SPECIES DIFFERENCES IN PCB TOXICODYNAMICS AND TOXICOKINETICS RELEVANT TO THE AROCLOR 1254 REFERENCE DOSE

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Introduction

Chemical-Specific Adjustment Factors (CSAFs) [1] and Data-Derived Extrapolation Factors (DDEFs) [2] can replace default uncertainty factors (UFs) for interspecies extrapolation (UF_A) and intraspecies variability (UF_H) commonly employed in the derivation of reference dose (RfD) human health risk values. For development of DDEFs, the default UFs are sub-divided into toxicokinetic (TK) and toxicodynamic (TD) components, representing 3-fold each (i.e., ~$10^{1/2}$). DDEFs for TK and TD are estimated using a variety of techniques from pharmacokinetic or biologically-based dose response models to relatively simple ratios quantitating animal-to-human sensitivity or human-to-human variability. Here, we generate potential DDEFs to replace the UF_A currently employed in the chronic oral RfD for the polychlorinated biphenyl (PCB) mixture, Aroclor 1254 [3]. The current Aroclor 1254 RfD is set by the US EPA at 2 x 10^{-5} mg/kg-d, based upon an oral-dosing study of rhesus monkeys (*Macaca mulatta*) [4,5]. The critical effects include various dermal lesions and decreased antibody response. The lowest dose employed (i.e., 0.005 mg/k-d) served as the LOAEL and this dose was divided by a composite UF of 300 to achieve the RfD. Of particular interest, the UF_A was reduced to 3 from the default of 10 due to, "… similarities in toxic responses and metabolism of PCBs between monkeys and humans and the general physiologic similarity between these species." [3] In this study, we analyze the necessity for a 3-fold UF_A in extrapolation to a human safe dose level. We also present new *in vitro* data which directly compares sensitivity of human and rhesus keratinocytes to induction of a biomarker for an early key event in the presumed mode of action (MOA). Following the DDEF guidelines, a quantitative extrapolation factor (EF) to replace the UF_A is derived.

Methods

Aroclor 1254, lot no. 122-078, was the same material used in several published studies [6,7]. The calculated dioxin toxic equivalency (TEQ) for the Aroclor lot used was ~21ppm (~70% due to PCB 126). Sources and purity analyses for the other chemicals have been described previously [8]. Neonatal foreskin normal human epidermal keratinocytes (NHEKs), purchased from Lonza (Walkersville, MD), were grown in keratinocyte-SFM (Invitrogen, Carlsbad, CA). Confluent fifth passage NHEKs were incubated in complete media (50 μg/ml bovine pituitary extract; 5 ng/ml EGF) for 48 h, changed to basal media (no supplements) for 24 h and then treated with chemicals in basal media for the time indicated or for 48 h in the dose-response studies [8]. Rhesus eyelid keratinocytes were purchased from Lonza. Confluent second passage rhesus keratinocytes were incubated in KBM Gold (Lonza) with the provided supplements, consisting of BPE, EGF, insulin hydrocortisone and epinephrine for 48 h, changed to basal keratinocyte –SFM for 24 h and then treated with vehicle (0.7% DMSO) or chemicals in keratinocyte-SFM for 48 h. Total mRNA was isolated using RNA Stat-60 (Tel-Test). Real-time PCR was carried out using the Roche LightCycler 480 and the LC480 SYBR Green I Master kit. Actin was used as the reference for sample normalization. Human primers for *CYP1A1* and *ACTIN* have been described previously [8]. The following primers (5'-3') were used for rhesus mRNAs: *CYP1A1*, ATCCCCCACAGCACCACAAGAGAC and TGCCCAAGCCAAAGAGAATCACCT; *ACTIN*, GCTGGCCGGGACCTGACTGACTA and CCGCCGTGGCCATCTCCTG. Relative quantification of the mRNA was determined using the calculated efficiencies and the previously described method [9]. Threshold modeling procedures are described in detail previously [8], except the new interspecies threshold model used here contrasts dose response data between human and rhesus for TCDD, PCB 126, and Aroclor 1254. Guidelines outlined in the EPA external review DDEF draft were followed to assess interspecies differences in TK and TD and to estimate interspecies TD EFs (i.e., EF_{AD}) [2]. Briefly, critical effects were identified and information regarding the MOA was assembled. Dose response models were constructed for the initial key event in the hypothesized MOA using *in vitro* cell cultures derived from the relevant target tissue. Species differences in TD

