CONTRIBUTION OF ORGANOCHLORINE PESTICIDES AND PCBS AS ANDROGEN RECEPTOR ANTAGONISTS INACID-TREATED - LIVER EXTRACTS OF HIGHER TROPHIC WILD ANIMALS

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Introduction

Feminization has been observed in wild animals upon exposure to various persistent organic pollutants (POPs). Organochlorine pesticides (OCPs) such as *p,p*'-DDE were found to affect male reproductive organs of alligators (*Alligator mississippiensis*) in Apopka Lake, USA [1] and *p,p*'-DDE and PCBs were also shown to be related to the decrease of testosterone concentration in the blood of Dall's porpoises (*Phocoenoides dalli*) [2]. However, only very few studies clearly showed the relationship between androgen receptor (AR) antagonistic activity and accumulated chemicals in wild animals.

Our previous study evaluated various endocrine-disrupting activities in pooled sulfuric acid-treated extracts of tissues from several high-trophic wild animal species using cell-based Chemically Activated Luciferase eXpression (CALUX) bioassays and detected potent AR antagonistic activities for Baikal seal (*Phoca sibirica*) but not for finless porpoise, raccoon dog and common cormorant [3]. Toxicity identification and evaluation (TIE) approach using AR-CALUX assay and chemical analysis showed that *p,p*'-DDE is an important contributor to AR antagonistic activity in Baikal seals. In this study, in order to generalize the results from the previous screening study and clarify the activity profiles of AR antagonists for a wider variety of wild animals, TIE was conducted on sulfuric acid-treated liver samples from seven high-trophic species, in view of (a) total AR antagonistic acitivities, (b) concentration of OCPs (DDTs, CHLs, HCHs etc.) and PCBs commonly found accumulated in many species [4,5], and (c) the contributions of these pollutants to the detected AR antagonistic activities.

Materials and methods

Sample collection

Livers of male specimens from seven animal species (striped dolphin (*Stenella coeruleoalba*) from Seto Inland Sea in 2003 (n = 5) [4], Stejneger's beaked whale (*Mesoplodon stejnegeri*) from Japan Sea in 2000-2003 (n = 6), finless porpoise (*Neophocaena phocaenoides*) from Omura Bay in 2005-2007 (n = 5), raccoon dog (*Nyctereutes procyonoides*) from Kanagawa in 2001 (n = 5) [5], common cormorant (*Phalacrocorax carbo*) from Biwa Lake in 2003 (n = 6), golden eagle (*Aquila chrysaetos*) from Iwate in 1993 (n = 1) and Steller's sea eagle (*Haliaeetus pelagicus*) from Hokkaido in 1999 (n = 1)) were collected. The samples were stored at -25 ºC until analysis.

Pretreatment of samples

Liver sample (11 g) was freeze-dried and extracted with acetone/hexane (1:1) in a high speed solvent extractor SE-100 (Mitsubishi Chemical Analytech., Japan). The extract was solvent-exchanged into 10 mL of hexane. A 4-mL portion of the solution was treated with sulfuric acid silica gel chromatography. Then the solvent was evaporated with Kuderna-Danish concentrator and with N_2 gas. One fourth of this concentrated solution was stored in 200 μL of hexane at 4 ºC until OCPs analysis, and the remaining three fourth was solvent-exchanged into 100 μL of DMSO and stored at 4 ºC until CALUX assay. For PCBs analysis, 5 mL of the crude extract was

treated using sulfric acid silica gel chromatography, gel permeation chromatography (GPC) and silica gel chromatography as the methods reported previously [4].

Instrumental analysis

GC-MS analyses for 19 OCPs —DDTs (*p,p*'-DDT, *p,p*'-DDD, *p,p*'-DDE, *o,p*'-DDT, *o,p*'-DDD, *o,p*'-DDE), CHLs (*trans*- and *cis*-chlordane, *trans*- and *cis*-nonachlor, oxychlordane), HCHs (α-, β-, γ-, δ-HCHs), HCB, methoxychlor, heptachlor and mirex— were performed using DB-17ms capillary column (J&W Scientific Inc.) in a GC6890-5973N system (Agilent Technologies Inc.). MS analyses were carried out in EI-SIM mode with ionization energy of 70 eV. PCBs analysis was conducted following procedures reported previously [4].

