INDUCTION OF ARYL HYDROCARBON RECEPTOR–MEDIATED AND ESTROGEN RECEPTOR–MEDIATED ACTIVITIES, AND MODULATION OF CELL PROLIFERATION BY DINAPHTHOFURANS

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Abstract—A group of heterocyclic aromatic compounds, dinaphthofurans (DNFs), recently have been identified as potentially significant contaminants in freshwater sediments. In the present study, a battery of in vitro assays was used for detection of toxic effects of DNFs that are potentially associated with endocrine disruption and tumor promotion. Dinaphthofurans were found to act as relatively potent inducers of aryl hydrocarbon receptor (AhR)–mediated activity in the chemical-activated luciferase reporter gene expression DR-CALUX assay. The relative AhR-inducing potencies of DNFs were similar or even higher than relative potencies of unsubstituted polycyclic aromatic hydrocarbons (PAHs), with dinaphtho[1,2-b;2′,3′-d]furan being the most potent AhR agonist. Two compounds, dinaphtho[2,1-b;2′,3′-d]furan and dinaphtho[1,2-b;1′,2′-d]furan, induced estrogen receptor (ER)–mediated activity in the estrogen receptor–mediated CALUX (the ER-CALUX) assay. Two types of potential tumor-promoting effects of DNFs were investigated, using in vitro bioassays for detection of inhibition of gap-junctional intercellular communication and detection of a release from contact inhibition. Although the acute inhibition of gap-junctional intercellular communication was not observed, all six tested DNFs were able to release rat liver epithelial WB-F344 cells from contact inhibition at concentrations as low as 100 nM. In summary, the present study indicated that DNFs can exert multiple biological effects in vitro, including induction of the AhR-mediated activity, release of cells from contact inhibition, and induction of ER-mediated activity.

Keywords—Aryl hydrocarbon receptor–mediated activity Estrogenicity Intercellular communication inhibition Heterocyclic polyaromatic hydrocarbons

INTRODUCTION

Polycyclic aromatic compounds (PAHs) are a broad group of environmental and food contaminants differing in structure and molecular weight. They include both unsubstituted and substituted PAHs, as well as heterocyclic aromatic compounds. The PAHs are known to elicit a host of toxic effects, including acute toxicity, developmental and reproductive toxicity, cytotoxicity, mutagenicity, and carcinogenicity [1,2]. Different PAHs have been detected in the environment only recently due to introducing specific in vitro bioassay-directed fractionation techniques and advanced chromatographic techniques [1]. The effect-directed analysis of sediment extracts collected in the region of Bitterfeld, Germany, identified several dinaphthofurans as a major cause for high induction potency of the aryl hydrocarbon receptor–dependent 7-ethoxyresorufin O-deethylase activity (EROD) in the rainbow trout liver RTL-W1 cell line [3,4]. It is likely that the presence of DNFs in the environment is related to the production of naphthol, a basic and high-volume production chemical for dye and other chemical industry [4]. The DNF potency to induce EROD activity was comparable to other PAHs, such as benz[a]pyrene. Although these findings suggested a potential ecotoxicological relevance of DNFs, currently there is no other information on mechanistic aspects of potential toxic effects of DNFs.