components were then calculated from ratios of EC50s or thresholds. These ratios served as candidates for an EF_{AD} to replace the TD portion of the UF_A, and adjusted RfDs were generated.

Results and Discussion

Mode of Action. Dermal lesions seen in rhesus orally-dosed with Aroclor 1254 grossly resemble signs of polychlorinated dibenzofuran (PCDF) poisoning observed during the Yusho and Yu-Cheng events [10]. These same lesions have been induced in rhesus with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [11], PCDFs [12], other Aroclors [13], and single co-planar PCB congeners [14]. Histologically, the rhesus dermal lesions exhibit pathologies highly similar to those of human chloracne induced by dioxins and PCDFs. Conserved histopathology includes: involution and/or disappearance of sebaceous glands; keratinization of the epidermal layer; and, sebaceous gland metaplasia [10]. It is widely-accepted that chloracne is specifically the consequence of sufficient exposure to potent and efficacious "dioxin-like" aryl hydrocarbon receptor (AHR) agonists [10]. AHR activation has been verified in human chloracne by demonstration of strong expression of the sensitive biomarker *CYP1A1* in lesions [15]. Due to the specificity of this response, a single MOA /mechanism is highly probable. Since both the CSAF and DDEF guidelines explicitly state that information regarding the entire MOA is not necessary to develop alternatives to default UFs [1, 2], we focused on the initial key event in the MOA for this critical effect, i.e., activation of the AHR pathway. Furthermore, there is a general scientific consensus implicating AHR activation as the initial key event for all "dioxin-like" toxicities [16]. This includes the "dioxin-like" immune suppression observed in the critical study for the Aroclor 1254 RfD. The key events downstream of AHR activation in the chloracne MOA have yet to be fully elucidated, but likely include: induction/repression of AHR-regulated genes in the target tissue; altered cellular differentiation; and, aberrant proliferation of keratinocytes [10].

Toxicokinetics. As defined by the US EPA, "[t]oxicokinetics is concerned with delivery of the biologically active chemical to the target tissue of interest." [2] The UF_A used for the Aroclor 1254 RfD was 3, reduced from the default of 10 partially based upon the assumption that rhesus and humans metabolize PCBs in a similar manner [3]. However, unique PCB congener profiles were apparent in rhesus tissues obtained from the critical study [17], suggesting considerable TK differences may exist between rhesus and other species. Based upon the fact that the MOA for the critical effect specifically involves "dioxin-like" toxicity, we will focus on potential TK differences for the most potent "dioxin-like" PCB congeners. Although the exact congener make-up of the Aroclor employed in the critical study is not known, analyses of the dioxin toxic equivalency (TEQ) of various Aroclor 1254 lots have determined that the co-planar congener PCB 126 makes up the majority of the TEQ [18]. Unfortunately, analytical methods were likely lacking at the time of the critical study to reliably detect this congener in tissue samples from exposed animals. Similarly, since the use of Aroclor 1254 was greatly reduced in the early 1970s, measurements of PCB 126 in workers at the time they were most highly exposed to Aroclor 1254 could not be made. However, some information may be gleaned from what is known about TCDD TK in monkeys, rodents, and humans. In rodents, TCDD TK models predict that basal and AHR-induced levels of hepatic Cyp1a2 are important for TCDD tissue distribution due to Cyp1a2-mediated hepatic sequestration of TCDD [19]. A similar phenomenon has been observed in rodents exposed to PCBs 126 and 169 [20]. In Cyp1a2 knockout mice, more TCDD is distributed to extra-hepatic tissues (e.g., skin) and knockouts exhibit increased sensitivity to some toxic endpoints [21]. Hepatic sequestration of TCDD appears to be lacking in *Macaca spp.* [22], possibly explaining their increased sensitivity to some toxic endpoints (e.g., dermal /developmental /reproductive effects), but not others (e.g., hepatotoxicity). Recent studies have revealed that macaque *CYP1A2* may be under the process of becoming a pseudogene [23]. Thus, macaques may lack a functional CYP1A2 protein entirely. This is in stark contrast to humans where CYP1A2 is the third most abundant CYP in the liver and hepatic CYP1A2 induction has been observed in humans highly exposed to TCDD and PCDFs [24]. Various "dioxin-like" compounds bind with comparable affinity to rat and human CYP1A2 including TCDD and PCB 126 [25]. Furthermore, TCDD and PCB 126 are capable of inducing *CYP1A2* expression to varying levels in human hepatocytes, although higher concentrations were required to achieve induction levels comparable to rat cells [26].

