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Effects on CYP1A of the polycyclic musk tonalide (AHTN) in single and co-exposure with benzo(a)pyrene and 3,3′-4,4′,5-pentachlorobiphenyl in the PLHC-1 fish cell line

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The aim of this study was to investigate the interaction of a common polycyclic musk fragrance, namely tonalide (AHTN), with the detoxification gene family CYP1A, by looking at both gene transcription (*cyp1a*) and enzyme activity (7-ethoxyresorufin-*O*-deethylase; EROD) in the PLHC-1 fish cell line. Time-dependent (6 and 24 h) exposure experiments with three doses of AHTN (1 nM, 10 nM and 2 μM) were performed. Co-exposure with known CYP1A inducers such as benzo[*a*]pyrene (B[*a*]P) and 3,3′-4,4′,5-pentachlorobiphenyl (PCB 126) was also investigated. A slight induction of *cyp1a* gene transcription was observed after 6 h, but not at 24 h. At 6 h, 40–45% of reduction of *cyp1a* transcription was observed in co-exposure with B[*a*]P and PCB 126 compared with single inducers. Complete recovery was observed after 24 h. No effect on EROD activity by AHTN was observed in either single exposure and co-exposure. AHTN seemed not to affect CYP1A at the gene or enzyme level, but in co-exposure with inducers ATHN seems able to reduce detoxification capability within a short time.

Keywords: tonalide; CYP1A; EROD; detoxification; co-exposure; PLHC-1

1. Introduction

Tonalide (7-acetyl-1,1,3,4,4,6-hexamethyl-tetrahydronaphtalene; AHTN) is a synthetic musk fragrance of a polycyclic nature widely used in detergents, cosmetics and personal care products. The major source of input of this compound into the aquatic environment is municipal waste discharge because in general musks are not completely removed during the waste treatment process [1]. AHTN is known to be a widespread contaminant, being detected in air [2], superficial waters (ranging from $ng \cdot L^{-1}$ to $-\mu g \cdot L^{-1}$) [3], marine waters ($ng \cdot L^{-1}$) [4] and sediments [5]. Its lipophilic nature ($\log K_{ow} = 5.70$) [6] suggests a high bioaccumulation potential for aquatic biota, with levels reported to be in the range of $\mu g \cdot kg^{-1}$ to $mg \cdot kg^{-1}$ in lipid bases [7–9].

The toxicity of AHTN to aquatic biota has been observed at higher concentrations than those present in the natural environment. A decrease in heart rate has been reported in zebrafish *Brachidanio rerio* larvae with an EC_{50} of 33 $\mu g \cdot L^{-1}$ [10]. Inhibition of larval development of the copepod *Nitocra spinipes* as result of pharmacological-mediated toxicity has been reported [11].

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Slight toxic effects have been observed for aquatic invertebrate species such as the mussel *Lamplis cardium* [1] from which risk characterisation currently defined AHTN as slightly toxic to aquatic organisms [12]. Both anti-oestrogenic and oestrogenic effects have been reported for AHTN. An anti-oestrogenic effect has been observed in zebrafish mediated mainly by the oestrogenic receptor ERγ [13], whereas induction of Erα and vitellogenin gene expression has been reported in male *Oryzias latipes* at concentrations of 500 $\mu g \cdot L^{-1}$ [14].

Based on evidence of species-specific bioaccumulation of AHTN, species-dependent metabolism has been assumed [6,8].

To address AHTN cellular pathways of toxicity studies have investigated its interaction with cellular metabolisation/detoxification systems.

Interference towards the main detoxification system, cytochrome P450, has been reported at the gene and enzyme levels. Specific P450 activities involved in the biosynthesis of oestrogens and androgens, as well as in the detoxification of xenobiotics such as CYP1A, were modulated by AHTN at different levels. CYP3A, P450 aromatase, CYP17 and CYP11β were modulated in carp exposed *in vitro*, by contrast, CYP1A-dependent 7-ethoxyresorufin-*O*-deethylase (EROD) activity does not seem to be modulated significantly compared with nitromusks such as as musk xylene and musk ketone [15]. Finally, at the gene level, *cyp3a* was significantly upregulated, although only slight induction of the *cyp1a* gene has been observed in a rainbow trout gonad cell line (RTG-2) [16].

However, a clear AHTN detoxification pathway remains uncertain, as does its interaction with other aquatic pollutants known to be detoxified by the P450 system. Some studies indicate that the toxicity of this compound can be influenced by additive and synergistic effects with other toxic chemicals [17]. A potential indirect role for AHTN in the detoxification of toxic aquatic pollutants that are known inducers of CYP1A might be of particular interest because of AHTN's weak toxic properties but the significant concentrations reached in both abiotic and biotic matrices [7–9].

