Relative Potencies of Halowax Mixtures and Individual Polychlorinated Naphthalenes (PCNs) to Induce Ah Receptor-Mediated Responses in the Rat Hepatoma H4IIE-Luc Cell Bioassay

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INTRODUCTION

Polychlorinated naphthalenes (PCNs) are ubiquitous environmental pollutants that are structurally similar to polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs). There are 75 possible PCN congeners with unique combinations of numbers and positions of chlorines. PCNs were produced as mixtures (*e.g.*, Halowaxes, Nibren waxes, Seekay waxes, and Clonacire waxes) for commercial applications such as protective coating materials, dielectric fluids, flame retardants, and even as fungicides (1).

The major mechanism of action for the toxicity of PCNs is related to their ability to bind to and activate the aryl hydrocarbon receptor (AhR), which is a cytosolic, ligand-activated transcription factor. Toxic effects mediated through the AhR are species-, sex-, and tissue-specific, and include among other pleiotropic effects a characteristic wasting syndrome, thymic atrophy, immunosuppression, liver enlargement and necrosis, hyperplasia, chloracne, numerous biochemical effects, carcinogenesis, teratogenesis, and death. Exposure to PCNs have long been known to be associated with chloracne and lethality in occupationally-exposed men. In most of these cases of chloracne and mortality, however, the possibility can not be ruled out that other contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or PCBs could have been present at sufficient concentrations to elicit these effects. Experimental exposures of animals to PCN mixtures have resulted in "X-disease" in cattle, induction of cytochrome P450 enzyme activities and mortalities in chickens and eider ducklings (2), P450 induction in immature male wistar rats (3) and three-spined stickleback (4).

Interest exists in assessing the risk posed by dioxin-like compounds, including PCNs, which are present as complex environmental mixtures. Development of mechanism-based cell bioassays to detect specific classes of compounds has enhanced the ability to screen such mixtures for dioxin-like activity. H4IIE-luciferase cells, which are stably transfected with an AhR-controlled luciferase reporter gene construct, respond specifically to AhR agonists. The purpose of the current study was to utilize the H4IIE-luciferase bioassay to determine relative potencies for 20 individual PCNs and 6 Halowax mixtures compared to TCDD as a standard reference compound.

MATERIALS AND METHODS

Halowax standards and 1,4-diCN were obtained from Accustandard Inc.. Halowaxes 1000,1001, and 1099 were 95% pure whereas Halowaxes 1014, 1051, and 1013 were 99% pure. 1,4-diCN was 98.3% pure. 2-monoCN was obtained from ICN Chemicals, Inc. (Irvine, CA) and was 99% pure. PCN congeners 1,2,4,5,6,8-hexaCN, a mixture of 1,2,3,4,6,7 and 1,2,3,5,6,7-hexaCN, 1,2,3,4,5,6,7-heptaCN, and octaCN were synthesized and purified at the Wallenberg Laboratory, Stockholm University, Sweden. PCN congeners 1,2,6,8 and 1,2,4,6-tetraCNs were synthesized at the National Institute for Resources and Environment, Tsukuba, Japan. Congener 1,2,6,8-tetraCN was 93% pure and had 1,2,3,6,8 and 1,2,4,5,6-pentaCNs and an unidentified hexaCN as impurities. Congener 1,2,4,6-tetraCN was 98% pure with 1,2,4,6,8-pentaCN and two triCNs as impurities. Other PCN congeners were obtained from Promochem (GmbH, Wesel, Germany) and were of >99% purity. Standards were analyzed for 2,3,7,8-tetrasubtituted PCDDs or PCDFs but were not detected (DL=100 pg/g) in the standards examined. At this level, the maximum possible response contributed by contaminants are 600-fold-below the detection limit of the bioassay.

Rat hepatoma cells stably transfected with an AhR-controlled luciferase reporter gene construct (H4IIE-luc) were cultured according to methods described previously (5) with the exception that 96-well culture ViewPlates (Packard Instruments, Meriden, CT) were used. After 24 h, the medium was changed to a medium containing 10% charcoal-stripped FBS (Hyclone, Logan, UT), and the cells were dosed with either no treatment (blanks), solvent only, TCDD, or the various samples to be tested in a volume of 1.25 µl. Cells were dosed in triplicate with TCDD in isooctane (0.1 - 30 pg/well) or test agents dissolved in isooctane (a minimum of 6 concentrations were tested). Luciferase activity was measured by incubating cells with LucLite™ reagent (Packard Instruments) for 20 min at room temperature. Light production, a measure of luciferase activity, was determined with a Dynatech ML 3000 luminometer at 30 C. Relative potencies were determined by probit analysis.

RESULTS AND DISCUSSION -

Individual PCN congeners

In general, full dose-reponse curves were obtained for more chlorinated congeners (*e.g.*, penta-, hexa-, and hepta-CNs) whereas most of the lesser chlorinated congeners as well as octa-CN were inactive. Note that the term “inactive” is specific to the conditions in this assay. Results for all congeners tested are summarized along with the greatest concentration tested and their maximum induction of luciferase normalized to the maximum elicited by TCDD **(Table 1**).

