Enantioselective effects of alpha-hexachlorocyclohexane (HCH) isomers on androgen receptor activity in vitro

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1. Introduction

Various pesticides are used in the agriculture around the world along with increasing demands of food. The public and government regulators continue to be concerned about the potential hazards posed to the health of wildlife and humans. Some of the most toxic and persistent organochlorine pesticides (OCPs) are no longer used in most developed countries following the ratification of the Stockholm Convention of the United Nations. However, these compounds are still used in some countries especially for the control of malaria and insecticides. After replacement of technical HCH with pure lindane during the 1960s, concentrations of \( \alpha \)-HCH and \( \beta \)-HCH, both of which are more persistent than lindane and inert as insecticides, were expected to decrease but experimental studies demonstrated higher stability.
of the α-isomer compared to γ-HCH, (Shen et al., 2004). But even the long half-life of the α-isomer does not provide sufficient explanation for its current high environmental concentrations, and existence of secondary sources of α-HCH has been proposed (Malaiyandi and Shah, 1984; Iwata et al., 1994).

From the toxicological point of view, α-HCH is the least explored isomer from all HCH isomers (Willet et al., 1998). It is considered to affect the central nervous system (Willet et al., 1998) but unlike lindane it has no or little effect on the gamma-aminobutyric acid GABA receptor (Nagata and Narahashi, 1995). α-HCH was also reported to cause liver cancer in mice and rats (Itō et al., 1975). Concerning the endocrine disruption, in vitro inhibitive effect of α-HCH on activated androgen receptor was reported (Schrader and Cooke, 2000). Specifically, α-HCH was shown to antagonize the androgen receptor (AR)-mediated effects of the natural ligand dihydrotestosterone, DHT (Roy et al., 2004). During the synthesis, two α-HCH enantiomers are formed as a racemic mixture (Willet et al., 1998) but to our knowledge all previous studies kept the 50% of α-HCH as a racemate, and toxicity of individual enantiomers has not been explored.

The present research aimed to study possible effects of isolated α-HCH enantiomers towards androgen receptor. The enantiomers were separated and concentrated using the semi-preparative HPLC and the relative potencies to interact with AR were determined (i) experimentally using the in vitro MDA-kb2 reporter gene assay, and by (ii) molecular modeling of interactions between HCH and AR.

2. Materials and methods

2.1. Chemicals

α-HCH (99% purity) was provided by Zbyněk Prokop and Jiri Damborsky (Loschmidt laboratories, Faculty of Science, Masaryk University, Brno, Czech Republic). 5α-Androstan-17β-ol-3-one (synonym 4,5α-dihydrotestosterone, DHT; CAS number 521-18-6, purity ≥99.0%) was purchased from Sigma–Aldrich. Other chemicals, solvents (the highest possible purity) and the components of the cell culture media were purchased from Sigma–Aldrich unless stated otherwise.

2.2. HPLC separation of the α-HCH enantiomers

Separation of the α-HCH enantiomers was performed using an Agilent 1100 series Chromatograph equipped with a UV–VIS diode array detector. Several chiral HPLC columns were tested and the best separation was achieved by using a CHIRALCEL OD-H column (cellulose tris-3,5-dimethylphenyl carbamate, 150 × 2.1 mm; Chiral Technologies Europe, 67404 Ilkirch – Cedex, FRANCE). Pure hexane (Pestanal, Fluka; for residual analysis) was used as a mobile phase (flow rate of 250 μL min⁻¹). Temperature of the column and the collector was kept at 25 °C; the analyses were detected at λ = 210 and 450 nm. After separation of the enantiomers, the solvent was evaporated, and individual enantiomers were dissolved in hexane. Concentrations were confirmed by external calibration curves. For toxicity testing, the enantiomers were dissolved in dimethylsulfoxide (DMSO; 10 mM stock solutions), a nontoxic solvent often used in biological studies.