The aim of this study was to investigate the interaction of AHTN with the detoxification gene family CYP1A by looking at gene transcription and EROD activity in the *Poeciliopsis lucida* hepatoma cell line (PLHC-1). AHTN was tested alone and in combination with the classical CYP1A inducers planar 3,3′-4,4′,5-pentachlorobiphenyl (IUPAC N° PCB 126) and benzo[*a*]pyrene (B[*a*]P).

The PLHC-1 fish cell line is commonly used in ecotoxicology to investigate toxicological pathways of both classical and emerging pollutants, including pharmaceutical and personal care products to which AHTN belongs [18]. With regard to the detoxification pathway, PLHC-1 expresses the gene for the aryl hydrocarbon receptor AhR [19] and by virtue of its high capability to metabolise xenobiotics, CYP1A induction has been extensively reported in relation to B[*a*]P and polychlorobiphenyl exposure [20–22].

2. Materials and methods

2.1. *Cell culture*

PLHC-1 cells (ATCC; LGC Promochem, Teddington, UK) were kindly provided by Dr Tvrtko Smital (Ruder Boskovich Institute, Zagreb, Croatia). They were grown in 75-cm^2 plastic flasks (PBI International) at 30 °C in 20 mL Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 5% fetal calf serum (Invitrogen). Cells were subcultured every 4 days by detaching with 1 mL per flask of 1:4 trypsin–EDTA solution (Gibco). One day prior to the experiments, cells were seeded in 6-well (*cyp1a* gene analysis) or 96-well (cytotoxicity, EROD activity) cell culture microtitre plates.

2.2. *Neutral Red assay*

Cell viability was assayed using Neutral Red [23]. Cells were grown in medium (200 μL·well^{-1}) for 24 h at a density of 25 × 10^4 cells·mL^{-1}. One hundred microlitres of medium was then removed and replaced with the same volume of medium containing serial dilutions (range of AHTN 0.005–500 μM LGC standards), 1 nM of PCB 126 and 0.1–10 μM of B[*a*]P (Sigma-Aldrich), singly and combined with 2 μM AHTN, all dissolved in dimethylsylfoxide (DMSO; maximum concentration 0.1%). Medium-only exposed cells and DMSO-exposed cells were used as controls. The plates were incubated for another 24 h. The medium was then removed and the plates washed with 200 μL phosphate-buffered saline (PBS) with the addition of 100 μL Neutral Red medium, containing 40 μg·mL^{-1} Neutral Red dissolved in DMEM. Cells were incubated for 2 h under culture conditions. The medium was then removed and the cells washed with 150 μL PBS. Then 100 μL Neutral Red destain solution (50% ethanol, 49% deionised water, 1% glacial acetic acid) was added and the plate was shaken for 10 min. The optical density (OD) of the Neutral Red extract was measured at 540 nm using a Victor3 microplate reader (Wallak).

2.3. *RNA extraction and Q-PCR*

Cells were grown in 4 mL·well^{-1} of medium for 24 h at a density of 45 × 10^4 cells·mL^{-1}. Two millilitres of medium was then removed and replaced with 2 mL of medium containing the tested chemicals at the following concentrations: for single exposure experiments, 1 nM, 10 nM and 2 μM AHTN, 1 nM PCB 126 and at 2 μM B[*a*]P; and for co-exposure experiments, 2 μM AHTN was combined with 1 nM PCB 126 or 2 μM B[*a*]P. All chemicals in single and combined exposures were dissolved in DMSO (0.1%). The plates were then incubated for 6 and 24 h. The medium was discarded and the plates washed twice with 1.2 mL PBS. Cells were recovered with a cell scraper and centrifuged for 10 min at 1200 ***g***. Pellets were stored at −80 °C.

Total RNA was isolated using a Qiagen RNeasy MiniKit (Qiagen) according to the manufacturer's protocol. RNA was treated with DNase from RNase-Free DNase set (Qiagen) to avoid any traces of genomic DNA. RNA concentrations were measured using a Shimadzu spectrophotometer at 260 nM. RNA quality was confirmed on a 1% agarose gel that showed discrete 18S and 28S rRNA bands. Total RNA (250 ng) was transcribed to cDNA using I-script cDNA Reverse Transcription Kit (BioRad).