Halowax Mixtures

Halowaxes 1000, 1001, and 1099, which were found to be inactive at the concentrations used in this study, are composed of mostly mono-, di-, tri-, and tetraCNs. However, halowaxes 1013, 1014, 1051, which were found to be active, are composed of mostly tetra- through octa-CNs.

Table 1. Summary of relative potencies and efficacy for individual PCN congeners

congener #	PCN	Relative Potency[1] at EC_{50}	Percent of TCDD max[2]	Highest Dose Tested (ng/well)[3]
2	2-CN	NQ		
5	1,4-DiCN	Slight activity	24	1250
6	1,5-DiCN	NQ	8	625
10	2,3-DiCN	NQ	7	625
17	1,2,7-TriCN	NQ	19	625
27	1,2,3,4-TetraCN	NQ		
33	1,2,4,6-TetraCN	NQ	18	1025
36	1,2,5,6-TetraCN	NQ	8	1.875
40	1,2,6,8-TetraCN	Slight activity		
48	2,3,6,7-TetraCN	NQ	14	12.5
53	1,2,3,5,8-PentaCN	NQ	7	12.5
54	1,2,3,6,7-PentaCN	1.69E-04	57	12.5
66	1,2,3,4,6,7-HexaCN	3.85E-03	85	12.5
67	1,2,3,5,6,7-HexaCN	1.00E-03	65	12.5
66/67	50:50 mix of 66/67	1.25E-03	75	155
68	1,2,3,5,6,8-HexaCN	1.53E-04	67	12.5
70	1,2,3,6,7,8-HexaCN	5.87E-04	83	12.5
71	1,2,4,5,6,8-HexaCN	NQ	9	127.5
73	1,2,3,4,5,6,7-HeptaCN	1.01E-03	70	128.75
74	1,2,3,4,5,6,8-HeptaCN	NQ	3	12.5
75	1,2,3,4,5,6,7,8-OctaCN	NQ	8	2.5

[1]NQ indicates that the relative potency was below the ability of this bioassay to quantify. However, congeners that were found to be "inactive" were arbitrarily assigned a relative potency of less than 1.0×10^{-7} (see Table 3)

[2]Percentage of the maximum response induced by TCDD

[3]Maximum concentrations tested in the bioassay varied for each congener and were limited by the mass of individual congeners that were available

In this investigation, relative potencies (REPs) were determined for the first time for individual PCN congeners using H4IIE-luc cells. REPs of the most potent congeners compared to TCDD were approximately 0.003. As a comparison, mammalian TEFs of some of the mono-*ortho* PCBs are in the range of 0.0001 - 0.0005. The most toxic non-*ortho* PCBs, such as 3,3',4,4',5-pentachlorobiphenyl (PCB #126), 3,3'4,4',5,5'-hexachlorobiphenyl (PCB #169), and 3,3',4,4'-tetrachlorobiphenyl (PCB #77), have mammalian TEFs of 0.1, 0.01, and 0.0001, respectively. Thus, the REPs of some PCN congeners are in the same range as some PCB congeners.

It is apparent from the REPs for hexa- and hepta-CNs that the position of chlorine is an important determinant of activity mediated by the AhR. For example, shifting the position of a single chlorine on hepta-CN #73 to become hepta-CN #74 or addition of a single chlorine to hepta-CN #73 to become octa-CN #75 eliminates all AhR-mediated activity. With other classes of halogenated aromatic hydrocarbons, TEFs generally correlate with the presence of lateral substitution and the absence of non-lateral substitution. As illustrated by the inactivity of 2,3,6,7-tetraCN, lateral substitution with PCNs is important, but not sufficient to cause AhR-mediated activity. As more molecular descriptors become available for individual PCNs, key features may be identified to provide a better understanding of potential structure-activity relationships.

The REPs derived in this study may not be appropriate for application to birds and fish, as evidenced by the different PCB TEFs for mammals, birds, and fish developed by the World Health Organization (6). For example, Engwall *et al.,* (2) observed EROD induction and lethality in chicken embryos with exposure to a mixture of HxCNs #66 and #67 (ED_{50} for EROD induction = 0.06 mg/kg egg) but when they tested 1,2,3,4,5,6,7-HpCN, they observed less EROD-induction (the greatest dose, 3 mg/kg, caused only 50% of the maximal induction caused by the HxCN mix). This discrepancy between mammalian (H4IIE cells) and avian (chicken egg)-derived REPs also occurs with PCBs as illustrated by the WHO TEFs of 0.0001 and 0.05 for 3,4,3'4'-tetrachlorinated biphenyl (PCB #77) for mammals and birds, respectively. Thus, there can be significant species-specific differences that must be considered before relative potencies (or TEFs) are applied to environmental data. In addtion, the REPs, like TEFs do not account for differences in toxicokinetics among congeners.

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