CALUX assay

AR antagonistic activity was evaluated with methods reported previously using U2OS-luc (human osteosarcoma) cells developed by BDS b.v. (Amsterdam, Netherlands) [3,6]. The cells were maintained in DMEM/F12 medium (1:1) with 7.5% FCS, plated for 1 day on 96-well microplates and exposed to DMEM/F12 medium (without phenol red) with 4.9% charcoal/dextran-treated FCS containing samples or chemicals in presence of EC_{50} level of dihydrotestosterone for 24 h. Thereafter cells were lysed, luciferin solution was added and luminescence intensity was measured. Sigmoid dose-response curves were made for chemicals by SigmaPlot 11.0 (Systat Software, Inc.). Extrapolation was done for compounds with relatively lower activity. The activities of the samples were expressed as Flutamide-equivalents. All measurements were done in 3 wells, and repeated 3 times at least. Cell viability was confirmed with MTT assay.

Results and discussion

AR antagonistic activities in wild animal liver extracts

AR antagonistic activities were detected in all the extracts from striped dolphin (*n*=5), Stejneger's beaked whale (*n*=6) and Steller's sea eagle (*n*=1) with means and ranges as: 38 (20-52), 47 (21-96) and 80 μg Flutamide-equivalents/g-lipid, respectively (Figure 1), while the activities were detected for limited individuals in finless porpoise (*n*=3), raccoon dog (*n*=3) and common cormorant (*n*=5).

Concentration of OCPs and PCBs in wild animal liver extracts

OCPs and PCBs were analyzed in individual liver extracts in which AR antagonistic activities could be detected. Among the target OCPs, *p,p*'-DDE had the highest concentrations in all the species except raccoon dog (Figure 2). Concentrations of *p,p*'-DDE in striped dolphin, Stejneger's beaked whale and Steller's sea eagle were higher than those in the other species [3-5]. Concentrations of *o,p*'-DDT and its metabolites were very low or not detectable. CHLs were the second most abundant group among the OCPs in marine species such as Steller's sea

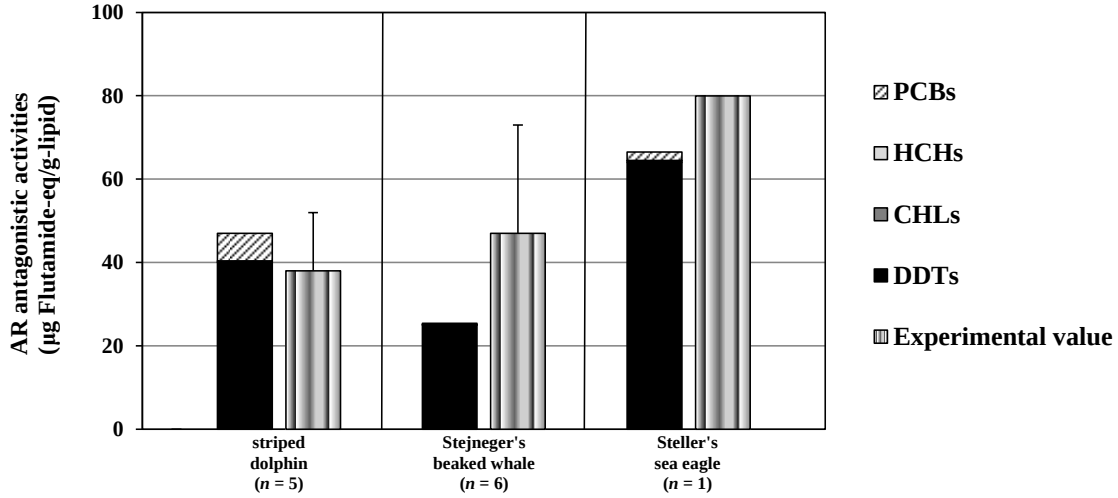

Figure 1. Experimental and theoretical values for AR antagonistic activities.

eagle, striped dolphin and finless porpoise, and had extremely high concentrations compared with the other compounds in raccoon dog [4,5]. Methoxychlor and heptachlor were not detected in all the species analyzed. Concentrations of mirex were comparable to those of HCB in cetaceans and raptors.

