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Repeated dose toxicity and relative potency of 1,2,3,4,6,7-hexachloronaphthalene (PCN 66) 1,2,3,5,6,7-hexachloronaphthalene (PCN 67) compared to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for induction of CYP1A1, CYP1A2 and thymic atrophy in female Harlan Sprague-Dawley rats

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Abstract

In this study we assessed the relative toxicity and potency of the chlorinated naphthalenes 1,2,3,4,6,7-hexachloronaphthalene (PCN 66) and 1,2,3,5,6,7-hexachloronaphthalene (PCN 67) relative to that of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Chemicals were administered in corn oil:acetone (99:1) by gavage to female Harlan Sprague-Dawley rats at dosages of 0 (vehicle), 500, 1500, 5000, 50000 and 500000 ng/kg (PCN 66 and PCN 67) and 1, 3, 10, 100, and 300 ng/kg (TCDD) for 2 weeks. Histopathologic changes were observed in the thymus, liver and lung of TCDD treated animals and in the liver and thymus of PCN treated animals. Significant increases in CYP1A1 and CYP1A2 associated enzyme activity were observed in all animals exposed to TCDD, PCN 66 and PCN 67. Dose response modeling of CYP1A1, CYP1A2 and thymic atrophy gave ranges of estimated relative potencies, as compared to TCDD, of 0.0015-0.0072, for PCN 66 and 0.00029-0.00067 for PCN 67. Given that PCN 66 and PCN 67 exposure resulted in biochemical and histopathologic changes similar to that seen with TCDD, this suggests that they should be included in the WHO Toxic Equivalency Factor (TEF) scheme, although the estimated relative potencies indicate that these hexachlorinated naphthalenes should not contribute greatly to the overall human body burden of dioxin-like activity.

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Keywords

1,2,3,4,6,7-hexachloronaphthalene (PCN 66); 1,2,3,5,6,7-hexachloronaphthalene (PCN 67); 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD); Toxic equivalency Factor; Relative Potency; TEF

1. Introduction

Polychlorinated naphthalenes (PCNs) are a family of two-ringed aromatic compounds, which contain one to eight chlorines per naphthalene and form 75 possible congeners (Falandysz 2003). PCN products are generally mixtures and were used in a variety of commercial applications including cable insulation, wood preservation, engine oil additives, electroplating masking compounds, capacitors, and refractive index testing oils and as a feedstock for dye production (WHO 2001). Production of PCNs has ceased due to substitutions of less toxic chemicals (Hayward 1998). PCNs are also formed during production of technical mixtures of chlorobiphenyls and can be found in various polychlorinated biphenyl formulations (Falandysz 2003). A variety of PCN mixtures were sold in the United States under the trade name Halowaxes; while in Europe they were sold under the trade names Nibren Waxes, Seekay Waxes and Clonacire Waxes, similar to PCB mixtures, the PCNs were sold as mixtures with different degrees of chlorination.

PCN exposure can occur through oral, inhalation, and dermal routes. Non-occupational exposure can result from air contamination near manufacturing sites, incineration of waste, and disposal of PCN containing items at landfills. PCNs have been detected in both urban and rural soils (Krauss and Wilcke 2003), waterway sediment (Brack et al. 2003; Kannan et al. 2001), aquifer water samples (Espadaler et al. 1997) and urban air (Helm and Bidleman 2003). As with other polychlorinated diaromatic hydrocarbons, PCNs are lipophilic compounds that persist in the environment and bioaccumulate in biological tissues (Falandysz 2003). Chlorinated naphthalenes have also been identified in fish (Ofstad et al. 1978), whale and seal tissue (Helm et al. 2002) representing potential dietary sources of these compounds.

Few studies examining human tissue concentrations of PCNs are available. PCN concentrations determined in pooled human milk from Sweden collected from 1972 and 1992 decreased from 3081 to 483 pg/g lipid over this 20 year period (Lunden and Noren 1998). In Sweden, concentrations of total PCNs ranged from 1000-4000 pg/g lipid in human adipose samples collected at autopsy with PCN 66 and 67 making up approximately 25-50% of the total (Weistrand and Noren 1998). More recently, PCN concentrations were measured in adipose tissue samples collected from 43 (14 male and 29 female) patients undergoing liposuction procedures in New York City (Kunisue et al. 2009). PCN congeners 1,2,3,4,6,7-hexachloronaphthalene (PCN 66) and 1,2,3,5,6,7-hexachloronaphthalene (PCN 67) made up approximately 16% of the total PCNs present at concentrations of approximately 100 and 47 pg /g lipid in males and females respectively. The PCN congeners 1,2,3,5,7- and 1,2,4,6,7-pentachloronaphthalene made up approximately 30% of the PCNs present in the adipose tissue from these tissues.

Occupational exposure to PCNs has been shown to produce illness similar to that caused by dioxin-like compounds. Workers from a cable manufacturing plant developed a high incidence of chloracne and liver disease associated with PCN exposure. Additional symptoms associated with PCN exposure include eye irritation, fatigue, headache, anemia, hematuria, impotency, anorexia, vomiting, and abdominal pain (HSDB 2011). Fatal cases of PCN toxicity have been associated with jaundice and hepatotoxicity (Hayward 1998). The carcinogenicity of PCNs has not been well studied, however, a cohort exposed to Halowax

was noted as having an increased incidence of gastrointestinal and respiratory neoplasms (Hayward 1998).

PCN exposure in animals results in toxicity similar to that reported in humans. Hyperkeratosis of rabbit ears and the skin of hairless mice was observed after PCN exposure (HSDB 2011). Fatal liver necrosis occurred in rabbits subcutaneously injected with a 15-ppm mixture of penta- and hexachloronaphthalene for up to 26 days (Hayward 1998). Rats fed mixtures of penta- and hexachloronaphthalenes every other day for 26 days developed swollen, vacuolated, and necrotic liver cells (HSDB 2011). Inhalation exposure to penta- and hexachloronaphthalene mixture to rats for 6 weeks resulted in hyalinization, swelling and slight granulation of the liver (Hayward 1998).

