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In Vitro and in Vivo Antiestrogenic Effects of Polycyclic Musks in Zebrafish

RICHARD H. M. M. SCHREURS,*† JULIETTE LEGLER,‡,† ELSA ARTOLA-GARICANO,† THEO L. SINNIGE,† PETER H. LANSER,† WILLEM SEINEN,† AND BART VAN DER BURG,*‡

Institute for Risk Assessment Sciences, Utrecht University, P.O. Box 80176, 3508 TD, Utrecht, The Netherlands, and Netherlands Institute for Developmental Biology, Uppsalaalan 8, 3584 CT, Utrecht, The Netherlands

The polycyclic musks 6-acetyl-1,1,2,4,4,7-hexamethylytetraine (AHTN) and 1,2,4,6,7-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-j-2-benzoyparan (HHCB). Polycyclic musks are used as fragrance ingredients in perfumes, soaps, and household cleaning products. They are known to be ubiquitously present in the aquatic environment, and because of their lipophilic nature, they tend to bioaccumulate in aquatic biota. In surface waters, concentrations between 1 ng/L and 5 μg/L have been found, depending mainly on the proportion of sewage effluents in the water. In fish, under normal environmental conditions, concentrations in the microgram per kilogram fresh weight (fw) range are found. In a previous study we showed that AHTN and HHCB exert mainly antiestrogenic effects on the human estrogen receptor α (ERα) and ERβ in an in vitro reporter gene assay. In the current study, we assessed the in vitro antiestrogenic effects of both musks on zebrafish ERs. Antagonism was observed on zfERα and more pronounced on the newly cloned zfERβ. Using a transgenic zebrafish assay, we studied antiestrogenicity of the musks in vivo. Dose-dependent antagonistic effects were observed at concentrations of 0.1 and 1 μM AHTN and HHCB. GC–MS analysis showed that the musks bioaccumulated in the fish, with internal concentrations (15–150 mg/kg fw) which were roughly 600 times higher than the nominal test doses. To our knowledge, this is the first time that environmental contaminants are shown to be antiestrogenic in an in vivo fish assay that focuses solely on ER-mediated effects. This makes the transgenic zebrafish assay a promising tool for the rapid detection of both estrogenic and antiestrogenic effects of chemicals in fish.

Introduction

Two chemicals that are ubiquitously present in the aquatic environment are the polycyclic musks 6-acetyl-1,1,2,4,4,7-

* Corresponding author phone: +31 30 2535018; fax: +31 30 2535077; e-mail: r.schreurs@iras.uu.nl.
† Utrecht University.
‡ Netherlands Institute for Developmental Biology.
§ Present address: Institute for Environmental Studies, Vrije Universiteit, De Boelelaan 1087, 1081 HV, Amsterdam, The Netherlands.

Materials and Methods

Chemicals and Reagents. 17β-Estradiol was purchased from Sigma (St. Louis, MO). HHCB and AHTN were kind gifts from International Flavours and Fragrances (IFF), Hilversum, The Netherlands, and PFW-Aroma Chemicals, Barneveld, The Netherlands, respectively. 4-Hydroxytamoxifen was a kind gift from Dr. A. Wakeling (Zeneca Pharmaceuticals, Macclesfield, U.K.).

Cell Culture. Human embryonal kidney 293 (HEK293) cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM)
and Ham's F12 medium (DF: Life Technologies Inc., Gaithersburg, MD) supplemented with 7.5% fetal calf serum (FCS) (Life Technologies Inc., Gaithersburg, MD) supplemented with 7.5% fetal calf serum (FCS) and Ham's F12 medium (DF; Life Technologies Inc., Gaithersburg, MD) supplemented with 7.5% fetal calf serum (FCS) and Ham's F12 medium (DF; Life Technologies Inc., Gaithersburg, MD) supplemented with 7.5% fetal calf serum (FCS) and Ham's F12 medium (DF; Life Technologies Inc., Gaithersburg, MD). The estrogen-responsive reporter gene construct (3xERE-TATA-Luc) is described in more detail elsewhere (12). The cloning of the full-length zebrafish (zf) ERα, zfERβ, and zfERγ and the insertion into the EcorO site of the multiple cloning site of the pSG5 expression vector (Stratagene) are described elsewhere (Zeinstra et al., manuscript in preparation).