Toxicodynamics. As defined by the US EPA, "[t]oxicodynamics describes the critical interaction of the active chemical moiety with the target site and the ensuing sequence of events leading to toxicity." [2] The rhesus monkey is highly sensitive to the toxic effects of dioxins and "dioxin-like" compounds. For example, ingestion of PCB-containing caulk was the suspected culprit in inducing severe "dioxin-like" toxicity and high mortality in rhesus monkeys housed in two separate facilities [27, 28]. This ultra-sensitive phenotype can be at least partially

explained by the occurrence of the same AHR ligand binding domain amino acid substitution in rhesus as in ultra-sensitive mouse and rat strains. This AHR genotype results in increased AHR affinity and subsequent toxicity for TCDD in rodents, and has not been identified in any human AHR sequence evaluated to date [29, 30]. Activation of the AHR pathway is the initial key event for the critical effects behind the Aroclor 1254 RfD. Previous investigations in our laboratory directly compared the *in vitro* response of human and rhesus hepatocytes for induction of the AHR activation biomarker, CYP1A1, following incubation with TCDD, PCB 126, or Aroclor 1254 [6]. We found that human hepatocytes were at least 2 orders of magnitude less sensitive to PCB 126- and Aroclor 1254-mediated CYP1A1 induction than rhesus cells. In this study, we expand upon previous work in hepatocytes to look at interspecies responses of keratinocytes, a sensitive cell type for one of the critical effects cited in the RfD. New dose response data for rhesus keratinocytes exposed to TCDD, 1,2,3,6,7,8-HxCDF, PCB 126, or Aroclor 1254, and human keratinocyte data previously described in Sutter *et al.* [8], are depicted in Figure 1. In addition, cells from human donors 1 and 5 were exposed to Aroclor 1254. For donor 1, Aroclor 1254 slightly induced CYP1A1 (< 1% of the maximal response achieved by TCDD) at only 10 and 30 µM. Although cells from donor 5 failed to respond to Aroclor 1254 at any concentration tested, they were responsive to TCDD, HxCDF, and PCB 126. Rhesus/human EC50 ratios for TCDD, HxCDF, and PCB 126 were 0.034, 0.038, and 0.00087, respectively. The interspecies ratio for PCB 126 is likely over-estimated because this congener acted as a partial agonist in human cells and not rhesus. Since we were unable to obtain a convergent model for human cells exposed to Aroclor 1254, we decided to focus on the bottom of the dose response curve by $\log_{10}$-transforming the response data and developed an interspecies threshold model similar to our previous threshold model. Figure 2A depicts the model used to directly estimate rhesus/human threshold ratios for TCDD and Aroclor 1254. TCDD and PCB 126 were also modeled simultaneously for rhesus (n=1) and human (n=4) donors (Figure 2B). Rhesus /human threshold ratios were 0.00068 (95% CI; 0.0004-0.0011) for Aroclor 1254 and 0.000095 (95% CI; 0.000048-0.00018) for PCB 126.