PCNs were sold as a variety of mixtures that ranged from those containing predominately low chlorinated mono and dichloronaphthalenes to mixtures containing predominately the octachloronaphthalenes. The more toxic mixtures are those containing predominately the penta- and hexachlorinated naphthalenes. These higher chlorinated mixtures induce biological effects similar to that of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) including induction of the liver cytochrome P450 -associated enzyme activities ethoxyresorufin-O-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH). In a rat hepatoma H-4-II cell line, the relative potency value of a mixture of PCN 66/67, compared to TCDD, was 0.002 and 0.003, based on measurement of cytochrome P450 -associated EROD and AHH, respectively (Hanberg et al. 1991). In another *in vitro* study relative potency estimates based on EROD induction were 0.00063 for PCN 66 and 0.00029 for PCN 67 (Villeneuve et al. 2000). Hexachlorinated naphthalenes can also induce the Aryl hydrocarbon (Ah) receptor dependent reporter gene activities (Behnisch et al. 2003). Given this profile of activity, ability to persist in the food chain (Falandysz 2003), and ability of PCN mixtures to produce toxicities similar to that of dioxins, PCNs have been considered for inclusion in the World Health Organization's (WHO) dioxin toxic equivalency factor (TEF) scheme (Van den Berg et al. 2006). While there have been studies of *in vitro* potency of individual congeners (Behnisch et al. 2003), there are no *in vivo* data available to estimate the relative potency for individual PCN congeners. The aim of this present study was to investigate the relative toxicity and potency of PCN 66 and PCN 67 for induction of toxicologic and biochemical endpoints in female Harlan Sprague-Dawley rats, compared to that of TCDD, after subacute (14 day) repeated exposure. Although production of PCNs has ceased in recent years due to substitutions of less toxic chemicals (Hayward 1998), the environmental contamination of PCNs and the detection of PCN 66 and PCN 67 in human tissue warrant further investigation of the *in vivo* toxicity and relative potency of these dioxin-like compounds. These data will provide for a better evaluation of the possible inclusion of PCN 66 and PCN 67 in the TEF scheme (Van den Berg et al. 2006).

2. Materials and Methods

PCN 66 (CAS No. 103426-96-6, Lot No.: 32467-79) and PCN 67 (CAS No. 103426-97-7; Lot # 32467-59) were obtained from Radian International (Austin, TX). TCDD (CAS No. 1746-01-6) was obtained from IIT Research Institute (Chicago, IL). PCN 66, PCN 67, and TCDD were formulated for gavage administration in corn oil (Spectrum, Gardena, CA) containing 1 percent acetone. Infrared spectrometry and nuclear magnetic resonance spectrometry were used to independently confirm the identity of each test chemical. Spectra were consistent with the structures of PCN 66 and PCN 67 although there was insufficient information to unambiguously differentiate the test compound from other possible symmetric hexachloronaphthalenes. The vendor stated purity (99.9% and 99.78 for PCN 66 and PCN 67, respectively) was checked by capillary gas chromatography. Each chromatogram showed one major peak. A 1:1000 dilution was analyzed to demonstrate that

an impurity at a level of 0.1% would have been detected. Consequently, the estimated purity for PCN 66 and PCN 67 was greater than 99.9%. Details about the physicochemical characterization of the materials used in the study is available from the National Toxicology Program Central Data Management group upon request (<http://ntp.niehs.nih.gov/go/contact>).

The studies were conducted for the National Toxicology Program at Battelle Columbus Laboratories (Columbus, OH) in accordance with Good Laboratory Practices, supported by NIEHS contract N01-ES-65406. Animal use was in accordance with the United States Public Health Service policy on humane care and use of laboratory animals and the Guide for the Care and Use of Laboratory Animals. Five-week-old female Harlan Sprague-Dawley rats (Harlan Sprague-Dawley Inc., Indianapolis, IN) were quarantined for 11-12 days. Animals were 52-53 days old on the first day of the studies. Feed (NTP-200 diet, Zeigler Brothers, Inc., Gardners, PA) and water were available *ad libitum*. Rats were housed 5 per cage. Before the study began, 10 females were randomly selected for parasite evaluation and gross observation for evidence of disease. All test for viral titers were negative.

PCN 66, PCN 67, and TCDD, formulated in corn oil/1% acetone as the vehicle, were administered by gavage to groups of five female rats per dosage group. Dosages of PCN 66 and PCN 67 were 500, 1500, 5000, 50000 and 500000 ng/kg, and dosages of TCDD were 1, 3, 10, 100, and 300 ng/kg. A group of 10 female rats received vehicle alone. Formulations or vehicle were administered to rats at a volume of 2.5 ml/kg (calculated based on the animal's most recent body weight) five days/week (ensuring two consecutive days before necropsy) for a total of 12 doses. Rats were observed twice daily for signs of mortality or moribundity and clinical observations were recorded daily. Body weights were recorded on all rats prior to the initiation of dosing on Study Day 1, after 7 days (Day 8), and on the day of study termination. Necropsies were performed on all rats. The thymus, right kidney, heart and lungs were weighed. Histopathology was performed on the liver, lung, thyroid gland and thymus. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4-6 μ m, and stained with hematoxylin and eosin. All of the above organs were examined to the lowest dose tested or to a no observed effect level. A semiquantitative grading scheme was used to evaluate the extent of the lesions in the tissue, generally using the criteria of Shackelford et al. (Shackelford et al. 2002) using five grades, as follows: no lesion (grade 0); minimal (grade 1); mild (grade 2); moderate (grade 3); and marked (grade 4). Thyroid follicular cell hypertrophy has been seen in previous studies of dioxin-like compounds. In those studies, follicular cell hypertrophy commonly occurred spontaneously in the control animals and thus some degree of hypertrophy was considered to be normal. Consequently, in those studies follicular cell hypertrophy was only diagnosed when at least half of the thyroid follicles in the glands were affected. In order to maintain consistency of diagnosis with the previous studies, all the thyroid glands were reviewed from the 14 day study using this criterion.

For determination of cytochrome P450 activities, microsomal suspensions were prepared from frozen liver tissue samples collected from 5 rats per group at study termination according to standard operating protocols developed at Battelle –Columbus based on the methods of Pearce et al (Pearce et al. 1996). The concentration of protein in each suspension was determined using the microtiter plate method of the Coomassie Protein Assay (Pierce Chemical. Co., Rockford, IL) with bovine serum albumin as the standard. Cytochrome P450 1A1 (CYP1A1)-associated 7-ethoxyresorufin-O-deethylase (EROD) and cytochrome P450 1A2 (CYP1A2)-associated acetanilide-4-hydroxylase (A4H) activity was determined in microsomal preparations by Battelle Toxicology Northwest Operations Richland, WA, USA under contract to the National Toxicology Program (NTP).

EROD activity was determined in microsomes isolated from frozen liver tissue according to standard operating protocols developed at Battelle –Columbus based on the methods described in Rutten et al and references therein (Rutten et al. 1992). For routine analysis of EROD, final concentrations of EROD assay components were 4.4 μ M 7 ethoxyresorufin, in 50 mM sodium phosphate buffer, pH 8.0. The final concentrations of hepatic microsomal protein were 0.21 mg/mL for controls and ranged from 0.021 to 0.085 mg/mL for treated samples. The reactions were initiated by the addition of NADPH to a final concentration of 1.4 mM and incubated at $37 \pm 1^\circ\text{C}$ for the required reaction time. Reactions were terminated by the addition of 100 μ L of diglyme. Resorufin production was determined using a CytoFluor II fluorescence microplate reader (Biosearch Instruments; Millipore Corp.; Bedford, MA) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Data are show as pmol/min per mg microsomal protein.