**Transient Transfections.** HEK293 cells were plated in 800 μl of phenol red-free DF medium containing 30 mM selenite, 10 μg/mL transferrin, and 0.2% BSA, supplemented with 5% dextran charcoal stripped FCS, at 8 × 10^5 cells per well of a 24-well plate (Costar). Cells were transfected using the calcium phosphate precipitation method (14) 30 h after plating.

A total amount of 2.0 μg of DNA/well was transfected, consisting of 0.6 μg of luciferase reporter plasmid, 0.6 μg of PDM-LacZ internal control plasmid, 0.6 μg of pBluescript SK, and 0.2 μg of ER expression plasmid. After an overnight incubation cells were given fresh media with or without test compounds (dissolved in ethanol, maximum 0.1% solvent).

After 24 h, the cells were lysed in 200 μl of lysis solution (1% Triton X-100, 25 mM glycylglycine, 15 mM MgSO₄, 4 mM EGTA, and 1 mM DTT). A 50 μL portion of cell lysate was transferred to a black 96-well plate to which 50 μl of luciferase substrate (LucLite reporter gene assay kit, Packard Instruments, Meriden, CT) was added. Luciferase activity was measured in a Topcount liquid scintillation counter (Packard Instruments) for 0.1 min per well. To correct for variations in transfection efficiencies, β-galactosidase activity was measured (15).

**Transgenic Zebrafish Assay.** The development of the transgenic zebrafish assay is described elsewhere (12). Exposure studies were carried out with homozygous F4 juvenile fish of 4–5 weeks of age. Fish (n = 5–6) were exposed for 96 h in 200 mL of acclimated tap water (26–27 °C) in beaker glasses. The compounds to be tested (dissolved in DMSO) were added to the water in a 1:10000 dilution. Fish were fed once daily with live brine shrimp (Artemia salinas). Half of the test medium was renewed daily. At the end of the exposure, fish were sacrificed in ice-water, transferred to Eppendorf vials, and immediately frozen at −80°C. To assay luciferase activity, Eppendorf vials containing whole fish were transferred to ice, 500 μL of cold lysis solution (1% Triton X-100, 25 mM glycylglycine, 15 mM MgSO₄, 4 mM EGTA, and 1 mM DTT, pH 7) was added, and the fish were homogenized using a micropestle (Eppendorf). After centrifugation at 12000 rpm for 15 min at 4°C, duplicate samples of 50 μL of supernatant were transferred to a black 96-well plate (Packard) to which 50 μL of luciferase substrate (LucLite reporter gene assay kit, Packard) was added. Luciferase activity was measured in a scintillation counter (Packard Topcount) for 0.1 min per well.

**Internal Exposure.** To assess the internal concentration of both musks in the zebrafish, six animals were exposed to 0.1 or 1 μM (i.e., 25.8 or 258 μg/L) musk for 1, 2, 3, or 4 days. Fish were exposed in 200 mL of acclimated tap water (26–27 °C) in beaker glasses. The compounds to be tested (dissolved in DMSO) were added to the water in a 1:10000 dilution. Fish were fed once daily with live brine shrimp (A. salinas). Each day, before half of the test medium was refreshed, water samples were taken. After exposure, fish were sacrificed, and cleaned up for GC–MS analysis. The same exposure experiment was done in the absence of zebrafish.

**Extraction and Cleanup of Fish Samples and Water Samples.** Fish sacrificed on ice were spiked with 1 μg of AHTN or HHCB as an internal standard, and ultrasonicated in a mixture of 10 mL of water and 4 mL of cyclohexane. Then 4 g of dry NaCl was added, and after centrifuging, the upper cyclohexane layer was transferred into a glass test tube. Again 4 mL of cyclohexane was added to the sample, and the procedure was repeated. After concentration under nitrogen to 1 mL, the organic layer was eluted on a silica column using a 6 mL mixture of cyclohexane and ethyl acetate (98:2 v/v). The eluate was concentrated under nitrogen and transferred into a vial for GC–MS analysis.

