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Differences in the action of lower and higher chlorinated polychlorinated naphthalene (PCN) congeners on estrogen dependent breast cancer cell line viability and apoptosis, and its correlation with Ahr and CYP1A1 expression.

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ABSTRACT

There are data showing that exposition to PCNs mixture increased incidence of gastrointestinal and respiratory neoplasms, but data regarding incidence of hormone-dependent cancer so far not shown.

The objective was to determine if exposure to single lower and higher chlorinated PCN congeners is associated with altered proliferation and apoptosis of estrogen dependent breast cancer cells, and whether such effects are related to induction of AhR and CYP1A1 protein expression. MCF-7 cells were exposed to PCN 34, 39, 42, 46, 48, 52, 53, 54, 66, 67, 70, 71, 73 and 74 at concentrations of 100–10,000 pg/ml. We evaluated the action of these PCN congeners on cell proliferation, DNA fragmentation and caspase-8,-9 activity. AhR and CYP1A1 protein expression and CYP1A1 activity was evaluated at a concentration of 1000 pg/ml. An opposite action of tri- to tetraCNs than of penta-to heptaCNs on cell proliferation and apoptosis was evident. Tetra PCNs increased cell proliferation, but had no effect on DNA fragmentation nor caspase activity. Fast induction of CYP1A1 protein expression under the influence of lower chlorinated PCNs suggests faster metabolism and a possible stimulatory action of locally formed metabolites on cell proliferation. None of the higher chlorinated PCNs affected cell proliferation but all higher chlorinated PCNs increased caspase-8 activity, and hexa PCNs also increased caspase-9 activity. The rapid activation of the Ah receptor and CYP1A1 protein expression by higher chlorinated PCNs point to their toxicity; however, it is not sufficient for potential carcinogenicity. Action of lower chlorinated naphthalenes metabolites should be explored.

1. Introduction

Polychlorinated naphthalenes (PCNs) are a group of two-ringed aromatic compounds that contain from one to eight chlorines per naphthalene, and can form 75 possible congeners (Falandysz, 2003). Halogenated naphthalenes, including brominated, chlorinated and mixed brominated/chlorinated derivatives of naphthalene, in addition to the better known halogenated derivatives of biphenyls (PCB), dibenzo-*p*-dioxins (PCDD) and dibenzofurans (PCDF), are all persistent contaminants of foods and the environment, with high toxicological potency at low doses over a lifetime exposure (Van den Berg et al., 2013; Falandysz et al., 2014). PCNs are sold as a variety of mixtures, which range from those containing predominately low-chlorinated mono- and dichloronaphthalenes, to mixtures primarily containing octachloronaphthalenes. The more toxic of these mixtures predominately contain penta- and hexachlorinated naphthalenes. These more highly chlorinated mixtures produce similar biological effects to that of 2,3,7,8-tetrachlorodibenzopdioxin (TCDD), including the induction of liver cytochrome P450-associated enzymes, ethoxyresorufin-O-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH) (Hooth et al., 2012). It has been demonstrated that these PCN mixtures are strong inducers of cytochrome P450s of the 1A family (CYP1A1) after either a single (Galoch et al., 2006) or repeated administration (Kilanowicz et al., 2009) in rats.

Genes encoding cytochrome P450s of the 1A family are well-known targets of the aryl hydrocarbon receptor (AhR), and their

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corresponding proteins may, therefore, serve as biomarkers for the toxicity of dioxins and dioxin-like chemicals. In addition, the induction of CYP1A1 and CYP1A2 have been mechanistically linked to carcinogenesis, since the uncoupling of the induced enzyme or increased metabolism of estrogens may result in the production of reactive oxygen species and oxidative DNA damage and/or enhanced induction of carcinogens, such as polycyclic hydrocarbons and arylamines (Matsumura, 2003; Nebert et al., 2000, 2004).

An increased incidence of gastrointestinal and respiratory neoplasms has been reported in a human exposed to Halowax (Hayward, 1998), however, they did not report incidences of hormone-dependent cancer. Data relating to the PCN cytotoxicity in the development of breast cancer are ambiguous. Kannan et al. (2000) examined the dioxin-like and estrogenic activities of a mixture of pollutants containing different PCNs and polychlorinated biphenyls (PCBs) in a sediment core collected from Tokyo Bay, Japan, describing the cytotoxicity of these mixtures on MCF-7 cells.