A4H activity was determined in microsomes isolated from frozen liver tissue according to standard operating protocols developed at Battelle –Columbus. For the A4H assays, fifty microliters of microsomal suspension (2 mg/mL in buffer) were preincubated for 5 minutes at $37 \pm 1^\circ\text{C}$ in a buffer containing 50 mM Tris (pH 7.5), 0.3 mM MgCl_2 , 0.6 mM NADPH, and 1 mg/mL bovine serum albumin in a total volume of 1 mL. Duplicate incubation mixtures were prepared for each sample. A third incubation mixture was also prepared from each sample and 10 μ L of 0.1 M NaHCO_3 was substituted for the 10- μ L aliquot of 60 mM NADPH to serve as a blank sample. Reactions were initiated by the addition of 25 μ L of 20 mM acetanilide (in acetone). Reactions were terminated after 20 minutes by the addition of 2.5 mL of ice-cold ethyl acetate containing 0.66 nmol of 3-hydroxyacetanilide (internal standard). Twenty microliters of an ethyl acetate extract of each sample were subsequently analyzed using reversed-phase HPLC to separate and quantitate the hydroxylated acetanilide derivatives. Data are show as nmol/min per mg microsomal protein.

For organ and body weight data, whose variances were considered homogeneous across test groups, as determined by Bartlett's test for homogeneity at the 0.05 level, tests for differences between the control and comparison groups were made using a Dunnett's test (Dunnett 1955). Cytochrome P450 data were analyzed using a one-way analysis of variance using PROC GLM in SAS 8.2 (SAS Institute, Inc.; Cary, NC) to test for treatment effects. The measurements in each group were compared to those obtained in the vehicle treated group using a Dunnett's test.

For the assessment of relative potencies, the cytochrome P450 enzyme data and thymic atrophy data were modeled using a Hill function (Toyoshiba et al. 2004) using the formula:

$$y = E_0 + \frac{(E_{\max})(\text{dose}^n)}{ED_{50}^n + \text{dose}^n} \quad (1)$$

where y is the observed response, E_0 is the background response, $E_{\max}$ is the maximum increase over background, ED_{50} is the half maximal dose, and n is the Hill (shape) parameter. Parameters were estimated using maximum likelihood techniques. For the P450 enzyme activity, data were modeled using both an "Independent parameter" model, which fit the data for TCDD, PCN 66 and PCN 67, resulting in separate estimates of E_0 , $E_{\max}$, ED_{50} and Hill parameter for each of the congeners, and also using a "common parameter" model where the data were fit under the assumption that E_0 , $E_{\max}$ and Hill parameter were shared between all data sets and only the ED_{50} for each chemical differed. Chi-square-based likelihood ratio tests were then used to evaluate the two models. The relative potency factor (RPF) of each congener at the ED_{50} is calculated as $ED_{50, \text{TCDD}} / ED_{50}$ for each PCN congener.

For the thymic atrophy incidence data, the independent model fit was too unstable and did not converge to give parameter estimates, so the data were only modeled using the common parameter model and fit under the assumption that background incidence is zero %, and that the maximum increase over the zero background is 100% incidence.

3. Results

All rats survived to the end of the study. The final mean body weight of rats exposed to 300 ng/kg TCDD was significantly lower ($p < 0.01$) than the controls but was within 10% of controls. (Table 1). The final mean body weights of rats exposed to 500,000 ng/kg PCN 66 or PCN 67 were significantly lower than the controls; 22% and 17% for PCN 66 and PCN67, respectively. All animals were clinically unremarkable with the exception of one rat exposed to 500,000 ng/kg PCN 66, which was thin at study termination.

3.1. Organ Weights

Absolute and relative thymus weights of rats exposed to TCDD generally decreased with increasing exposure concentration (Table 1). Absolute and relative thymus weights of rats exposed to 300 ng/kg TCDD were decreased 37% and 33%, respectively. Similarly absolute and relative thymus weights were also significantly decreased in rats exposed to 500,000 ng/kg PCN 66 or PCN 67. Rats exposed to the high dose of PCN 66 had a 66% and 57% reduction in absolute and relative thymic weights, respectively. Rats exposed to the high dose of PCN 67 had a 44% and 33% reduction in absolute and relative thymic weights, respectively.

Mean absolute weight of the heart was 10% lower than controls in rats treated with 300ng/kg TCDD. In rats treated with 500,000 ng/kg PCN66, mean absolute weight of the heart, lung and right kidney were 25%, 24% and 21% lower than controls, respectively. In rats treated with 500,000 ng/kg PCN67, mean absolute weight of the heart, lung and right kidney were 22%, 23% and 13% lower than controls, respectively. These effects were considered to be related to the reduction in final mean body weights observed at the highest dose for TCDD, PCN66 and PCN67.

3.2. Histopathology

In rats exposed to TCDD, treatment-related findings were present in the liver, lung and thymus (Table 2, Figure 1). Diffuse hepatocellular hypertrophy was observed in rats exposed to 300 ng/kg TCDD and was characterized by diffuse enlargement of hepatocytes, with cytoplasm that was lacy, eosinophilic, and granular. Minimal focal to multifocal hepatic necrosis was present in two out of 10 control rats and two out of 5 high dose rats. Focal, and multifocal necrosis has been seen in control rats and may be due to hypoxic change secondary to impaired blood flow (Thoolen et al. 2010). Suppurative inflammation of the liver was diagnosed in two rats treated with 300 ng/kg TCDD; neither had a concurrent diagnosis of necrosis. However, since suppurative inflammation of the liver is rare in control rats, this was considered to be exposure-related. Hepatic hematopoietic cell proliferation was reduced in incidence in rats exposed to 100 ng/kg and higher TCDD when compared to controls. Minimal thymic atrophy was present in rats exposed to 10 ng/kg and higher TCDD; 80% of the rats were affected in the high dose group. In the lung (alveoli), histiocytic cell infiltrates were present in 3/5 rats exposed to 300 ng/kg TCDD compared to 2/10 control rats; in addition, the average severity among high-dose rats was increased (1.7 in TCDD-exposed rats compared to 1.0 in controls).