Epidemiological studies indicate that reproductive dysfunctions and carcinogenesis can be associated with environmental chemical contamination. Many of these adverse effects are associated with nongenotoxic (epigenetic) events, including activation of intracellular receptors and other signal transduction pathways and perturbations of intercellular communication [5]. Polycyclic aromatic hydrocarbons have been shown to interfere with various endocrine pathways affecting, for example, estrogen receptor (ER)– or androgen receptor–mediated signaling [6–10]. The ER-mediated disorders due to xenobiotics have been revealed as one of the major mechanisms of endocrine disruption [11]. Disruption of ER signaling by PAHs also has been related to AhR activation [9]. Dioxin-like toxicity, mediated by activation of AhR, has been described as a set of adverse effects including increased capacity to activate promutagens (as a consequence of induction of cytochrome P4501A enzymes), tumor promotion, immunotoxicity, and an adverse impact on reproduction [12]. Although dioxin-like activity is attributed mostly to the persistent polyhalogenated aromatic hydrocarbons, several studies showed that PAHs and some heterocyclic polyaromatic compounds also elicit relatively strong AhR activation in both mammalian and fish cellular models [13–15]. Importantly, PAHs and not persistent polyhalogenated aromatics have been found to be the prevalent contributors to the AhR-mediated activity in vitro in many river sediment samples, for example, in the Czech Republic [16,17]. Despite the fact that PAHs and heterocyclic polyaromatic compounds are more readily metabolized than...
polyhalogenated compounds, a chronic exposure to PAHs might result at least in some of dioxin-like effects [18].

Imbalance in tissue homeostasis due to disruption of cell-to-cell communication or proliferation/apoptosis balance, has been linked to growth and developmental diseases, such as cancer. Inhibition of gap-junctional intercellular communication (GJIC) plays an important role in these mechanisms [19] and in vitro determination of this effect has been found as a suitable tool for detection of many tumor promoters and carcinogens [19]. A release from contact inhibition is another suitable tool for detection of many tumor promoters and in vitro determination of this effect has been found as a part of effects mechanism known to be associated with the process of carcinogenesis, and it has been suggested to be a part of effects of model tumor promoters, such as 12-cinogenesis, and it has been suggested to be a part of effects mechanism known to be associated with the process of carcinogens [19]. A release from contact inhibition is another suitable tool for detection of many tumor promoters and in vitro determination of this effect has been found as a part of effects mechanism known to be associated with the process of carcinogenesis, and it has been suggested to be a part of effects mechanism known to be associated with the process of carcinogens [19].

In the present study, a battery of in vitro assays was used for detection of potential effects of DNFs associated with endocrine disruption and tumor promotion. The AhR-mediated activity of DNFs was studied in the chemical-activated luciferase expression (DR-CALUX) assay, using rat hepatoma H4IIE cells stably transfected with AhR-inducible luciferase reporter gene [27]. The ER-mediated CALUX (ER-CALUX) assay employing the T47D.Luc transgenic cell line derived from the human breast carcinoma cell line T47D [28] was used to determine ER-inducing potencies relative to 17β-estradiol. Effects of DNFs on GJIC and contact inhibition were studied using rat liver epithelial stem-like WB-F344 cells [21,23,26]. To our knowledge, this is the first comprehensive study of specific in vitro toxic effects of a series of DNFs, environmental toxicants originally identified in sediment samples [4].

**MATERIALS AND METHODS**

**Chemicals**

Dinaphtho[1,2-b;2′,1′-d]furan (DN[1,2-b;2′,1′-d]F, CAS 239–69–0), dinaphtho[2,3-b;2′,3′-d]furan (DN[2,3-b;2′,3′-d]F, CAS 242–31–3), dinaphtho[2,1-b;2′,3′-d]furan (DN[2,1-b;2′,3′-d]F, CAS 204–91–1), dinaphtho[1,2-b;1′,2′-d]furan (DN[1,2-b;1′,2′-d]F, CAS 207–93–2), and dinaphtho[2,1-b;1′,2′-d]furan (DN[2,1-b;1′,2′-d]F, CAS 194–63–8) were supplied by Chiron (Trondheim, Norway). Stock solutions were prepared in dimethylsulfoxide (DMSO) Merck, Darmstadt, Germany) and stored in the dark. Chemical structures of DNFs under study are shown in Figure 1. The 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was from Cambridge Isotope Laboratories (Andover, MA, USA). All other chemicals were provided by Sigma-Aldrich (Prague, Czech Republic).

