NON-AH RECEPTOR MECHANISMS UNDERLYING IMMUNOTOXICITY AND NEUROTOXICITY

STRUCTURE ACTIVITY RELATIONSHIPS BETWEEN SELECTED *ortho*-SUBSTITUTED POLYCHLORINATED BIPHENYLS TOWARD ACTIVATION OF RYANODINE RECEPTOR TYPE 1 - TEQS FOR NON-DIOXIN LIKE PCB CONGENERS

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Introduction

Polychlorinated biphenyls (PCBs) along with polychlorinated dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF) are halogenated aromatic hydrocarbons (HAHs). PCBs were synthesized and marketed as Aroclor mixtures, which were widely used in industries. The high lipophilicity and chemical resistance of these chemicals lead to worldwide contamination and accumulation in biota[1, 2]. Due to the diverse structures and difference in metabolic degradation of these chemicals, complex mixtures of PCB congeners with various concentrations have been identified in biological samples[3-6].

Certain dioxin-like PCB congeners have been shown to cause toxicity similar to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most potent congener in the PCDD family. The toxicity responses include chloracne, wasting syndrome, increase incidence of soft tissue sarcomas, immunotoxicity, reproductive toxicity, developmental toxicity, disruption of endocrine pathways, hepatotoxicity, and thymic and splenic atrophy. Recent studies have suggested that these toxic responses might be mediated through a common pathway which involves binding of HAHs to the aryl hydrocarbon (Ah) receptor[7]. Among the structures examined, 2,3,7,8-TCDD has been shown to have the highest affinity toward the Ah receptor[8-10]. As a result, relative toxicity of dioxin-like PCB, PCDD and PCDF congeners have been ranked according to their relative binding affinity to the Ah receptor. The most potent agonist to the Ah receptor, 2,3,7,8-TCDD has been assigned with 1.0 toxic equivalency factor (TEF) and TEFs for various PCB congeners have been calculated according to their relative binding affinity to the Ah receptor[10]. Based on the induction of aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin o-deethylase (EROD) activity in hepatocytes[11], or immunotoxicity measured with B6C3F1 mice immunized with trinitrophenyl-lipopolysaccharide (TNP-LPS)[12], several modifications on the TEF values of PCBs as well as other HAHs have been suggested on risk assessment of HAHs in complex mixtures.

In 1997, the World Health Organization held a meeting to derive consensus TEFs for PCDDs, PCDFs and dioxin-like PCBs for human, fish and wildlife risk assessment[13]. The TEF concept is based on a major assumption: at low dose, toxic equivalent (TEQ) concentration of HAH mixtures is additive, but no synergism nor antagonism, among the individual congeners. Several limitations on the TEF concepts have been identified[14]. In most of the

biological/environmental samples, relatively low concentrations of dioxin-like PCB congeners have been found, compared to non-dioxin like PCB congeners with low TEF. As a result of partial agonistic or antagonistic property of certain PCB congeners, non-additive interactions of PCB mixtures have been reported[14]. TEQ values are based *in vivo* and *in vitro* studies in biotic system. Influence in physiochemical factors that governed actual uptake of these chemicals from abiotic to biotic systems is not determined[15]. Due to the fact that TEFs are derived from Ah receptor-mediated mechanism, modulations through non-Ah receptor-mediated mechanisms are not considered. The meeting has concluded that the TEF concept is the most plausible and feasible approach for risk assessment of dioxin-like HAHs. However, the uses of TEF on non-dioxin-like PCB congeners, which are *di-ortho* or higher *ortho*-substituted, are excluded due to insufficient *in vivo* evidence[13]. Since *ortho*-substituted PCB congeners collectively represent a significant component of PCB mixtures found in environmental samples and biological tissues, it is urgent to develop new approaches for risk assessment of *ortho*-substituted PCB congeners.

Methods and Materials

Membrane Preparations. Membrane vesicles enriched in RyR1 were isolated from fast-twitch (white) skeletal muscles obtained from back of male New Zealand White rabbits, according to the method of Saito et al[16]. Briefly, muscle was grounded and then homogenized in ice-cold homogenization buffer consisting of 5mM imidazole-HCl, pH7.4, 0.3M sucrose, 10μM/ml leupeptin and 100μM phenylmethylsulfonyl fluoride. The junctional SR fraction was isolated by differential centrifugation and further purified by discontinuous sucrose gradient.

[^{3}H]Ryanodine Binding Assays. Specific binding of [^{3}H]ryanodine to skeletal microsomes was performed as previously reported[17], with slight modifications. Briefly, The ability of selected PCB congeners to dose-dependently alter [^{3}H]ryanodine binding to skeletal microsomes was examined by measuring the specific binding of 1nM [^{3}H]ryanodine to 12μg microsomal protein with the presence of 10nM to 100μM PCB in assay buffer consisting of 140mM KCl, 15mM NaCl, 20mM HEPES, 10% sucrose, 50μM $CaCl_2$, pH7.4, with a final volume of 500μl. Non-specific binding was obtained by addition of 1000-fold excess of cold ryanodine in the assay.