Water samples of 3 mL were extracted using 4 mL of cyclohexane. The organic layer was spiked with either 1 μg of AHTN or HHCB, concentrated under nitrogen, and transferred into a vial for GC–MS analysis.

**GC–MS Analysis.** AHTN and HHCB concentrations in all extracts were analyzed on a Carlo Erba 5300 GC (Milan, Italy) equipped with a split/splitless injector, a 30 m × 0.25 mm (0.25 μm film thickness) fused silica DB-5MS column (J&W Scientific, Folsom, CA), and a QMD 1000 mass spectrometer (Carlo Erba Instruments, Milan, Italy). Analyses were carried out by splitless injection of 1 μL at 225 °C. The column temperature was maintained at 90 °C for 1 min, and raised by 30 °C/min to 150 °C followed by 4 °C/min to 210 °C. The mass spectrometer was operated using selected ion monitoring (SIM), for m/z 243. Unknown concentrations were quantified with the peak area ratio of the compound to be quantified and the internal standard, using a standard curve. The recovery of this method is 100%.

**Data Analysis.** Luciferase activity per well was measured as light units. In the in vitro experiments, each concentration was analyzed in triplicate. The luciferase activity per well was divided by the concomitant β-galactosidase activity. In the transgenic zebrafish assay five to six fish per concentration were measured. From these values, the fold induction was calculated by dividing the mean value in exposed and nonexposed wells or fish. ERE-luc activity as a percentage of estradiol at 100% is shown in Figure 1. Results of both musks in the zebrafish, six animals were exposed to 0.1 or 1 μM (i.e., 25.8 or 258 μg/L) musk for 1, 2, 3, or 4 days. Fish were exposed in 200 mL of acclimated tap water (26–27 °C) in beaker glasses. The compounds to be tested (dissolved in DMSO) were added to the water in a 1:10000 dilution. Fish were fed once daily with live brine shrimp (A. salinas). Each day, before half of the test medium was refreshed, water samples were taken. After exposure, fish were sacrificed, and cleaned up for GC–MS analysis. The same exposure experiment was done in the absence of zebrafish.

**Human and Zebrafish ER Transactivation by Estradiol in a Transiently Transfected HEK293 Reporter Cell Line.** To compare the human and zebrafish ERs for E2-induced transcriptional activation, we used the HEK293 cell line. This cell line lacks significant endogenous levels of ER, can be easily transfected, and has been shown to be highly responsive to estrogens in transient transfections (16). Cells were transiently transfected with one of the tested ER expression plasmids and a reporter construct, consisting of three EREs upstream from a TATA box in front of luciferase cDNA.

Dose–response curves of E2 are shown in Figure 1. Transcriptional activity at hERα was about 1 order of
magnitude higher than that of hERβ (Figure 1A), with EC50 values of 0.009 and 0.10 nM, respectively. When the zebrafish ERs were compared, ERç was the most responsive to E2, suggesting an important role in estrogen regulation (Figure 1B). Zebrafish ERR and ERâ were about equally sensitive, but less sensitive than zfERç. We found EC50 values of 0.38, 0.73, and 0.09 nM for zfERR, zfERö, and zfERç, respectively.

Comparison of human and zebrafish ERs revealed that E2-induced transcriptional activity of the human ERγ was about 40 times higher than its zebrafish counterpart, while human ERâ was only about 7 times more responsive than zfERç. Together with the small difference in sensitivity between zfERR and zfERö, in contrast to the human ERs, these findings may suggest that zfERç, compared to its ERç counterpart, is relatively more sensitive to estrogens than hERç. These findings are consistent with another study (9). In addition, we used the dose–response curves to determine the submaximal E2 dose for each receptor subtype. At this concentration the response can be influenced most sensitively and can therefore be used in competition experiments, to assess antiestrogenicity.