**Cell culture**

The rat hepatoma H4IIEGud.Luc1.1 cells (BDS, Amsterdam, The Netherlands) were grown in the alpha modification of minimal essential medium (α-[MEM] Sigma-Aldrich), supplemented with 10% of heat-inactivated fetal bovine serum (Sigma-Aldrich). The WB-F344 rat liver epithelial cells [29] were grown in modified Eagle’s MEM with 50% increased concentrations of essential and nonessential amino acids, and supplemented with pyruvate (110 mg/L), 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), and 5% heat-inactivated fetal bovine serum. Only the cells at passage levels 15 to 22 were used throughout the study. Human breast carcinoma T47D.Luc cells (BDS) were grown in a 1:1 mixture of Dulbecco’s modified MEM and Ham’s F-12 nutrient mixture supplemented with 5% fetal bovine serum. The H4IIEGud.Luc1.1 and WB-F344 cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C. The T47D.Luc cells were maintained in a humidified atmosphere of 7.5% CO₂ at 37°C. Cells routinely were grown in 75-cm² flasks and subcultured twice a week. All tissue culture reagents were obtained from Sigma-Aldrich.

**Detection of AhR-mediated activity**

The rat hepatoma H4IIEGud.Luc1.1 cell line, stably transfected with a luciferase reporter gene under the control of
dioxin responsive elements, was used to detect for AhR-mediated activity in the DR-CALUX assay [27,30]. The assays were performed in 96-well cell culture plates. Briefly, 24 h after seeding at split ratio 1:2, cells (at 90–100% confluency) were exposed to the tested or reference compounds (TCDD) dissolved in dimethylsulfoxide. Maximum concentrations of DMSO did not exceed 0.4% (v/v). Following 24-h exposure, the medium was aspirated, cells were washed with phosphate-buffered saline (PBS), and luciferase was extracted with the low salt lysis buffer (10 mM Tris, 2 mM DTT, 2 mM 1,2-diamin cyclic hexane-N,N,N'-N'-tetraacetic acid, pH 7.8). The plates were frozen at −80°C and luciferase expression was then measured in a microplate luminometer using the Luciferase Monitoring Kit (Labsystems, Oy, Helsinki, Finland). Cytotoxicity was not observed using concentrations up to 10 µM.

Detection of estrogenicity

Human breast carcinoma T47D.Luc cells stably transfected with pEREetaLuc plasmid [28] were used for detection of ER-mediated activity in the ER-CALUX assay. The assay was performed in 96-well cell culture plates. Initial seeding density was 5 × 10^5 cells per well in 100 µL experimental medium prepared from phenol red-free Dulbecco’s modified MEM/F-12 containing dextran/charcoal-stripped serum. After 24 h, the media was changed and fresh media added for another 24 h. The cells were dosed with test chemicals or 17β-estradiol calibration standards (positive control) prepared in fresh media and incubated for 24 h. The concentrations of DMSO did not exceed 0.1% v/v. After exposure, the media were aspirated and cells were washed with PBS and lysed with the low-salt lysis buffer. The plates were frozen at −80°C and the luciferase activity was determined as described above. No cytotoxic effects of DNFs were observed.

Assessment of cell proliferation

The effects of DNFs on proliferation of confluent WB-F344 rat liver epithelial cells were determined as described previously [24]. Briefly, cells were seeded at an initial concentration of 30,000 cells/cm² in four-well cell-culture plates (Nunc, Roskilde, Denmark) and grown until they reached an approximate confluency (after 72 h). The media were then changed for fresh (supplemented with 5% fetal bovine serum) and the cells were exposed to test compounds dissolved in DMSO for another 72 h. The final concentration of DMSO did not exceed 0.1% (v/v) in any of the samples. The media with test compounds were changed daily, in order to ensure that the cells would receive adequate amount of nutrients to enable proliferation at high cell densities. Following the exposure, the media were removed and cells were harvested with trypsin and counted with a Coulter Counter (Model ZM, Coulter Electronics, Luton, UK). Cells were washed with PBS and fixed in 70% ethanol at 4°C overnight.

