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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, 50,000 and 500,000 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.

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 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.

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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 to 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, 50,000 and 500,000 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 μm, 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. (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. (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