In the rats exposed to PCN 66 and PCN 67, treatment-related findings were present only in the liver and thymus. Diffuse hepatocellular hypertrophy similar to that observed in the TCDD-exposed rats was significantly increased in PCN rats exposed to 500,000 ng/kg and

was of a severity equivalent to that observed in the TCDD-exposed rats treated with 300 ng/kg. Hepatocellular diffuse micro- and macrovesicular fatty change (Figure 1A) was significantly increased in PCN 66 groups exposed to 50,000 ng/kg or higher and in the high dose group of PCN 67-exposed rats. Hepatocellular diffuse fatty changes were not observed in the controls or TCDD treated rats. Focal to multifocal hepatocellular necrosis, associated with acute inflammation, was significantly increased in rats exposed to 500,000 ng/kg PCN 67 compared to controls (Figure 1B). In addition, even though the severity of the necrosis was less in the affected PCN 67-exposed rats than in control rats, suppurative inflammation was associated with the necrosis in five of the rats in the 500,000 ng/kg PCN 67 group and in one rat exposed to 50,000 ng/kg PCN 66. There was a reduction in the incidence of hepatic hematopoietic cell proliferation in PCN 66 and PCN 67 rats exposed to the high dose compared to controls.

The incidence of thymic atrophy was significantly increased in rats treated with 500,000 ng/kg PCN 66 and PCN 67 (Table 2, Figure 1C, 1D). However, the severity of atrophy (mild to moderate) was greater than in the TCDD-exposed rats, and was considered to be responsible for the observed reduction in group mean absolute and relative thymus weights. Minimal thymic atrophy was also present in occasional rats in lower PCN 66 groups (two exposed to 50,000 ng/kg and one exposed to 5,000 ng/kg). The thymic atrophy data for all three chemicals was fit to the Hill model simultaneously under the assumption of common background expression, maximum increase over background and shape parameter (Table 3). The only parameter varying between the congeners was the ED₅₀. Under these conditions, the ED₅₀s for thymic atrophy for TCDD, PCN 66, and PCN 67 were 35, 18,792, and 108,126 ng/kg, respectively. Using the ratio of ED₅₀ for TCDD divided by the ED₅₀ of the test chemical the relative potency for thymic atrophy for PCN 66 and PCN 67 compared to TCDD are 0.0072 and 0.00032 respectively (Table 3).

Thyroid follicular cell hypertrophy was observed in both control animals and animals treated with TCDD, PCN 66 and PCN67 (Figure 1E, 1F). The severity varied from minimal to mild in control animals and from minimal to moderate in treated animals. While not statistically significant, the increased incidence of follicular cell hypertrophy in the high dose group of TCDD may have been related to treatment (Table 2). There did not appear to be any effect of PCN treatment on the thyroid in the study.

3.3. Cytochrome P450 Enzymes

Following treatment with TCDD, PCN 66 or PCN 67 there was a significant dose dependent induction of hepatic cytochrome P450 activity (Table 4, Figure 2). Hepatic CYP1A1 associated EROD activity was significantly increased in all treatment groups exposed to TCDD or PCN 66 and in groups exposed to 1500 ng/kg or higher PCN 67. Both PCN 66 and PCN 67 induced EROD activity similar to that of TCDD. Hepatic EROD activity was increased 74-fold in rats exposed to 100 ng/kg TCDD, 71-fold in rats exposed to 50,000 ng/kg PCN 66 and 73-fold in rats exposed to 500,000 ng/kg PCN 67.

The EROD activity data for the three congeners were initially modeled independently (Table 5). Parameter estimates for E₀, E_{max} and Hill parameter were similar for TCDD, PCN 66 and PCN 67. The shape parameter *n* was in all cases >1.5 indicating a non-linear response. The estimated ED₅₀ for TCDD was 11 ng/kg. This is similar to that observed following 13 weeks of treatment with TCDD (5 ng/kg)(Toyoshiba et al. 2004). The ED₅₀s for the PCN 66 and PCN 67 were higher (6633 and 37800 ng/kg, respectively), which is expected based on their lower binding affinity for the AhR. Relative potency factors (RPFs) for PCN 66 and PCN 67 based on the independently modeled ED₅₀s were 0.0017 and 0.00029.

We then modeled the CYP1A1 data for all three compounds simultaneously under the assumption of common background expression, maximum increase over background and shape parameter (Table 5). The only parameter varying between the congeners was the half maximal dose (ED₅₀). Under these conditions, the ED₅₀ for TCDD was still 11 ng/kg, and the shape parameter was again >1.5. Maximum likelihood ratio analysis of the “Independent” and “same shape” models could not reject the hypothesis that the two models gave equivalent fits to the data ($p=0.8$), and therefore, it is appropriate to use a simpler model to compare the dose response under the assumption of same shape. Under these conditions of same shape, the relative potency can be described simply as the ratio of the ED₅₀s with that of TCDD. Under these conditions, the RPFs for PCN 66 and PCN 67 were 0.0015 and 0.00036, respectively.

CYP1A2-associated hepatic A4H activity was significantly increased in rats exposed to 10 ng/kg or higher TCDD and in rats exposed to 50,000 ng/kg or higher PCN 66 or PCN 67 (Table 4, Figure 2). Maximum observed increase in activity for CYP1A2 by TCDD, PCN 66 and PCN 67 was 3.5, 3.9 and 2.5 fold, respectively. The estimated ED₅₀ for induction of CYP1A2 by TCDD was 24 ng/kg (Table 6). In contrast to the CYP1A1 activity, the shape of the dose response curves for PCN 66 and PCN 67 were highly non-linear in comparison to TCDD. ED₅₀ based RPFs for PCN 66 and PCN 67 under these conditions were 0.0041 and 0.00061, respectively (Table 6). When the data were modeled under the assumption of same shape, maximum likelihood ratio analyses indicated that the fits were significantly worse ($p=0.0073$). This is likely driven by the higher Hill shape parameters observed for PCN 66 and PCN 67 compared to TCDD and low $E_{\max}$ value for the independent parameter model for PCN 67 (Table 6). However an RPF can still be calculated based on comparison of ED₅₀ estimates, using an assumption of “same shape” as used in the WHO TEF scheme. This is done by forcing the models for TCDD, PCN 66 and PCN 67 to share a common parameter for background expression, maximum increase over background and shape parameter. Under these model assumptions the RPFs for PCN 66 and PCN 67, for CYP1A2 were approximately 2-fold lower, 0.0022 and 0.00032, respectively (Table 6, Figure 2).

4. Discussion

The purpose of this study was to determine the relative potency values of PCN 66 and PCN 67 relative to TCDD. PCN-related effects in the present study included reduced weight gain with the related observation of thinness, decreased absolute and relative thymus weights, thymic atrophy, hepatic pathology, and induction of the hepatic CYP1A1 and CYP1A2. Liver pathology and induction of cytochrome P450 enzymes have been previously reported after exposure to chlorinated naphthalenes (Hayward 1998). It has been proposed that highly chlorinated naphthalenes with chlorine substitutions at the 2, 3, 6, and 7 positions were the strongest activators of the Ah receptor and subsequent inducers of EROD activity (Campbell et al. 1983). In the study of Campbell et al., the hexachlorinated naphthalene 1,2,3,4,5,6-hexachloronaphthalene was a potent inducer, although less so than 1,2,3,4,5,6,7-heptachloronaphthalene. Cytochrome P450 induction in the present study appeared maximal at either the highest or next to highest dose for all compounds evaluated. Furthermore, the degree of response was similar between PCNs and TCDD (71 to 74 fold induction in EROD and 2 to 4 fold induction in A4H activity).