Cell cycle analysis

Fixed cells were washed once with PBS and resuspended in 0.5 ml of Vindelov solution (1M Tris-HCl–pH 8.0; 0.1% Triton X-100 [Sigma], v/v; 10 mM NaCl; propidium iodide 50 µg/ml; RNase A 50 Kunitz units/ml) and incubated at 37°C for 30 min. The cells were then analyzed on a FACS Calibur (Franklin Lakes, NJ, USA), using 488-nm (15 mW) air-cooled argon-ion laser for propidium iodide excitation, and CELLQuest® software for data acquisition (Becton Dickinson, San Jose, CA, USA). A minimum of 15,000 events was collected per sample. Data were analyzed using ModFit LT Version 2.0 software (Verity Software House, Topsham, ME, USA).

GJIC inhibition

The assay was performed as described previously [26,31]. The confluent WB-F344 cells, grown in 24-well plates, were exposed to DNFs (up to 50 µM concentration), TPA (20 nM, positive control), or DMSO (negative control) for 30 min. After the exposure, the cells were washed twice with 0.5 × PBS; fluorescent dye was added (lucifer yellow 0.05% w/v in PBS) and the cells were scanned using a surgical blade. After 4 min, the cells were washed twice with 0.5 × PBS and fixed with 4% formaldehyde (v/v). The migration of the dye from the scrape line was evaluated using an epifluorescence microscope (Nikon, Nikon, Japan). Three independent experiments were carried out in duplicate and at least three scrapes per well were evaluated.

Data analysis

For each compound tested, relative AhR- and ER-inducing potencies were defined as the ability to induce luciferase activity using concentration-response curves. Their relative induction equivalency factors (IEFs) were estimated as described previously [32]. Cell proliferation data were expressed as means ± standard deviation for at least three independent repeats and analyzed by Student t-test, or by the nonparametric Mann-Whitney U-test and Kruskal-Wallis analysis of variance. A p-value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

AhR-mediated activity

Environmental compounds that elicit toxic effects similar to that of polychlorinated dioxins are of concern due to their high chronic toxicity to organisms of different evolutionary levels [33]. It has been suggested that a majority of dioxin-like effects are mediated through AhR [34], and activation of AhR is one of the most important modes of action of PAHs [1,35]. In vitro determination of this adverse effect frequently is performed by measurement of AhR-dependent cytochrome P4501A activity (using 7-ethoxyresorufin as a model substrate, [36]), or by detection of reporter gene luciferase activity under control of activated AhR in various hepatoma cell lines [14,27,37,38]. In this study, activation of AhR by DNFs was determined in the DR-CALUX assay using a rat hepatoma H4IIE cell line stably transfected with luciferase reporter construct under control of dioxin responsive elements [30]. Five DNFs under study induced a relatively strong AhR-inducing activity (Fig. 2, Table 1), with 25% effective concentration (EC25) between 1 and 100 nM. The relative potencies expressed as IEFs were around only three orders of magnitude lower than the reference toxicant TCDD; DN[1,2-b;1-9-d]F was found to be the most potent AhR inducer with IEF 1.15 × 10^-2 . The sixth compound, DN[2,1-b;1-9-d]F; also induced AhR-mediated luciferase activity, however only at concentrations ≥3 µM.

Although PAHs do not elicit a full spectrum of dioxin-like toxicity due to their ready metabolism, they repeatedly have been reported to be significant contributors to the overall AhR-mediated activity in various environmental samples [16,37]. Relative AhR-inducing potencies of DNFs are comparable with the reported potencies of benzo[a]fluoranthene and dibenzo[a,h]anthracene, the most potent AhR inducers among
In vitro effects of dinaphthofurans (DNFs) on aryl hydrocarbon receptor (AhR)–mediated and estrogen receptor (ER)–mediated activities, contact inhibition, and gap-junctional intercellular communication (GJIC), 12-O-tetradecanoylphorbol-13-acetate (TPA); dinaphtho (DN).