Inhibition of E2-Induced Transactivation of Human and Zebrafish ER by AHTN and HHCB. To assess the antiestrogenic effects of AHTN and HHCB, we dosed the transiently transfected HEK293 cells with different concentrations of musk, and a submaximal concentration of estradiol. A submaximal dose of 0.01 nM E2 was used for hERR-mediated experiments, a dose of 0.1 nM was used for hERö and zfERö, and 1 nM E2 was used for zfERR and zfERç. AHTN and HHCB alone showed a marginal transcriptional activation of the human ERγ, at the highest test concentration of 10μM. This was also previously shown in stably transfected HEK293 cells (5). Neither of the musks could stimulate transcriptional activity of the zebrafish ERs and the human ERö (data not shown). As a positive control for antiestrogenicity in this experiment, the well-known antiestrogen 4-hydroxytamoxifen (OHT) was used at a concentration of 0.01 μM. OHT strongly inhibited the E2-stimulated transcriptional activation of all tested estrogen receptors (Figure 2). A clear dose-dependent and significant suppression of E2 FIGURE 1. Activation of transcription of hERα and hERβ (A) and zfERR, zfERö, and zfERç (B) by 17β-estradiol in transiently transfected HEK293 cells. Results are expressed as a percentage of maximal E2 induction of each receptor subtype. Values represent means ± SEM from three independent experiments with each concentration measured in triplicate.

FIGURE 2. Repression of transcription of hERα (A), hERβ (B), zfERR (C), zfERö (D), or zfERç (E) by OH-tamoxifen (black bar; 0.01 μM), AHTN (white bars; 0.1–10 μM), and HHCB (gray bars; 0.1–10 μM) in transiently transfected HEK293 cells. Results are expressed as a percentage of submaximal E2 induction of each receptor subtype (hERα, 0.01 nM; hERβ, 0.1 nM; zfERR, 1 nM; zfERö, 1 nM; zfERç, 0.1 nM). Values represent mean ± SEM from three independent experiments with each concentration measured in triplicate. Key: *, P < 0.05; **, P < 0.01; ***, P < 0.001 (by one-way ANOVA and LSD for differences between E2 treatment alone and E2 + compound).
induction by AHTN and HHCB was shown toward human ERβ and zebrafish ERγ (Figure 2B,E). A weak antagonistic effect could be observed on human ERα and zRFERβ only at the highest test concentration of 10 nM (Figure 2A,D), whereas no effect was seen at zRFERα (Figure 2C). Co-administration of the musks with higher concentrations of E2 abolished the antagonistic effects of the musks, which suggests a competitive interaction at the level of the ER (data not shown).

**Inhibition of E2-Induced Transactivation by AHTN and HHCB in Transgenic Zebrafish.** Since both musks antagonized E2-induced transcription on the zebrafish ERβ and ERγ in vitro, we wished to examine whether these antiestrogenic effects could also be observed in vivo. Therefore, we used a transgenic zebrafish assay, a rapid and specific in vivo assay for the detection of (xeno)estrogens (Figure 3). The detection limit of the assay is about 0.3 nM E2, and the EC50 is about 10 nM.

**TABLE 1. Concentrations of AHTN and HHCB Determined in Test Water during the Internal Exposure Experiment**

<table>
<thead>
<tr>
<th>compd</th>
<th>t = 0 h</th>
<th>t = 24 h</th>
<th>t = 96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHTN</td>
<td>25.8</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>258</td>
<td>229</td>
<td>196</td>
</tr>
<tr>
<td>HHCB</td>
<td>25.8</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>258</td>
<td>176</td>
<td>158</td>
</tr>
</tbody>
</table>

*See Materials and Methods for details. Concentrations are expressed as milligrams per liter.*

**FIGURE 4. Internal concentrations of AHTN and HHCB at nominal test concentrations of 0.1 (bottom part of graph) and 1 μM (top part of graph) during the 4-day internal exposure experiment. Results are expressed as milligrams of musk per kilogram fresh weight. Values represent means ± SEM from 5–6 fish. Key: *, P < 0.05; **, P < 0.01 (by Student’s t test for differences between day 1 and the following days).**