Histopathologic effects of PCN 66 and 67 in the present study were observed in the liver (hepatocellular hypertrophy, fatty change, necrosis, and inflammation) and thymus (atrophy) and were consistent with effects observed for TCDD (hepatocellular hypertrophy and thymic atrophy) and previous studies of TCDD (De Waal et al. 1993). Effects of PCNs on hepatic fatty change, necrosis, and inflammation were not seen with TCDD in the present study. Although the lungs and thyroid glands are reported targets of TCDD and related chemicals

(Sewall et al. 1995; Tritscher et al. 2000; Walker et al. 2007), treatment-related effects on these organs were not observed (i.e., alveolar-bronchiolar metaplasia and thyroid follicular cell hypertrophy and hyperplasia, respectively), probably due to the short duration of exposure in this study.

Dose-response analysis of CYP1A-associated P450 activity confirmed that *in vivo*, the PCNs exhibit similar induction to that of TCDD, indicating that these are dioxin-like compounds (Figure 2). For CYP1A1 and CYP1A2 the dose responses were similar for PCN66 and PCN 67 albeit at a lower potency than for TCDD. The estimated relative potencies for CYP1A1 induction by PCN 66 and PCN 67 (0.0015 and 0.00036, respectively) (Table 6), were similar to earlier *in vitro* estimates of EROD induction in H4IIE liver cells of 0.0015–0.00026, and 0.00031–0.00027 for PCN 66 and PCN 67 respectively (Villeneuve et al. 2000), and 0.0012–0.0017, and 0.000038–0.000061, for PCN 66 and PCN 67 respectively, using a CALUX assay (Behnisch 2003)

PCN 66 and PCN 67 are structurally related to TCDD, bioaccumulate, bind to the Ah receptor and exhibit both biochemical and histopathologic effects similar to TCDD. As such they meet all the criteria required for inclusion in the WHO toxic equivalency factor scheme (Van den Berg et al. 2006). *In vitro* data are not normally weighted very highly in the WHO determination of TEFs, due to uncertainties about extrapolation of RPFs from *in vitro* to *in vivo*. While the effects of PCN mixtures has been reported before, this paper is the first to report *in vivo* relative potency data for specific PCN congeners and confirms the prior relative potencies for PCN 66 and PCN 67 observed from *in vitro* studies.

Although PCN 66 and PCN 67 exposure results in physiologic changes similar to that seen after TCDD exposure, the relative potency indicates that these hexachlorinated naphthalenes should not contribute greatly to the overall body burden of dioxin like compounds in the general population. In exposure assessments, PCN 66/PCN 67 cannot be readily separated in biological samples so estimates of PCN 66 or PCN 67 in food and human adipose are usually presented as being combined PCN 66/PCN 67. PCN 66/PCN 67 contributed about 0.5% to the total TEQ in human milk samples in which PCDDs, PCDFs, and non-ortho and mono-ortho PCBs were also measured (Lunden and Noren 1998). In human adipose tissue and liver, PCN 66/PCN 67 contributed 25 times less to the total TCDD “toxic equivalents” (TEQ) than non-ortho and mono-ortho PCBs (PCDDs and PCDFs were not measured) (Weistrand and Noren 1998). The levels of PCN 66/67 in adipose tissue from Canadians are about 1 ng/g fat in adipose tissue. Using a relative potency value of 0.003 this would convert to about 3 ng “TEQ-PCN”/kg fat in adipose tissue. The background levels of PCDDs/PCDFs/PCBs in the US range from about 10–20 ng TEQ/kg lipid (Patterson et al. 1994).

More recent studies from a small population in New York City found the PCN congeners PCN 66 and PCN 67 present in adipose tissue (Kunisue et al. 2009) and plasma (Horii et al. 2003). In adipose tissue PCN 66/PCN 67 was found at concentrations of approximately 100 and 47 pg/g lipid in males and females respectively. Assuming TEFs of 0.003 for the PCNs and 25% body fat, this population has approximately human body burdens of PCNs of 0.04–0.075 ngTEQ/kg. In the general population, the background body burden of dioxin equivalents is estimated at approximately 2–5 ng TEQ/kg for the PCDD/Fs and PCBs (Lorber et al. 2009). Thus, the PCNs would contribute approximately 1–3% of the total TEQ body burden in this population. PCN 66/PCN 67 was found at concentrations ranging from below detection limits to approximately 179 pg/g lipid in plasma from New York State employees and National Guard personnel who worked at the World Trade Center (WTC) in the weeks after the collapse of the buildings. Using a relative potency value of 0.003, this results in TEQ estimates from PCN 66/PCN 67 up to 0.54 pg TEQ/kg plasma lipid. In this population, the TEQ from the PCDD/Fs and PCBs ranged from approximately 20–60 pg

TEQ/kg plasma lipid. These data suggest that the PCNs make up approximately 1% of the total TEQ in the population who were working at the WTC.

The contribution of these two PCNs to the total TCDD equivalents (TEQ) in a broad sampling of foods has recently been estimated using a RPF of 0.004 as a surrogate TEF for the PCN 66/PCN 67 combined (Fernandes et al. 2011). Based on this RPF, although PCN 66/PCN 67 are the highest contributors to the total TEQ activity in food from the PCNs, the PCN class as a whole is only 0.14pg/kg/month compared to a 12 pg TEQ/kg/month for PCDD/F and PCBs or about 1% of the monthly intake (Fernandes et al. 2011). Interestingly, this estimated TEQ intake is similar to the estimated contribution of the PCNs to present TEQ body burdens in the US presented here. Consequently, while PCNs should be included in the WHO TEF scheme, the hexachlorinated naphthalenes may only contribute a few percent to the overall intake or body burden of dioxin like compounds in the general population.