Real-time PCR (q-PCR) was used to evaluate *cyp1a* gene expression, as described previously [24]. Specific primers were designed using IDTDNA (www.idtdna.com). 18S rRNA was used as a housekeeping gene based on high stability. Primer sequences for PLHC-1 *r18S* were kindly provided by Jovica Loncar (Ruder Boskovic Institute, Zagreb, Croatia).
cyp1a Fw: 5′-GCATTTGGCGTGCTCGAAGAAA-3′;
Rev 5′-TTGCAGATGTGCTCCTCCAACA-3′

$$\text{Efficiency curve } y = -3.294 \text{ Log } (x) + 33.29 \text{ E} = 101.2\% \; r^2 = 0.993$$

18S Fw: 5′-CCTTTAACGAGGATCCATTGGA-3′;
Rev 5′-CGAGCTTTTTAACTGCAGCAACT-3′

$$\text{Efficiency curve } y = -3.572 \text{ Log } (x) + 15.98 \text{ E} = 90.5\% \; r^2 = 0.993$$

q-PCR was performed using a Stratagene Mx 3000 P thermal cycler. Each amplification reaction contained 12.5 μL SYBR green mix (BioRad), 1 μL cDNA and 0.75 μL forward and reverse primers at 10 μM, in 25 μL total volume. The cycling parameters were: 3 min denaturing at 95 °C, 40 cycles at 95 °C for 15 s, annealing at 55 °C for 45 s, and elongation at 72 °C for 1 min. All primer pairs gave a single dissociation peak in all reactions, and no amplification occurred in

reactions without template. The amplification products were run on 1% agarose gel stained with ethidium bromide (Sigma Aldrich) to verify the size and PCR specificity. Data were analysed by the $\Delta\Delta$Ct method [25].

2.4. *EROD activity*

EROD activity was assayed using the microplate method, as reported previously [26]. Cells were grown in 200 μL·well^{-1} of medium for 24 h at a density of 45×10^4 cells·mL^{-1}. One hundred microlitres of medium was then removed and replaced with same volume of tested chemicals at the following final concentrations: 0.2 μM, 2 μM and 4 μM AHTN, 1 nM PCB 126 and 0.2 μM B[*a*]P. For co-exposure experiments 0.2 μM AHTN was combined with 1 nM PCB 126 and 0.2 μM B[*a*]P. All were dissolved in DMSO (maximum concentration 0.1%). Cells were incubated for 6 or 24 h and then washed with 200 μL PBS followed by the addition of 100 μL of 2 μM 7-ethoxyresorufin (7-ER) dissolved in phosphate buffer pH 8.0. The kinetics of resorufin formation were monitored for 10 min using a Victor3 microplate reader (Wallak) at $\lambda_{Excitation} = 530$ nm and $\lambda_{Emission} = 590$ nm. Protein content was quantified by photometric assay [27] using bovine serum albumin as the standard (0–0.5 mg·L^{-1}, $r^2 = 0.9728$).

2.5. *Data analysis*

Each experiment was performed at least three times in triplicates (three wells/concentrations). Untreated cells and cells with DMSO were used as controls. Comparison among treatments was made by the non-parametric Mann–Whitney–Wilcoxon rank sum test, taking $p = 0.05$ as the significance cut-off. Concerning cytotoxicity, the EC_{50} of AHTN was calculated by fitting the OD of a typical experiment to a classical sigmoidal dose–response model. Data were evaluated graphically and statistically using GraphPad Prism5 software.

3. Results

3.1. *Cytotoxicity*

At 24 h, AHTN caused a reduction in cell viability with an LC_{50} of 35.76 μM. Concentrations of AHTN <10 μM were non-toxic (see Supplementary materials, available online only). No cytotoxic effects have been observed for the tested concentrations of 1 nM PCB 126 and up to 10 μM B[*a*]P both singly and in combination with AHTN (see Supplementary materials, available online only).

3.2. *Effects on CYP1A*

After 6 h of exposure, AHTN causes a dose-dependent slight induction of *cyp1a* gene transcription compared with controls (2.3 ± 0.07-fold at 2 μM) (Figure 1). After 24 h, no differences in *cyp1a* gene transcription were observed (Figure 1).

Regarding the two CYP1A inducers, both PCB 126 and B[*a*]P caused a significant induction of *cyp1a* transcription at 6 and 24 h, higher at 6 h for PCB 126 than at 24 h, and the opposite for B[*a*]P (Figure 2).

Co-exposure experiments at 6 h showed that AHTN caused a significant reduction in *cyp1a* transcription levels as 45% for PCB 126 and at 40% for B[*a*]P compared with single inducers exposure. No significant reduction was observed at 24 h (Figure 2).