2.3. Cell culture

The effects towards AR were tested using the human breast carcinoma cell line MDA-kb2 stably transfected with the luciferase gene under the control of AR (Wilson et al., 2002). The cell line was routinely cultured in L-15 Leibovitz medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator under atmospheric conditions (no external addition of CO₂). Before the experiment, cells were trypsinized, mixed with L-15 Leibovitz medium supplemented with 10% dialyzed FBS (serum steroids removed) and seeded into 96-well plates at a density of 10000 cells well⁻¹. After the 24-h pre-incubation, cells were exposed in three replicates to the solvent DMSO, 1 mM DHT (positive control) or a range of HCH concentrations (either without or in the presence of DHT). Maximum DMSO concentration in the test system was 1% v/v and it had no effect on the cell viability or reporter luciferase expression. After 24-h exposure, the medium was removed, the cells were washed with the phosphate-buffered saline (PBS), lysed for 30 min at room temperature by addition of 25 μL of lysing buffer per well (Promega E1531). Luminescence (activity of the reporter luciferase) was measured using the flash mode with a multivell plate reader (Luminoscan Ascent, Thermofisher Scientific Inc., Waltham, MA, USA) by use of a luciferase assay reagent injected to a dispenser to each well just before luminescence measurement. Luciferase assay reagent consisted of 20 mM Tricine, 1.07 mM Mg(NO₃)₂Mg(OH)₂, 2.67 mM MgSO₄·7H₂O, 0.1 mM EDTA disodium salt, 33.3 mM dithiothreitol, 270 μM of coenzyme A, 470 μM luciferin, and 530 μM of ATP in redistilled water, pH = 7.8. Viability of cells was determined using a neutral red method (Freyberger and Schmuck, 2005; Benisek et al., 2008). Neutral red (0.5 mg mL⁻¹) was added to each well and the microplate was incubated at 37 °C for 1 h. Medium was then removed, cells lysed with 1% acetic acid in 50% ethanol and absorbance at 570 nm was measured (only viable cells accumulated neutral red). Effects of both enantiomers and the racemic HCH mixture were tested in three independent experiments and each exposure variant was tested in three replicated wells. Results are presented as means ± SEM of N = 3 independent experiments.

2.4. Molecular modeling of the HCH binding to AR

The binding affinity of HCH to AR was studied by molecular modeling using an AutoDock software – file PDB ID: 2Q7I. This file contains ligand binding domain (LBD) of the AR, activation function 2 (AF2) and testosterone. Various isomers of HCH were docked into 2Q7I, and binding affinities were calculated either in the presence or the absence of AF2 and testosterone. Inside docking calculations, MMFF94 force field (Halgren, 1996) was used for energy minimization of ligand molecules (HCH). Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. This type of docking is referred to as a rigid body docking. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added. Affinity (grid) maps of 70 × 70 × 70 Å grid points and 0.375 Å spacing were generated using the Autogrid program (Morris et al., 1998). Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method (Solis and Wets, 1981). Each docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250 000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied.

2.5. Statistics

Responses of treatments and controls were compared using the one-way analysis of variance (ANOVA) followed by the Dunnet’s multiple range test. Differences between the effects of individual enantiomers used at the same concentrations were analyzed by Student’s t-test. For all statistics, p-values less than 0.05 were considered statistically significant. Calculations were performed in Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA).
3. Results and discussion

The separation of $\alpha$-HCH enantiomers performed in this study was inspired by a previously suggested protocol (Champion et al., 2004), in which a chiral column CHIRALCEL OJ was used. For separation we used a chiral column (Vetter et al., 1997; Jantunen and Bidleman, 1998) rather than enantioselective capillaries (Wiberg et al., 1998). Several chiral columns were tested in our experiments for their ability to separate enantiomers and the optimal separation was obtained with the CHIRALCEL OD-H column (Fig. 1) using experimental conditions described in Section 2. Since we were not able to fully determine the absolute configuration of the separated enantiomers, they are further labeled En1 and En2 (enantiomers 1 and 2) considering their retention time from the column.