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References

- Behnisch PA, Hosoe K, Sakai S. Brominated dioxin-like compounds: in vitro assessment in comparison to classical dioxin-like compounds and other polyaromatic compounds. *Environ Int.* 2003; 29:861–877. [PubMed: 12850102]
- Brack W, Kind T, Schrader S, Moder M, Schuurmann G. Polychlorinated naphthalenes in sediments from the industrial region of Bitterfeld. *Environ Pollut.* 2003; 121:81–85. [PubMed: 12475064]
- Campbell MA, Bandiera S, Robertson L, Parkinson A, Safe S. Hepta-, hexa-, tetra- and dichloronaphthalene congeners as inducers of hepatic microsomal drug-metabolizing enzymes. *Toxicology.* 1983; 26:193–205. [PubMed: 6857695]
- De Waal EJ, Rademakers LH, Schuurman HJ, Van Loveren H, Vos JG. Ultrastructure of the cortical epithelium of the rat thymus after in vivo exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Arch Toxicol.* 1993; 67:558–564. [PubMed: 8285855]
- Dunnett CW. A multiple comparison procedure for comparing several treatments with a control. *J Am Stat Assoc.* 1955; 50:1096–1121.
- Espadaler I, Eljarrat E, Caixach J, Rivera J, Marti I, Ventura F. Assessment of polychlorinated naphthalenes in aquifer samples for drinking water purposes. *Rapid Commun Mass Spectrom.* 1997; 11:410–414. [PubMed: 9069644]
- Falandysz J. Chloronaphthalenes as food-chain contaminants: a review. *Food Addit Contam.* 2003; 20:995–1014. [PubMed: 14668151]
- Fernandes AR, Tlustos C, Rose M, Smith F, Carr M, Panton S. Polychlorinated naphthalenes (PCNs) in Irish foods: Occurrence and human dietary exposure. *Chemosphere.* 2011; 85:322–328. [PubMed: 21783225]
- Hanberg A, Stahlberg M, Georgellis A, de Wit C, Ahlberg UG. Swedish dioxin survey: evaluation of the H-4-II E bioassay for screening environmental samples for dioxin-like enzyme induction. *Pharmacol Toxicol.* 1991; 69:442–449. [PubMed: 1766920]
- Hayward D. Identification of bioaccumulating polychlorinated naphthalenes and their toxicological significance. *Environ Res.* 1998; 76:1–18. [PubMed: 9466892]

- Helm PA, Bidleman TF. Current combustion-related sources contribute to polychlorinated naphthalene and dioxin-like polychlorinated biphenyl levels and profiles in air in Toronto, Canada. *Environ Sci Technol*. 2003; 37:1075–1082. [PubMed: 12680657]
- Helm PA, Bidleman TF, Stern GA, Koczanski K. Polychlorinated naphthalenes and coplanar polychlorinated biphenyls in beluga whale (*Delphinapterus leucas*) and ringed seal (*Phoca hispida*) from the eastern Canadian Arctic. *Environ Pollut*. 2002; 119:69–78. [PubMed: 12125730]
- Horii Y, Jiang Q, Hanari N, Lam PK, Yamashita N, Jansing R, Aldous KM, Mauer MP, Eadon GA, Kannan K. Polychlorinated dibenzo-p-dioxins, dibenzofurans, biphenyls, and naphthalenes in plasma of workers deployed at the World Trade Center after the collapse. *Environ Sci Technol*. 2003; 44:5188–5194. [PubMed: 20455569]
- HSDB. 2011. <http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?/temp/~Ga29hN:1>
- Kannan K, Kober JL, Kang YS, Masunaga S, Nakanishi J, Ostaszewski A, Giesy JP. Polychlorinated naphthalenes, biphenyls, dibenzo-p-dioxins, and dibenzofurans as well as polycyclic aromatic hydrocarbons and alkylphenols in sediment from the Detroit and Rouge Rivers, Michigan, USA. *Environ Toxicol Chem*. 2001; 20:1878–1889. [PubMed: 11521813]
- Krauss M, Wilcke W. Polychlorinated naphthalenes in urban soils: analysis, concentrations, and relation to other persistent organic pollutants. *Environ Pollut*. 2003; 122:75–89. [PubMed: 12535597]
- Kunisue T, Johnson-Restrepo B, Hilker DR, Aldous KM, Kannan K. Polychlorinated naphthalenes in human adipose tissue from New York, USA. *Environ Pollut*. 2009; 157:910–915. [PubMed: 19084307]
- Lorber M, Patterson D, Huwe J, Kahn H. Evaluation of background exposures of Americans to dioxin-like compounds in the 1990s and the 2000s. *Chemosphere*. 2009; 77:640–651. [PubMed: 19733382]
- Lunden A, Noren K. Polychlorinated naphthalenes and other organochlorine contaminants in Swedish human milk, 1972–1992. *Arch Environ Contam Toxicol*. 1998; 34:414–423. [PubMed: 9543513]
- Ofstad EB, Lunde G, Martinsen K, Rygg B. Chlorinated aromatic hydrocarbons in fish from an area polluted by industrial effluents. *Sci Total Environ*. 1978; 10:219–230. [PubMed: 734441]
- Patterson DG Jr, Todd GD, Turner WE, Maggio V, Alexander LR, Needham LL. Levels of non-ortho-substituted (coplanar), mono- and di-ortho-substituted polychlorinated biphenyls, dibenzo-p-dioxins, and dibenzofurans in human serum and adipose tissue. *Environ Health Perspect*. 1994; 102(Suppl 1):195–204. [PubMed: 8187709]
- Pearce RE, McIntyre CJ, Madan A, Sanzgiri U, Draper AJ, Bullock PL, Cook DC, Burton LA, Latham J, Nevins C, Parkinson A. Effects of freezing, thawing, and storing human liver microsomes on cytochrome P450 activity. *Arch Biochem Biophys*. 1996; 331:145–169. [PubMed: 8660694]
- Rutten AA, Falke HE, Catsburg JF, Wortelboer HM, Blaauboer BJ, Doorn L, van Leeuwen FX, Theelen R, Rietjens IM. Interlaboratory comparison of microsomal ethoxyresorufin and pentoxyresorufin O-dealkylation determinations: standardization of assay conditions. *Arch Toxicol*. 1992; 66:237–244. [PubMed: 1514921]
- Sewall CH, Flagler N, Vanden Heuvel JP, Clark GC, Tritscher AM, Maronpot RM, Lucier GW. Alterations in thyroid function in female Sprague-Dawley rats following chronic treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Appl Pharmacol*. 1995; 132:237–244. [PubMed: 7540335]
- Shackelford C, Long G, Wolf J, Okerberg C, Herbert R. Qualitative and quantitative analysis of nonneoplastic lesions in toxicology studies. *Toxicol Pathol*. 2002; 30:93–96. [PubMed: 11890482]
- Thoolen B, Maronpot RR, Harada T, Nyska A, Rousseaux C, Nolte T, Malarkey DE, Kaufmann W, Kuttler K, Deschl U, Nakae D, Gregson R, Vinlove MP, Brix AE, Singh B, Belpoggi F, Ward JM. Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. *Toxicol Pathol*. 2010; 38:5S–81S. [PubMed: 21191096]
- Toyoshiba H, Walker NJ, Bailer AJ, Portier CJ. Evaluation of toxic equivalency factors for induction of cytochromes P450 CYP1A1 and CYP1A2 enzyme activity by dioxin-like compounds. *Toxicol Appl Pharmacol*. 2004; 194:156–168. [PubMed: 14736496]

