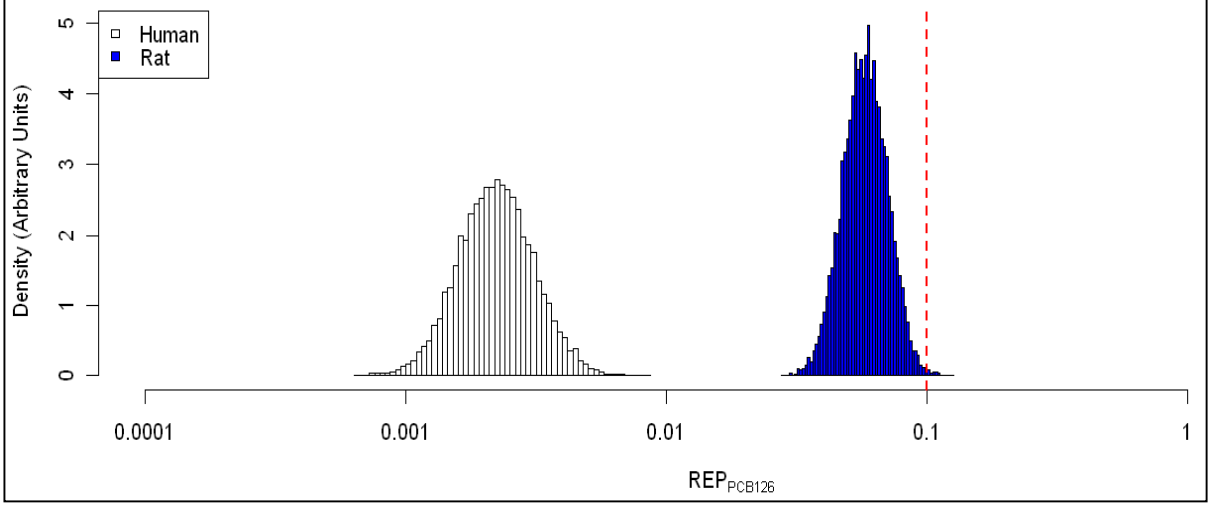


Figure 3. Inter-species and *in vitro-in vivo* extrapolation parallelogram supplemented with data from current study