Environmental levels, degradation and changes in the enantiomeric ratio of HCH enantiomers has been subject of several research papers (Klobes et al., 1998; Covaci et al., 2004; Muller and Kohler, 2004) but to our knowledge, this is the first study, which attempted to investigate biological effects of isolated $\alpha$-HCH enantiomers. First, the activity of the racemic $\alpha$-HCH towards the AR was tested using the MDA-kb2 cellular reporter gene assay. No direct activation of the AR by $\alpha$-HCH was found up to 50 $\mu$M (data not shown), which corresponded to the previously reported results (Roy et al., 2004). Interaction of $\alpha$-HCH with DHT, the natural ligand of AR, was then studied. Selected 1 nM DHT concentration caused approximately 50% luciferase induction (Fig. 2a), which allowed assessment of both stimulatory and inhibitory effects of $\alpha$-HCH. As shown in Fig. 2b, racemic $\alpha$-HCH significantly suppressed the AR activation by DHT in a concentration-dependent manner and two higher $\alpha$-HCH concentrations (10 $\mu$M and 50 $\mu$M) caused complete inhibition of reporter luciferase. No significant cytotoxicity was detected after exposures to $\alpha$-HCH alone or in combination with DHT (Fig. 2b; circle symbols).

Pronounced anti-androgenic effects of individual $\alpha$-HCH enantiomers En1 and En2 on the DHT-induced AR activation are shown in Fig. 3. While En1 suppressed the AR activity within the same concentrations as racemate (10–50 $\mu$M), En2 appeared to be more effective at lower 2 $\mu$M concentration. On the other hand variable responses were observed at higher concentrations with partial recovery at 50 $\mu$M, which was confirmed by repeated experiments (Fig. 3).

The original hypothesis tested in our experiments considered possible interference of $\alpha$-HCH and DHT with the binding site at AR. Molecular modeling results (Table 1) suggested that most HCH isomers may directly interact with the AR binding site. However, the frequency of successful docking for $\alpha$-HCH enantiomers appeared to be greater (50–60%) than for other HCH.
diastereomers, which may partially explain the α-HCH anti-androgenicity observed in the present work and previously (Roy et al., 2004). Interestingly, molecular modeling also suggested weak but still significant differences between the estimated free energy of binding to the AR of two α-HCH enantiomers (Table 1), which could explain slight differences between enantiomers observed in vitro (Fig. 3).

Nevertheless, α-HCH could also affect various upstream signaling events, which control the AR action. Some of the known upstream AR regulators are for example protein kinase A (Nazareth and Weigel, 1996), mitogen-activated protein kinases MAPKs, and phosphatidylinositol 3-kinase/Akt signaling (Jia et al., 2004). Recently, the controlling role of other less explored co-repressors/co-activators has been described (Askew et al., 2009). Modulations of these regulatory pathways by persistent organic compounds have been documented at pesticides (Tessier and Matsumura, 2015) and significant role of the up-stream regulators has been discussed (Benisek et al., 2008, 2011). More research efforts are thus needed to fully understand cellular toxicity mechanisms of organic toxicants including α-HCH.

In summary, various insecticides were shown to have enantioselective effects related to their major targets such as acetylcholinesterase including – for example – isocarbophos (Lin et al., 2008), fipronil (Wilson et al., 2008), chloramidophos (Zhou et al., 2007) or acetophenate (Xu et al., 2008). Recently, enantioselective effects towards non-target cellular regulators such as estrogen receptor has been documented for α,p’-DDT (Wang et al., 2009) and bifenthrin (Wang et al., 2007). Our results for the first time demonstrate anti-androgenic action of different α-HCH stereoisomers. Although the original hypothesis expected pronounced differences between the two α-HCH isomers, we were able to demonstrate rather minor – but still significant – differences. Molecular modeling suggested that α-HCH interaction with AR could be responsible for the effects but several up-stream regulatory events could also be affected. Further research should address both in vivo relevance and molecular mechanisms of α-HCH anti-androgenicity with special respect to its high environmental persistence.

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References


Table 1

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Est. free energy of binding (kcal mol⁻¹)</th>
<th>Est. inhibition constant, Kᵢ (µM)</th>
<th>Electrostatic energy (kcal mol⁻¹)</th>
<th>Total intermolec. energy (kcal mol⁻¹)</th>
<th>Frequency (%)</th>
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<td>5.86</td>
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<td>54.46</td>
<td>5.46</td>
<td>10.14</td>
<td>5.70</td>
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a Testosterone (TES) binding energy to androgen receptor in the presence of AF2.