- Tritscher AM, Mahler J, Portier CJ, Lucier GW, Walker NJ. Induction of lung lesions in female rats following chronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Pathol.* 2000; 28:761–769. [PubMed: 11127289]
- Van den Berg M, Birnbaum LS, Denison M, De Vito M, Farland W, Feeley M, Fiedler H, Hakansson H, Hanberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N, Peterson RE. The 2005 World Health Organization reevaluation of human and Mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicol Sci.* 2006; 93:223–241. [PubMed: 16829543]
- Villeneuve DL, Kannan K, Khim JS, Falandysz J, Nikiforov VA, Blankenship AL, Giesy JP. Relative potencies of individual polychlorinated naphthalenes to induce dioxin-like responses in fish and mammalian in vitro bioassays. *Arch Environ Contam Toxicol.* 2000; 39:273–281. [PubMed: 10948276]
- Walker NJ, Yoshizawa K, Miller RA, Brix AE, Sells DM, Jokinen MP, Wyde ME, Easterling M, Nyska A. Pulmonary lesions in female Harlan Sprague-Dawley rats following two-year oral treatment with dioxin-like compounds. *Toxicol Pathol.* 2007; 35:880–889. [PubMed: 18098034]
- Weistrand C, Noren K. Polychlorinated naphthalenes and other organochlorine contaminants in human adipose and liver tissue. *J Toxicol Environ Health A.* 1998; 53:293–311. [PubMed: 9490327]
- WHO. Concise International Chemical Assessment Document. Vol. 34. World Health Organization; 2001. CHLORINATED NAPHTHALENES.

Figure 1.

Selected representative histopathological lesions noted in the study

A. Micro- and macrovesicular vacuolation (fatty change) in a female rat exposed to a high dose (500,000 ng/kg) of PCN 66. H&E, at magnification ×20.

B. Acute inflammation (arrows) in a female rat exposed to high dose (500,000 ng/kg) of PCN 67. H&E, at magnification ×20.

C. Moderate thymic atrophy in a female rat exposed to high dose (500,000 ng/kg) of PCN 66. Note depletion of lymphocytes and thinning of the cortex (arrows). H&E, at magnification ×4. Compare with control thymus in Figure D.

D. Thymus of a female common concurrent control rat from the study. Note the normal thickness of the cortex. H&E, at magnification ×4.

E. Minimal thyroid follicular cell hypertrophy in a female rat exposed to high dose (500,000 ng/kg) of PCN 66. Note the cuboidal aspect of the epithelium lining the follicles (arrows). H&E, at magnification ×40. Compare with control thymus in Figure F.

F. Thyroid of a female common concurrent control rat from the study. Note the flattened aspect of the epithelium lining the follicles (arrows). H&E, at magnification ×40.

Figure 2.
Dose response modeling of (A) hepatic CYP1A1-associated EROD activity (pmol/min/mg)
(B) hepatic CYP1A2-associated A4H activity (nmol/min/mg) for TCDD, PCN 66 and
PCN67 under the assumption of common dose response parameters but differing ED50s.

Body Weights and Thymus Weights for the 14-Day Relative Potency Study of PCN 66 and PCN 67 in Female Harlan Sprague-Dawley Rats

Table 1

Dose (ng/kg)	Mean Body Weight ^a (g)		Final Weight ^b (%)	Thymus Weights ^d (g)		% of Controls
	Initial	Final		Absolute	Relative ^c	
Control ^d	159 ± 2	207 ± 4	48 ± 4	-	0.406 ± 0.012	1.968 ± 0.058
TCDD ^e						
1	164 ± 3	207 ± 6	43 ± 4	100	0.484 ± 0.039	2.326 ± 0.139
3	163 ± 4	208 ± 5	45 ± 2	100	0.436 ± 0.024	2.107 ± 0.153
10	162 ± 3	208 ± 7	46 ± 5	100	0.379 ± 0.034	1.828 ± 0.173
100	161 ± 4	208 ± 7	47 ± 4	100	0.391 ± 0.046	1.863 ± 0.165
300	161 ± 4	191 ± 7	30 ± 4 ^{**}	92	0.255 ± 0.032 ^{**}	1.321 ± 0.119 ^{**}
PCN 66 ^e						
500	158 ± 2	209 ± 5	52 ± 4	100	0.417 ± 0.026	1.989 ± 0.093
1,500	157 ± 2	209 ± 3	51 ± 4	100	0.391 ± 0.026	1.872 ± 0.109
5,000	156 ± 3	198 ± 7	42 ± 4	96	0.381 ± 0.015	1.925 ± 0.076
50,000	159 ± 3	204 ± 5	45 ± 3	99	0.383 ± 0.010	1.897 ± 0.046
500,000	156 ± 4	162 ± 7 ^{**}	6 ± 4 ^{**}	78	0.138 ± 0.007 ^{**}	0.852 ± 0.017 ^{**}
PCN 67 ^e						
500	159 ± 4	203 ± 4	44 ± 2	98	0.376 ± 0.023	1.862 ± 0.117
1,500	158 ± 3	206 ± 3	48 ± 4	100	0.432 ± 0.014	2.100 ± 0.079
5,000	157 ± 1	204 ± 5	48 ± 4	99	0.397 ± 0.042	1.944 ± 0.201
50,000	161 ± 3	210 ± 3	49 ± 2	101	0.397 ± 0.025	1.891 ± 0.104
500,000	157 ± 3	172 ± 3 ^{**}	15 ± 4 ^{**}	83	0.228 ± 0.014 ^{**}	1.327 ± 0.083 ^{**}

^aWeights and weight changes are given as mean ± standard error.

^bFinal weight relative to controls

^cMean of the individual ratios of organ weight (mg) to body weight (grams)

^dn=10

^en=5

** Significantly different (p < 0.01) from the control group by Williams' or Dunnett's test

Table 2
Incidence and Average Severity of Selected Treatment-Related Microscopic Findings in Female Sprague-Dawley Rats.