Figure 1

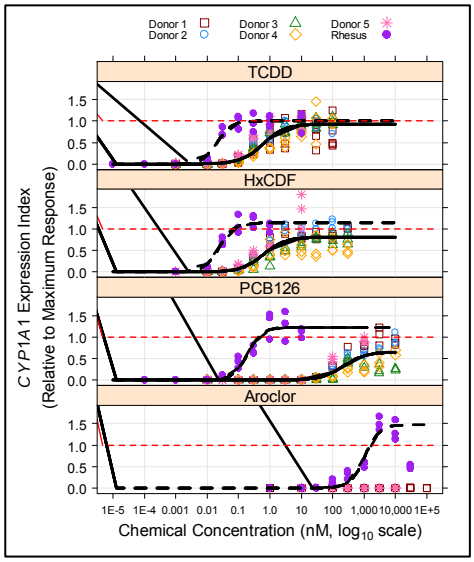
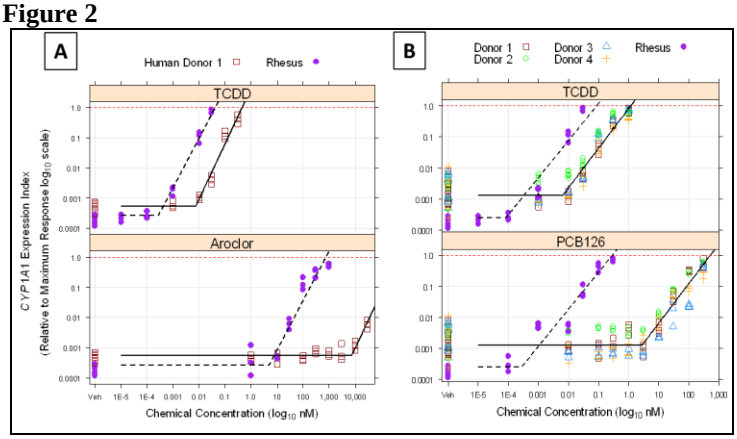


Figure 2



New RfD Estimation. Following the DDEF guidelines [2], we have examined the critical effects for the Aroclor 1254 RfD and gathered qualitative and quantitative information relevant to the UF_A. AHR activation was determined to be the initial key event in the MOA for the critical effect. In regards to interspecies TK differences, it is clear there is no basis for an assumption that the average human will accumulate the most potent "dioxin-like" congeners present in Aroclor 1254 (i.e., PCB 126) at the target tissue to a greater extent than rhesus monkeys. However, quantitative data are lacking, so we have elected to take a conservative approach and leave the TK portion of the UF_A at a default of 3. On the other hand, clear species differences for TD were found in keratinocytes and hepatocytes for a sensitive biomarker of the initial key event in the MOA for the critical effect. Using the most conservative estimate of EF_{AD} (i.e., the upper bound threshold ratio for Aroclor 1254 of 0.0011),

an adjusted RfD was calculated as follows: 2.0 x 10^{-5} mg/kg-d ÷ 0.0011 (EF_{AD}) = **1.8 x 10^{-2} mg /kg-d.** Thus, after adjustment for only interspecies TD differences, the safe dose from chronic oral exposure to Aroclor 1254 is ~900 fold higher than that suggested by the current RfD in IRIS. This finding is consistent with negative epidemiological studies of capacitor workers highly exposed to Aroclor 1254, with body burdens often exceeding those of the monkeys used in the critical study [31, 32]. Although "dioxin-like" AHR activation (i.e., CYP1A1/2 induction) has been described in PCDD- and PCDF-exposed humans exhibiting chloracne [15, 24], there is no evidence that this key event was produced in Aroclor 1254-exposed capacitor workers. Thus, the EF_{AD} derived here is consistent with the US EPA draft DDEF guidance [2] that states, "[q]uantitatively, DDEF values for UF_A components might be less than 1 if humans are less sensitive."

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