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

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ABSTRACT

Alpha-hexachlorocyclohexane (alpha-HCH), a part of the HCH pesticide mixture, is one of the most widespread persistent organic pollutants. Interestingly, only limited number of studies addressed the toxicity of alpha-HCH and the effects of its individual optical isomers have not been investigated in detail. In the present study we separated two alpha-HCH enantiomers by preparative HPLC and studied their activities towards androgen receptor (AR) using the MDA-kb2 cell line stably transfected with the luciferase reporter gene under the control of AR. There was no direct effect of alpha-HCH on AR but both isomers significantly suppressed the activity of AR in co-exposure with the natural ligand dihydrotestosterone in a concentration-dependent manner. One of the enantiomers appeared to be more active at lower concentration, which was also supported by the molecular modeling calculations with AR that showed a slight difference in estimated free energy of binding and inhibition constant between two enantiomers. Although studies with other pesticides demonstrated strong enantioselective differences in toxicity, the present research shows rather minor differences in modulations of AR by both alpha-HCH enantiomers. For the first time, enantioselective effects of alpha-HCH were demonstrated and the results suggest interaction with multiple regulatory events controlling the AR activity. Full elucidation of the toxicity mechanism will require further research.

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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 other endemic diseases (Maffei et al., 2009; Mansour, 2009). OCPs are persistent in the environment with half-lives ranging from months to years and may accumulate to the levels causing adverse effects in animals (Mingelgrin and Nasser, 2006).

Chirality is an attribute of natural as well as anthropogenic compounds including pesticides (Muller and Kohler, 2004; Liu et al., 2005). Synthesis of pesticides containing stereogenic center usually results in a mixture of stereoisomers. Once they are released into the environment, the enantiomeric ratio may be changed by the enantiospecific or –selective breakdown (Muller and Kohler, 2004; Smith, 2009). Although the different effects of enantiomers are known at some pesticides, there is still a lack of information about their toxicity and fate in the environment (Muller and Kohler, 2004; Smith, 2009).

Hexachlorocyclohexane (HCH) has been one of the most frequently used organochlorine pesticides after the World War II. Originally, HCH was used as a technical mixture of six isomers, containing approximately 10% of the effective pesticide γ-HCH (lindane). The mixture was composed primarily of α-HCH and β-HCH, both of which are more persistent than lindane and inert as insecticides. After replacement of technical HCH with pure lindane during the 1960s, concentrations of α-HCH were expected to decrease but experimental studies demonstrated higher stability

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of the alpha-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 (Willett et al., 1998). It is considered to affect the central nervous system (Willett 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 (Ito 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 (Willett et al., 1998) but to our knowledge all previous studies addressed the toxicity 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 Zbynek 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 Illkirch – 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 analytes 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 CO_2). 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 nM 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 multiwell plate reader (Luminoscan Ascent, Thermo Fisher Scientific Inc., Waltham, MA, USA) by use of a luciferase assay reagent injected by a dispenser to each well just before luminescene measurement. Luciferase assay reagent consisted of 20 mM Tricine, 1.07 mM $Mg(CO_3)Mg(OH)_2$, 2.67 mM $MgSO_4{\cdot}7H_2O$, 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 250000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quarternion 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).

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