Ca^{2+} Transport Measurement. Net Ca^{2+} efflux from skeletal microsomes was measured with the metallochromic dye antipyrylazo III with the use of a diode array spectrophotometer (model 8542, Hewlett Packard, Palo Alto, CA), as reported previously[17]. The Ca^{2+} transport buffer consisted of 18.5mM K-MOPS, pH7.0, 92.5mM KCl, 7.5mM Na-pyrophosphate, 250μM antipyrylazo III, 1mM Mg-ATP, 20μg/ml creatine phosphokinase, 5mM phosphocreatine and 40μg skeletal microsome with a final volume of 1.2ml. Calcium release rate after the addition of PCB was determined by linear regression of the kinetic data. Rate data was calibrated by bolus addition of standard Ca^{2+} at the end of each assay.

Data Analysis. Potency and efficacy of selected PCB congeners were analyzed by using ENZFITTER computer program.

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Results and Discussion

Epidemiological studies have suggested that perinatal exposures to PCBs are correlated to cognitive deficits in children[18-20]. Significantly higher level of *ortho*-substituted PCBs has been reported in the caudate nucleus of patient with Parkinson's disease[21]. Studies on non-Ah receptor-mediated effects of non-dioxin-like PCB congeners have implicated the neurotoxic responses induced by *ortho*-substituted PCBs. Mechanistic studies on neurotoxicity induced by *ortho*-substituted was first performed by Seegal and coworkers[22, 23]. Structure activity relationships between selected PCB congeners and cellular catecholamine levels in neurons have been examined both *in vitro* using rat pheochromocytoma cells (PC12) and *in vivo* using nonhuman primates. Recent studies have further demonstrated that disruption of Ca^{2+} signaling may be the underlying mechanism for certain non-Ah receptor-mediated responses of *ortho*-substituted PCB congeners[24, 25].

We have provided direct evidence for a highly selective interaction between selected *ortho*-substituted PCB congeners and the immunophilin FKBP12/ryanodine receptor complex (FKBP12/RyR)[26, 27]. Together with the inositol 1,4,5-trisphosphate receptors (IP_3R), RyR complex are broadly expressed both in excitable and non-excitable cells. They are localized within specific regions of the sarcoplasmic or endoplasmic reticulum (SR/ER) stores. The RyR complex functions as a Ca^{2+} induced Ca^{2+} release (CICR) channel, whereas the IP_3R releases Ca^{2+} from store through the PLC-IP_3 signaling cascade. In the CNS, all three genetic isoforms of RyR are widely expressed. Recent studies with hippocampal neuronal culture have shown that localized Ca^{2+} efflux through RyR is related to spine formation and elongation, suggesting the role of RyR in neurodevelopment and neuroplasticity[28]. Although the detailed pathway by which *ortho*-substituted PCB congeners alter Ca^{2+} signaling in neuron are still needed to be elucidated, the stringent requirements of the structure of PCB congeners on alteration of Ca^{2+} signaling have suggested an receptor-mediated pathway.

To further understand the mechanism(s) by which PCBs disrupt Ca^{2+} signaling, we have performed a study detailing structure activity relationships between thirty-one PCB congeners and twelve PCB metabolites towards activation of ryanodine receptor type 1 (RyR1, expressed in skeletal muscle, brain and B lymphocytes). The dose response relationships between selected PCB congeners and activity of RyR1 were determined by a radioligand binding assay using [^{3}H]ryanodine (the conformation selective probe specific for ryanodine receptors) and microsomal preparations enriched in RyR1 isolated from rabbit fast-twitch skeletal muscle. Our results have revealed that the *ortho*- and *meta*-chloro-substitutions on the biphenyl structure of PCBs are the most important determinants of efficacy towards RyR1. Among the thirty-one congeners tested, fourteen environmentally relevant congeners were examined. PCB 18, PCB 52 and PCB 187 are highly efficacious towards activation of RyR1, while PCB 41, PCB 101 and PCB 110 are with intermediate efficacy. Of all the tested congeners, PCB 136 (2,2',3,3',6,6'-hexachlorobiphenyl) is the most efficacious and potent congener (EC_{50} = 575 nM). These results provide valuable information on predicting the potency or efficacy of environmentally relevant PCB congeners/metabolites on disruption of cellular Ca^{2+} signaling. We propose the use of efficacy of PCB towards activation of RyR1 and hence disruption of Ca^{2+} signaling, a non-Ah receptor-mediated mechanism, on the risk assessment of *ortho*-substituted PCB congeners.

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