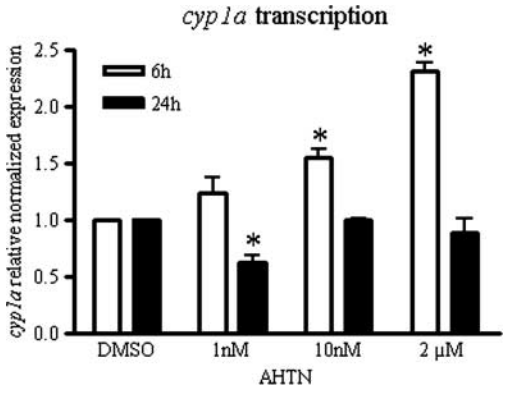

Figure 1. Transcription levels of *cyp*1*a*/*18S* in PLHC-1, exposed for 6 and 24 h to tonalide (AHTN), with respect to control (dimethylsufoxide; DMSO). Results are mean ± SD ($N = 3$).
*Significant difference with respect to control, $p < 0.05$.

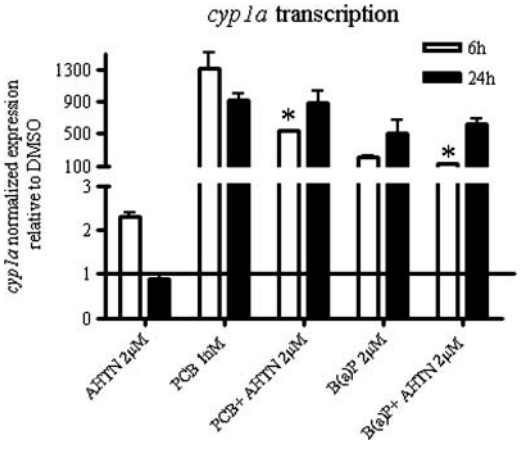

Figure 2. Transcription levels of *cyp*1*a*/*18S* in PLHC-1, exposed for 6 and 24 h to tonlide (AHTN), 3,3′-4,4′,5-pentachlorobiphenyl (PCB 126) and benzo(a)pyrene [B(a)P] alone and in combination, with respect to control (dimethylsufoxide; DMSO). Results are mean ± SD ($N = 3$).
*Significant difference of the pollutant alone with respect to the combination, $p < 0.05$.

At the enzyme level, AHTN caused a slight increase in EROD activity at both 6 and 24 h, with a maximum at the highest concentrations (2–4 µM) (Table 1). Both CYP1A inducers significantly increased EROD activity at 6 and 24 h, with the activity being higher for PCB 126 (1 nM) than for B[*a*]P (0.2 µM) (Table 2).

Table 1. 7-Ethoxyresorufin-*O*-deethylase (EROD) activity in PLHC-1 exposed to tonalide (AHTN) for 6 and 24 h.

	EROD activity	
	6 h	24 h
Untreated	1.78 ± 0.62	1.42 ± 0.82
Dimethylsulfoxide	1.90 ± 0.39	0.93 ± 0.34
AHTN 0.2 µM	1.69 ± 0.16	1.93 ± 0.17
AHTN 2 µM	2.32 ± 0.79	2.18 ± 0.01
AHTN 4 µM	3.23 ± 1.76	3.40 ± 1.16

Note: Values are given as pmol·min^{-1}·mg protein^{-1} ± SD of at least three different experiments in triplicate.

Table 2. 7-Ethoxyresorufin-*O*-deethylase (EROD) activity in PLHC-1 upon exposure to 3,3′-4,4′,5-pentachlorobiphenyl (PCB 126; 1 nM) and benzo[*a*]pyrene (B[*a*]P; 0.2 μM) singly and in combination with tonalide (AHTN; 0.2 μM) at 6 and 24 h.

	EROD activity	
	6 h	24 h
Untreated	1.78 ± 0.62	1.42 ± 0.82
Dimethylsulfoxide	1.90 ± 0.39	0.93 ± 0.34
PCB 126	54.23 ± 3.36	123.31 ± 17.97
B[*a*]P	27.99 ± 0.8	86.88 ± 39.94
PCB 126 + AHTN	51.86 ± 2.94	118.52 ± 14.59
B[*a*]P + AHTN	28.81 ± 4.76	67.12 ± 5.31

Note: Values are given as pmol·min^{-1}·mg protein^{-1} ± SD of at least three different experiments in triplicate.

Co-exposure did not affect EROD activity in comparison with exposure to single inducers (Table 2).

4. Discussion

The aim of this study was to investigate the interaction of AHTN with the CYP1A family by looking at gene transcription and EROD activity in an immortalised cell line, PLHC-1.