Table 1. Effects of dinaphthofurans (DNFs) on aryl hydrocarbon receptor (AhR)–mediated induction of luciferase reporter gene in H4IIEpGudLuc1.1 rat hepatoma cell line by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and concentration of selected DNF = inducing the 25% of maximum TCDD-induced luciferase activity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>AhR IEFs</th>
<th>ER IEFs</th>
<th>Increase of cell numbers</th>
<th>Inh. GJIC (EC50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDD</td>
<td>1</td>
<td>1</td>
<td>100 pM$^c$</td>
<td>NI$^d$</td>
</tr>
<tr>
<td>TPA</td>
<td>1</td>
<td>1</td>
<td>7.6 nM</td>
<td>NI</td>
</tr>
<tr>
<td>DN[1,2-b;1',2'-d]F</td>
<td>7.8E-04</td>
<td>7.40E-06</td>
<td>100 nM</td>
<td>NI</td>
</tr>
<tr>
<td>DN[2,3-b;2',3'-d]F</td>
<td>2.78E-04</td>
<td>NI</td>
<td>100 nM</td>
<td>NI</td>
</tr>
<tr>
<td>DN[1,2-b;2',1'-d]F</td>
<td>8.16E-04</td>
<td>NI</td>
<td>100 nM</td>
<td>NI</td>
</tr>
<tr>
<td>DN[2,1-b;2',3'-d]F</td>
<td>1.10E-03</td>
<td>1.04E-07</td>
<td>100 nM</td>
<td>NI</td>
</tr>
<tr>
<td>DN[1,2-b;2',3'-d]F</td>
<td>1.15E-02</td>
<td>NI</td>
<td>10 nM</td>
<td>NI</td>
</tr>
<tr>
<td>DN[2,1-b;1',2'-d]F</td>
<td>4.52E-07</td>
<td>WI</td>
<td>100 nM</td>
<td>NI</td>
</tr>
</tbody>
</table>

$^a$ The numbers represent induction equivalency factors (IEFs) calculated as the ratio between the 25% effective concentration (EC25) of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and concentration of selected DNF = inducing the 25% of maximum TCDD-induced luciferase activity.

$^b$ The number represent IEFs calculated as the ratio between the 25% effective concentration (EC25) of 17β-estradiol and concentration of selected DNF = inducing the 25% of maximum 17β-estradiol–induced luciferase activity. WI = No induction of luciferase activity observed up to 4 μM concentration of a given compound; WI = Significant (p < 0.05), but too low induction of luciferase activity did not allow the calculation of IEFs.

$^c$ Minimum concentrations inducing a significant (p < 0.05) increase in cell numbers of confluent WB-F344 cells.

$^d$ NI = Not inhibiting GJIC in concentration range up to 50 μM. All the experiments were repeated three times in triplicate.

Fig. 2. Aryl hydrocarbon receptor–mediated induction of luciferase reporter gene in H4IIEpGudLuc1.1 rat hepatoma cell line by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), dinaphtho[1,2-b;1',1'-d]furan, dinaphtho[2,1-b;2',3'-d]furan, and dinaphtho[2,3-b;2',3'-d]furan (A), and by TCDD, dinaphtho[1,2-b;2',3'-d]furan, dinaphtho[1,2-b;1',2'-d]furan, and dinaphtho[2,1-b;1',2'-d]furan (B), after 24-h exposure. The data shown here are representative of three independent experiments; error bars represent standard deviation from the mean value.

PAHs, determined in the same in vitro bioassay [14]. These results are in accordance with previous findings [4] that some of DNFs such as DN[1,2-b;1',2'-d]F and DN[2,1-b;2',3'-d]F were recognized to be potent inducers of EROD activity in the rainbow trout liver RTL-W1 cell line. Both compounds were detected in sediments collected in industrially-polluted German sites and identified as significant contributors to the overall EROD induction potency of sediment extracts [3,4]. Therefore, despite the limited data available on concentrations of DNFs found in the environment, the AhR activation should be considered a potential significant toxic mode of action of DNFs. Indeed, further studies including in vivo effects of DNFs, their toxicokinetics, and bioavailability characteristics need to be performed to support this hypothesis.