**Discussion**

In this study we were mainly interested in the in vitro and in vivo antiestrogenic effects of two polycyclic musks (AHTN and HHCB), which are ubiquitously present in the aquatic environment. For the in vitro experiments, we used the ERα and ERβ subtypes cloned from human and zebrafish, and also the novel zebrafish ERγ (9). Next to ERα and ERβ, fish have a third ER subtype (ER2 or ERγ), which has been recently discovered in the Atlantic croaker (17), goldfish (18), and the zebrafish (10, 11, 19; see also Callard et al., unpublished results; GenBank Nos. AAK16740, AAK16741, and AAK16742). Comparison of the overall amino acid sequences of the three receptors indicates that each zER protein was generated by a distinct gene (11).
the ligand binding domains of the human and the zebrafish ER$_\alpha$ reveals a homology of 55%. The percentage of identical amino acid residues of the human and the zebrafish ER$_\alpha$ is 57%, and that of hER$\alpha$ and zfER$\alpha$ is 61% (11). These alignment results taken together with phylogenetic analysis clearly indicated that zfER$\alpha$ belongs to the ER$_\alpha$ subgroup and that both zfER$\alpha$ and zER$\alpha$ belong to the ER$\beta$ subgroup (11).

The zebrafish ER subtypes, which have been cloned in our laboratory, have been shown to bind estradiol in a receptor-binding assay (Zeinstra et al., manuscript in preparation). This was partly to be expected, since all three zebrafish ERs contain the same specific amino acids for estradiol binding as are found in the human ER$_\alpha$, i.e., Glu353, Arg394, and His524 in hER$_\alpha$ (20). In the present results we show that zebrafish ER$_\alpha$ is the most sensitive to estradiol, which was also shown in other studies (9, 11). Furthermore, the difference between E2-stimulated transactivation of human ER$_\alpha$ and ER$\beta$ was not shown in the zebrafish situation, where ER$_\alpha$ and ER$\beta$ are about equally potent and ER$\alpha$ is the most responsive transactivator. Overall, zebrafish ER$_\alpha$ and ER$\beta$ are less sensitive to E2 than their human counterparts, which also has been shown for rainbow trout ERs (21). This may explain the fact that both musk compounds do not show any agonistic effect on the three zebrafish ERs. On the human ER$_\alpha$, the transactivation potential of which is minimally a factor of 10 higher than those of the zebrafish ERs, a marginal agonism has been shown in stably transfected HEK293 cells (5). On the other hand, antioestrogenic effects of AHTN and HHCB on the human ERs have been recently shown (5). In the present study we also showed a dose-dependent antagonism on zfER$\alpha$, already starting at 100 nM and which is almost as strong as that on hER$\alpha$. Of the three zebrafish ERs, zfER$\alpha$ showed the highest susceptibility for suppression of E2 induction by OHT and both musks.

In our in vivo transgenic zebrafish study, at test concentrations of 0.1 and 1 µM AHTN or HHCB, a significant repression of E2-induced transactivation was observed, especially at the EC50 E2 concentration. This repression is quite strong, namely, down to 20% of the E2 induction for both compounds at the highest test dose.

The nominal water concentrations used in this in vivo study (0.01, 0.1, and 1 µM musk, i.e., 2.58, 25.8, or 258 µg/L musk) were comparable to the nominal concentrations used in standard fish toxicity studies. In a 36-day early-life-stage test (OECD 210) with fathead minnow (Pimephales promelas) no observed effect concentrations (NOECs) were found of 35 µg of AHTN/L and 68 µg of HHCB/L. In a 21-day growth test (OECD 204) with bluegill sunfish (Lepomis macrochirus) NOECs were found of 89 µg of AHTN/L and 93 µg of HHCB/L (22). The actual concentrations at which we observe antioestrogenic effects are around or below the no-observed-effect levels from these studies. That is, no developmental disorders were or will be observed at the concentrations used in our transgenic zebrafish assay.