Regarding cytotoxicity, AHTN was one order of magnitude more toxic than the other musk fragrances tested, such as musk xylene (MX) for PLHC-1 (LC_{50} = 35.76 and 123.6 μM, respectively) [24]. The same difference was also observed in another cell line, rainbow trout liver RTL-W1 [17], confirming the highest AHTN cytotoxicity observed among musks. In Europe, use of AHTN is higher than the use of nitromusks such as MX, at 358 t per year of AHTN compared with 67 t of MX (OSPAR data for 2000) [28]. Cytotoxic effects in cell lines often correlate with *in vivo* acute fish toxicity [18]. More caution in the use of AHTN in household and personal care products might thus be recommended based on the potential adverse effects at higher biological levels.

The limited involvement of CYP1A in AHTN metabolism in fish reported to date seems to be confirmed by the slight modulation of *cyp1a* gene transcription observed with AHTN in PLHC-1.

The significant reduction in short-term co-exposure with PCB 126 and B[*a*]P (6 h; 45–40%) suggests an indirect effect of AHTN towards CYP1A. AHTN does not appear to act on basal levels of *cyp1a* transcription, but is able to significantly reduce the induction caused by PCB 126 and B[*a*]P. With regard to EROD activity, no interaction of AHTN is observed at 6 or 24 h, with either single or co-exposure. Consequently, the alteration at the transcriptional level by AHTN does not seem to interfere with this CYP1A-related enzyme. This is in agreement with reports in other aquatic species such as carp. A greater ability of nitromusks to interact with CYP1A compared with polycyclic musks has been observed [15]. AHTN showed only slight inhibition of EROD activity compared with other musks. Again this inhibition has been reported in midge larvae (*Chironomus riparius*) [29]. In the same study, upon exposure to piperonyl butoxyde, a CYP450 inhibitor, greater AHTN bioaccumulation was observed, suggesting the involvement of other enzymes in AHTN metabolisation.

Moreover, in rat microsomes, AHTN seems not to alter EROD activity [30], thus confirming the hypothesis that it is not metabolised by CYP1A.

Based on the overall data at both the gene and enzyme level, a possible role of AHTN as a competitive antagonist of the AhR can be thus hypothesised.

Other environmental contaminants have shown similar behaviour in co-exposure experiments, for example, the polybrominated diphenyl ethers PBDE 85 and PCB 105 and PCB 128 [31,32]. A competitive antagonist role towards AhR has been hypothesised for PBDE 85 based on the results of a co-exposure experiment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. In agreement our observations for AHTN, PBDE 85, despite its relatively high affinity for AhR, does not bind to the receptor, resulting in only weak induction of EROD [31].

Also PCB 105 and PCB 128 do not induce EROD activity or CYP1A protein expression, but in a co-exposure experiment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, they inhibit specific binding of this compound to AhR [32].

Concerning the complete recovery in *cyp1a* transcription by AHTN, a possible explanation could be complete metabolism of the compound. In fish, AHTN is metabolised at a turnover rate of approximately one third per day, and is excreted in the form of metabolites [33]. Nevertheless, this hypothesis needs further confirmation, because information on the metabolism and the behaviour of AHTN metabolites remains scarce.

The sensitivity of the PLHC-1 CYP1A system to classical CYP1A inducers is confirmed by the significant induction of *cyp1a* transcription and EROD observed with PCB 126 (1 nM) and B[*a*]P (0.2 and 2 μM for gene and enzyme, respectively). The two compounds strongly induce the transcription of *cyp1a* and EROD activity, as shown in previous studies [18,20–22]. Induction of *cyp1a* transcription by PCB 126 and B[*a*]P follows an opposite trend as a function of time. B[*a*]P causes an increase in transcription between 6 and 24 h, whereas for PCB 126, transcription decreases over time. A hypothesis of different kinetics for the two inducers at the selected doses has been confirmed [20].

5. Conclusions

This study provides information on the interaction of AHTN with the CYP1A system in fish, in both single exposure and co-exposure with classical aquatic pollutants and potent CYP1A inducers. Despite this, AHTN seems not to interfere significantly with CYP1A at the basal level; on co-exposure with inducers, some indirect effects are evident in the short-term because AHTN significantly reduces the repsonse of CYP1A to them. The reduced responsiveness of CYP1A to toxic inducers might increases their bioaccumulation and toxicity. Therefore, under conditions of co-exposure, unexpected adverse effects might occur, especially in areas chemically impacted by a mixture of toxic pollutants. The use of co-exposure experiments proved a great investigative tool to clarify the interaction and behaviour of emerging contaminants with respect to important cellular defence systems.

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