**Estrogenic activity**

In recent years, much attention has been devoted to adverse effects of chemicals on endocrine pathways, especially on compounds that mimic or inhibit the effects of endogenous estrogens. The action of xenoestrogens has been linked to various physiologic and morphologic reproduction disorders in wildlife species, and a number of assays have been developed in an effort to identify the compounds responsible for endocrine disruption [39]. In the present study, modulation of ER-mediated activity by DNFs was investigated using the ER-CALUX reporter gene assay. Two of the test compounds, DN[1,2-b;1',2'-d]F and DN[2,1-b;2',3'-d]F, induced a high luciferase activity at concentrations ≥ 100 nM (Fig. 3). The stron-
gest ER inducer, DN[1,2-b;1',2'-d]F, increased ER-mediated gene expression by more than 180%, as compared to the maximal response due to 17β-estradiol treatment. The DN[2,1-b; 1',2'-d]F showed a low ER-inducing activity, which did not exceed 15% of the maximum activity induced by the reference compound, and other DNFs had no effect (Table 1). The ER-mediated potencies of DN[1,2-b;1',2'-d]F and DN[2,1-b;2',3'-d]F document their relatively strong estrogenic effect in vitro when compared with relative potencies of unsubstituted PAHs, such as benzo[a]pyrene and benzo[a]anthracene [6,8,40], or with low-chlorinated PCBs and hydroxy-PCB congeners [41,42]. The concentrations of polyaromatic xenoestrogens that are required to observe a significant ER activation often are rather high, as compared to physiological ER ligands. Nevertheless, recently it has been shown that weak environmental estrogens can, at least in vitro, produce significant mixture effects when combined at concentrations below their NOECs [43]. Therefore, as DNFs likely are to contribute to a complex mixture exposure by ER-activating compounds present in contaminated sediments, this fact should be taken into consideration when assessing their potential significance as xenoestrogens.

Effects on GJIC inhibition and cell proliferation of liver epithelial cells

Gap junctions are membrane channels that permit the transfer of small water-soluble molecules (<1 kDa) between the adjacent cells [19]. Most tumor cells have a reduced ability to communicate among themselves and/or with surrounding normal cells, confirming the importance of intact GJIC in growth control and suggesting that inhibition of GJIC is a typical property of tumor promoters [19]. When looking for potential in vitro effects of DNFs that could play a role in tumor promotion, DNFs were found not to inhibit GJIC at concentrations up to 50 μM in WB-F344 cells (Table 1). It is known from a previous study that PAHs with higher molecular weight possess only low or no GJIC inhibition potency [26].

Using the same cellular model, we investigated whether DNFs are able to mimic another effect of tumor promoters, a release of confluent cell culture from growth restraints induced by contact inhibition. Although this effect is far less studied than inhibition of GJIC, it has been reported to be induced by both model tumor promoters (e.g., TPA) and environmental contaminants known as powerful tumor promoters, such as TCDD [20,21]. It was found that all six DNFs significantly increased cell numbers in confluent WB-F344 cells, at concentrations ≥100 nM (Fig. 4). The effective concentration for cell proliferation of the most potent DN[1,2-b;2',3'-d]F was 10 nM (Table 1). Interestingly, this compound also was the most potent AhR inducer in the DR-CALUX assay. The increased cell numbers mostly corresponded with an increased percentage of cells in S-phase (Fig. 5), suggesting a higher proliferative rate to be responsible for the increase in cell numbers. The potential role for AhR in the regulation of growth in certain cell types, such as in hepatocytes and hepatocyte-like cells, is supported, for example, by the fact that a decreased AhR or AhR nuclear translocator content in murine hepatoma cells slows down their proliferation rate [44]. Recent data seem to suggest that TCDD could release hepatic oval cells from contact inhibition, rather than to directly stimulate cell proliferation, because TCDD has been reported not to stimulate proliferation of subconfluent, serum-deprived WB-F344 cells [23]. A comparative study of a large series of PAHs, including some potent carcinogens, revealed that AhR-inducing PAHs, such as benzo[a]pyrene, benzo[a]anthracene, or chrysene, can induce a release from contact inhibition [24].