Due to the composite nature of PCN mixtures, our knowledge regarding the environmentally relevant properties of all 75 CN congeners is limited (Falandysz et al., 2014). To the best of our knowledge, there have been no studies which have investigated the effect of single PCN congeners on progression of breast cancer cells. In this study we plan to determine the effect of single PCN on the progression of breast cancer. MCF-7 breast cancer cells were exposed to PCN 34, 39, 42, 46, 48, 52, 53, 54, 66, 67, 70, 71, 73 or 74 for 72 h. As endpoints, we evaluated action on cell proliferation, apoptosis measured by caspase activity and DNA fragmentation, and activation of AhR protein expression, CYP1A1 activity and protein expression.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM; without phenol red), charcoal-dextran and insulin were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Foetal bovine serum (FBS; heat inactivated), penicillin, streptomycin and trypsin-EDTA were obtained from PAA Laboratories GmbH (Colbe, Germany). Phosphate-buffered saline (PBS) was purchased from Biomed (Lublin, Poland). PCN 34, 39, 42, 46, 48, 52, 53, 54, 66, 67, 70, 71, 73 and 74 were dissolved in dimethyl sulphoxide (DMSO). The final concentration of DMSO in the medium was $\leq$0.1%.

2.2. Cell culture

MCF-7 human breast cancer cells (ATCC, Manassas, VA, USA; passage No. 151) were routinely cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 IU/ml of penicillin and 100 μg of streptomycin. Cells were grown in 75 cm^2 tissue culture dishes (Nunc, Roskilde, Denmark) in a 37 °C incubator with a humidified mixture of 5% CO_2 and 95% air. Twenty-four hours before each experiment, the medium was removed and replaced with DMEM (without phenol red) supplemented with 5% dextran-coated, charcoal-treated FBS (5% DC-FBS) to exclude any estrogenic effects caused by the medium. The cells were then plated in the same medium and allowed to attach overnight. Cells were exposed to the individual PCNs as described below.

2.3. XTT cell viability assay

Cells were seeded into 96-well culture plates at a density of 2.5×10^4 cells/well and allowed to attach overnight. After 24 h, the medium was changed and cells were treated for an additional 48 h with individual PCN congeners at concentrations of 100, 500, 1000 and 10,000 pg/ml. The corresponding molar concentration equivalents were 0.37594, 1.8797, 3.7594 and 37.594 nM for tetra PCN 34, 39, 42, 46 and 48, respectively; 0.33, 1.66, 3.33 and 33.29 nM for penta PCN 52, 53 and 54, respectively; 0.30, 1.49, 2.99 and 29.85 nM for hexa PCN 66, 67, 70 and 71 respectively; and 0.27, 1.35, 2.71 and 27.06 nM for hepta PCN 73 and 74, respectively. Culture medium and medium containing DMSO were used as controls.

The XTT sodium salt (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt) assay was used to measure the viability of cells. Tetrazolium salts are reduced to formazan by mitochondrial succinate dehydrogenase, an enzyme that is only active in cells with an intact metabolism and respiratory chain. The formazan can be quantified photometrically and correlated with the metabolic activity and number of viable cells. The XTT assay (Xenometrix, Allschwil, Switzerland) was performed according to the manufacturer's instructions after a 48-h incubation with the individual PCN congeners. The reduction of XTT in the cultures was determined after a 2-h incubation by measuring the absorbance at 450 nm using an absorbance microplate reader (ELx808; Bio-Tek Instruments,Inc. Winooski, VT, USA). All samples were run in triplicate in the same assay.

2.4. DNA fragmentation assay

The concentration-dependent effects of individual PCN congeners on cell apoptosis were measured by detecting DNA fragmentation, determined using a Cellular DNA Fragmentation ELISA kit (Roche Applied Science, Mannheim, Germany). This assay is based on the quantitative detection of bromodeoxyuridine (BrdU)-labelled DNA fragments. Cells were seeded into 96-well culture plates at a density of 7.0×10^5 cells/well. After exposure to BrdU for 18 h, cells were treated for 48 h with the individual PCN congeners at concentrations ranging from 100 to 10,000 pg/ml. The culture medium was used as a control. After 24 h, DNA fragmentation was determined according to the manufacturer's instructions. Absorbance values were measured spectrophotometrically at 450 nm using an ELISA reader ELx808 (BioTek). Six replicates of each sample were run in the same assay.

2.5. Caspase-8 and caspase-9 activity

The activities of caspase-8 and -9 were measured after 24 h of exposure, using the fluorescent substrates Ac-IETD-AMC and Ac-LEHD-AFC, respectively. Cells were lysed in caspase assay buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol and 10 mM DTT) and incubated on ice for 10 min. Cell lysates were then incubated at 37 °C with the appropriate caspase substrate at a final concentration of 10 μM. The amount of fluorescent product was monitored every 30 min for 5 h using a fluorescence microplate reader (FLx800; Bio-Tek Instruments) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm for caspase-8, and an excitation wavelength of 400 nm and an emission wavelength of 505 nm for caspase-9. Culture medium alone was used as a control for non-specific binding. All samples were run in quadruplicate in the same assay. After 5 h, 90 μg of fluorescamine in 100 μl of acetonitrile was added to each well to stop the reaction and to determine the protein concentration in each well (400 nm excitation filter and 460 nm emission filter).

2.6. Western blot analysis of AhR and CYP1A1

MCF-7 cells were plated into 48-well plates at a density of 1×10^5 cells/well. On the following day, the medium was changed