Furthermore, the nominal concentrations at which an antioestrogenic effect is observed are roughly 25 to more than 1000 times higher than the concentrations found in the environment. Under normal environmental conditions, in river and seawater, levels of musk in the nanogram per liter range are found (reviewed in refs 23 and 24). The higher the input of sewage water, the higher the concentration of musk that is found (3). Maximum levels in the low microgram per liter range have been found in water samples from lakes with a high input of sewage water. Higher concentrations have been found in sewage water effluents and sewage settlement ponds, with maximal levels in eel of 19.2 mg/kg fw (cited in ref 25). In eel under natural conditions mean levels of 43 µg/kg fw were found (cited in ref 3). In other fish species under natural conditions, comparable or lower concentrations are found, depending largely on the lipid content (reviewed in refs 23 and 24). The internal concentration that we have found at the lowest observed effect concentration for antioestrogenicity in vivo (i.e., 0.1 µM) is about 15.8 mg of AHTN/kg fw and 18.9 mg of HHCB/kg fw. These concentrations can be reached in fish from ponds of sewage treatment plants (24, 25) and might induce antioestrogenic effects in this particular situation. Under normal environmental conditions, the concentrations of the musks in receiving waters are too low to cause antioestrogenic effects.

Concentrations in the fish depend on the toxicokinetics and the biotransformation of the compound in the fish. The high antagonistic potency in vivo may be due to its high lipophilicity and thus to its strong bioaccumulation. From our GC–MS results it is clear that both musks are indeed bioaccumulating. In the zebrafish, concentrations have been found which are roughly a factor 600 higher than the nominal test dose. Internal concentrations stay roughly the same during the 96 h in vivo experiment, which means that the equilibrium has been reached within 1 day. Butte et al. (26) showed that for both polycyclic musks the steady state for uptake in zebrafish was reached in about 10 h. Concentrations in the fish also depend on the fate of the compound in the aquarium. When AHTN or HHCB is added to beaker glasses without fish, immediately after the first dosing, 15–30% has disappeared, and at the end of the 96 h experiment, 25–40% has disappeared. Compounds may sorb to the glass wall and detritus, or degradation of the compounds may take place. HHCB can be transformed into HHCB lactone by autoxidation, which can also occur as a biotransformation reaction (27). Nevertheless, a significant amount of musk has been accumulated in the fish, resulting in low actual water concentrations at the end of the exposure experiment, and also in the observed antioestrogenic effects.

The antioestrogenic effects seen in the in vivo transgenic zebrafish assay are probably for the greater part mediated by the zfER$\alpha$, which is the most responsive to estradiol in our in vitro assays. Also, the strongest antagonism by the musks was seen at this receptor subtype. However, it cannot be ruled out that the other two ER subtypes also play a role in the netto effect. Comparison between the in vitro system and the in vivo test system is possible because the measured end point (luciferase protein) is induced according to the netto effect. Comparison between the in vivo system and the in vivo test system is possible because the measured end point (luciferase protein) is induced according to the
antiestrogenic CYP1A inducer 3,3',4,4'-tetrachlorobiphenyl (TCB) shows both potentiation and reduction of nonylphenol-induced Vtg synthesis, depending on doses and temporal exposure sequence. Another in vivo study shows both potentiation and repression of E2-induced Vtg synthesis by the CYP1A inducer β-naphthoflavone, which has been shown to be antiestrogenic in vitro (33). These studies assessed antiestrogenicity which is both ER- and AhR-mediated. Only a few in vivo fish studies showed antagonism that was postulated to be solely ER-dependent. Tilapia showed decreased E2-induced Vtg levels after tamoxifen exposure (34). Similar results were found in exposed female Tilapia by Lazier et al. (35). Recently, Panter et al. (36) exposed fathead minnows via the water. However, no dose-dependent antagonism was found when fish were exposed to ethynylestradiol and the antiestrogen ZM189,154. Using an AhR-specific CALUX assay, we showed no in vitro (ant)agonistic effects of the musks on the human AhR (unpublished data). Furthermore, AHTN is not a CYP1A inducer (37). We conclude that, in our in vivo assay, the observed antiestrogenic effects are not AhR-mediated, but solely ER-mediated. This makes the transgenic zebrafish assay a suitable tool for the rapid detection of both estrogenic and antiestrogenic effects of chemicals in an in vivo fish model.

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