Fig. 4. Modulation of cell proliferation by selected dinaphthofurans in WB-F344 cells assessed by counting cell numbers. Cells were cultured for 72 h prior to exposition, and then treated for another 72 h with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or the tested dinaphthofurans in a daily applied fresh medium. Cells were then trypsinized and counted on a Coulter Counter (Coulter Electronics, Luton, UK). Cell numbers (×1,000) are expressed as mean ± standard deviation of at least three independent experiments run in duplicates. * = A significant difference between control (0.1% dimethylsulfoxide [DMSO]) and treated samples (p < 0.05).
In vitro effects of dinaphthofurans

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Fig. 5. Modulation of cell cycle by selected dinaphthofurans in WB-F344 cells assessed by measuring percentage of cells in S-phase of cell cycle. Cells were cultured for 72 h prior to exposition, and then treated for another 72 h with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or the tested dinaphthofurans in a daily applied fresh medium. Cells were then trypsinized and fixed in ethanol for a subsequent flow cytometric analysis of DNA content. The cell-cycle analysis was performed on a FACSCalibur cytometer equipped with CellQuest and ModFit software (Becton Dickinson, San Jose, CA, USA). The percentage of cells in S-phase is expressed as mean ± standard deviation of at least three independent experiments run in duplicates. * = A significant difference between control (0.1% dimethylsulfoxide [DMSO]) and treated samples (p < 0.05).

These findings seem to support the hypothesis that AhR activation might be involved in the observed effects of DNFs on confluent cell population. From an ecotoxicological point of view, these data seem to indicate that DNFs might elicit effects potentially associated with tumor promotion at low concentrations.

CONCLUSION

Dinaphthofurans are newly discovered aquatic contaminants of potential environmental relevance. In this study, DNFs were shown able to modulate various toxic endpoints in vitro. Their relative AhR-inducing potencies are similar to or even higher than IEF values reported for PAHs [6,13,14]. Because the majority of toxic effects of dioxins are supposed to be associated with AhR activation, DNFs as relatively potent AhR inducers might be considered compounds posing a serious threat to the environment. Two DNFs, DN[1,2-b;1',2'-d]F and DN[2,1-b;2',3'-d]F, were found to be relatively potent inducers of ER-mediated activity. Their relative potencies were comparable to other polyaromatic compounds shown to be estrogenic in vitro, such as some PAHs or PCBs and their hydroxylated derivatives. This seems to suggest a possible role in endocrine disruption. Potential tumor-promoting effects of DNFs were investigated in two in vitro bioassays. Though acute inhibition of GJIC was not observed, all six DNFs were able to release cells from contact inhibition at concentrations as low as 10 to 100 nM, which were comparable to unsubstituted PAHs [24].

In general, PAHs and other polycyclic aromatic compounds occur at high concentration at many contaminated sites [33] and, in some cases, they might have higher ecotoxicological significance than persistent polyhalogenated aromatic hydrocarbons [16,17]. Significance of DNFs as environmental contaminants is highlighted by the fact that some of them have been identified at relatively high concentrations in aquatic sediment samples, probably originating from naphthol production [4]. The present study indicated that DNF can exert multiple biological effects and that AhR activation should be considered a potential significant toxic mode of action of DNFs. Future studies should investigate the toxicokinetics and bioavailability of DNFs in aquatic systems.

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