Chemical Dosage (ng/kg)	Control			TCDD				PCN 66				PCN 67			
	0	1	3	10	30	50	100	300	500	1,500	5,000	50,000	500,000	500,000	500,000
Liver ^a	10	0	5	5	5	5	5	5	5	5	5	5	5	5	5
Hypertrophy, hepatocellular ^b	0	-	0	0	0	0	0	4 ^{**} (1.0) ^c	0	0	0	0	4 ^{**} (1.5)	-	3 [*] (1.0)
Fatty change ^b	0	-	0	0	0	0	0	0	1 (1.0)	1 (1.0)	1 (1.0)	3 [*] (1.0)	5 ^{**} (2.2)	-	5 ^{**} (3.2)
Necrosis ^b	2 (2.0)	-	0	1 (3.0)	0	2 (1.0)	0	2 (1.0)	0	0	0	2 (1.0)	0	-	5 ^{**} (1.2)
Inflammation, suppurative ^b	0	-	0	0	0	2 (1.0)	0	2 (1.0)	0	0	0	1 (1.0)	0	-	5 ^{**} (1.2)
Hematopoietic cell proliferation ^b	6 (1.0)	-	2 (1.0)	2 (1.0)	0 [*]	0 [*]	0 [*]	3 (1.0)	4 (1.0)	4 (1.0)	2 (1.0)	5 (1.0)	0 [*]	-	1 (1.0)
Lung ^a	10	0	0	0	5	5	5	5	0	0	0	0	5	0	5
Infiltrate, cellular, histiocytic ^b	2 (1.0)	-	-	-	1 (1.0)	3 (1.7)	0	0	-	-	-	-	0	-	0
Thymus ^a	10	0	5	5	5	5	5	5	0	5	5	5	5	0	5
Atrophy ^b	0	-	0	2 (1.0)	2 (1.0)	4 ^{**} (1.0)	0	0	-	0	1 (1.0)	2 (1.0)	5 ^{**} (3.0)	-	5 ^{**} (2.0)
Thyroid ^a	10	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Follicular cell hypertrophy ^b	2 (1.5)	2 (1.0)	0	1 (1.0)	1 (1.0)	4 (2.0)	0	2 (1.5)	1 (1.0)	3 (2.3)	2 (1.0)	1 (1.0)	2 (1.5)	1 (2.0)	2 (2.0)

^a Number of animals with tissue examined microscopically;

^b Number of animals with lesion, - = Not examined;

^c Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

* Significantly different (p < 0.05) from the control group by the Fishers exact test

** Significantly different (p < 0.01) from the control group by the Fishers exact test

Table 3

Dose response modeling of thymic atrophy

Common parameter model ^a			
	TCDD	PCN 66	PCN 67
E ₀ (% incidence)	0		
E _{max}	100		
Shape	0.88		
ED ₅₀ (ng/kg)	35.1	18792	108126
RPF-PCN 66 ^b		0.0072	
RPF-PCN 67 ^b			0.00032

^aThe entire dataset was modeled under the assumption that there is a common E₀, shape and E_{max} parameter for all three chemicals. In this case the background incidence was fixed at zero and the E_{max} was assumed to be a maximum of 100% incidence.

^bRelative Potency Factor (RPF) calculated as ED₅₀-TCDD/ED₅₀-PCN

Table 4

EROD and A4H Activity for the 14-Day Relative Potency Study of PCN 66 and PCN 67 in Female Harlan Sprague-Dawley Rats

Dosage (ng/kg)	EROD ^a	A4H ^b
Vehicle	28.3 ± 1.25	0.523 ± 0.042
TCDD		
1	52.80 ± 3.80**	0.527 ± 0.015
3	227.80 ± 39.02**	0.623 ± 0.060
10	892.80 ± 77.86**	0.781 ± 0.095*
100	2088.0 ± 63.20**	1.826 ± 0.166**
300	1876.0 ± 105.10**	1.738 ± 0.075**
PCN 66		
500	40.34 ± 3.68*	0.389 ± 0.016
1,500	154.40 ± 13.43**	0.457 ± 0.025
5,000	641.40 ± 22.22**	0.845 ± 0.044
50,000	2020.0 ± 119.46**	2.062 ± 0.097*
500,000	1602.0 ± 143.09**	1.278 ± 0.132*
PCN 67		
500	32.10 ± 1.99	0.517 ± 0.042
1,500	48.02 ± 4.69**	0.524 ± 0.046
5,000	111.96 ± 15.65**	0.509 ± 0.026
50,000	1332.0 ± 186.50**	1.199 ± 0.153*
500,000	2070.0 ± 89.11**	1.298 ± 0.065*

^a pmol/min/mg microsomal protein

^b nmol/min/mg microsomal protein

* Significantly different (p = 0.05) from the control group by Shirley's or Dunn's test

** Significantly different (p = 0.01) from the control group by Shirley's or Dunn's test

Table 5

Dose response modeling of CYP1A1 activity

	Independent parameter model ^a			Common parameter model ^b
	TCDD	PCN 66	PCN 67	
E ₀ (pmol/min/mg)	27.8	27.1	28.4	28.1
E _{max} (pmol/min/mg)	1949	1752	2046	1879
Shape	1.78	1.82	1.62	1.76
ED ₅₀ (ng/kg)	11.06	6633	37800	10.97 ^c
RPF-PCN 66 ^d		0.0017		0.0015
RPF-PCN 67 ^d			0.00029	0.00036
P value ^e			0.80	

^a Each curve had independent parameter estimates using the Hill model

^b The entire dataset was modeled under the assumption that there is a common E₀, shape and E_{max} parameter for all three chemicals.

^c ED₅₀ for TCDD dataset only

^d Calculated as ED₅₀TCDD/ED₅₀PCN

^e Likelihood ratio test comparing the Independent parameter and common parameter models

Table 6

Dose response modeling of CYP1A2 activity

	Independent parameter model ^a			Common parameter model ^b
	TCDD	PCN 66	PCN 67	
E ₀ (nmol/min/mg)	0.530	0.448	0.512	0.501
E _{max} (nmol/min/mg)	1.293	1.153	0.780	1.094
Shape	1.65	4.34	4.16	3.30
ED ₅₀ (ng/kg)	24	5828	35067	14.1 ^c
RPF-PCN 66 ^d		0.0041		0.0022
RPF-PCN 67 ^d			0.00067	0.00032
P value ^e			0.0073	

^a Each curve had independent parameter estimates using the Hill model

^b The entire dataset was modeled under the assumption that there is a common E₀, shape and E_{max} parameter for all three chemicals.

^c ED₅₀ for TCDD dataset only

^d Calculated as ED₅₀TCDD/ED₅₀PCN

^e Likelihood ratio test comparing the Independent parameter and common parameter models

Table 7Summary table of estimated relative potencies ^a for PCN 66 and PCN 67

	PCN 66	PCN 67
CYP1A1-Independent parameter model	0.0017	0.00029
CYP1A2- Independent parameter model	0.0041	0.00067
CYP1A1-Common parameter model	0.0015	0.00036
CYP1A2- Common parameter model	0.0022	0.00032
Thymic atrophy -Common parameter model	0.0072	0.00032

^aCalculated as ED₅₀TCDD/ED₅₀PCN