AR antagonistic potencies of OCPs and PCBs

AR-CALUX assay was carried out for 13 OCPs and 9 PCBs (Table 1). *p,p*'-DDE showed the highest potency. *p,p*'-DDD and -DDT have shown lower potencies than *p,p*'-DDE [7]. CHLs also exerted AR antagonists in order of *cis*- > *trans*-chlordane > oxychlordane > *cis*- > *trans*-nonachlor. Among HCHs, α- and β-HCHs showed higher potencies. The potencies of PCB128, -118, -138, -153 and -180 were high among target POPs, in agreement with the results from a previous study [6], whereas PCB99, -149, -187 and -183 were found for the first time to have relatively high potencies.

Contribution of OCPs and PCBs to AR antagonistic activities

The theoretical AR antagonistic activities in wild animal liver extracts, calculated based on the concentrations of representative OCPs and PCBs and their IC_{25}-based REP values, were compared with the experimental (CALUX-derived) values (Figure 1). The contribution of OCPs and PCBs to the experimental AR antagonistic activities was 124% and 83% for striped dolphin and Steller's sea eagle, respectively and the main contributor was *p,p*'-DDE. For Stejneger's beaked whale, finless porpoise and golden eagle, the contribution was 54%, 54% and 44%, respectively and *p,p*'-DDE was the major contributor. The contribution of PCBs was to some extent confirmed for striped dolphin and finless porpoise (17% for both species). For raccoon dog and common cormorant, most activities could not be explained by the target OCPs and PCBs (of which oxychlordane and *p,p*'-DDE was the most important contributor in each species). This result suggests that some other unknown compounds contribute to AR antagonistic activities in these higher trophic terrestrial animals.

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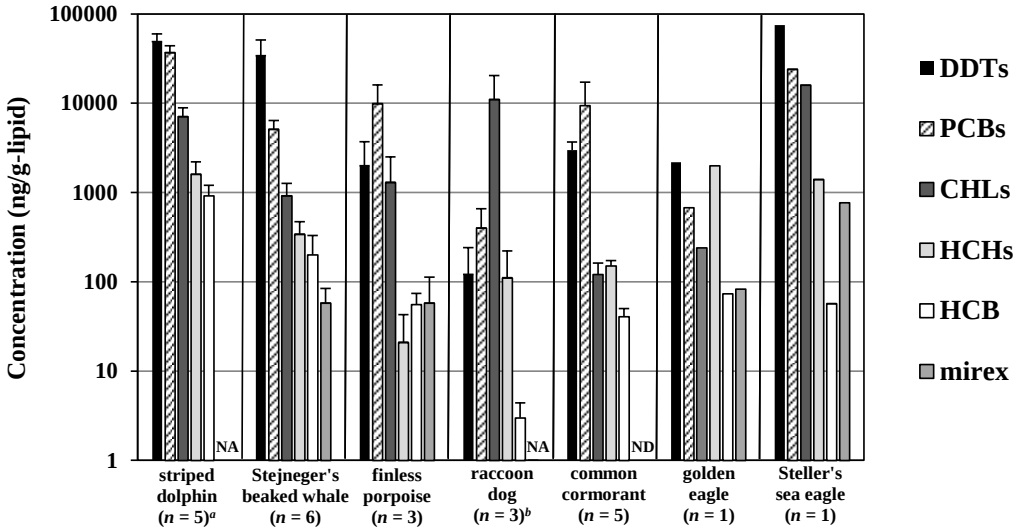

Figure 2. OCPs and PCBs concentration in male liver extracts of seven wild animal species (NA: not available, ND: not detactable, *a*: cited from ref [4], *b*: cited from ref [5]).

Table 1. AR antagonistic REP values for OCPs and PCBs[a].

	AR antagonistic REP (weight base) IC_{25}		AR antagonistic REP (weight base) IC_{25}
Standard			
Flutamide	1		
OCPs		PCBs	
p,*p*'-DDT	0.19	PCB99	0.16
p,*p*'-DDD	0.28	PCB118	0.31
p,*p*'-DDE	0.87	PCB128	0.84
trans-Chlordane	0.074	PCB138	0.15
cis-Chlordane	0.19	PCB149	0.11
trans-Nonachlor	0.015	PCB153	0.047
cis-Nonachlor	0.024	PCB180	0.034
Oxychlordane	0.048	PCB183	0.060
α-HCH	0.15	PCB187	0.077
β-HCH	0.10		
γ-HCH	0.057		
HCB	0.057		
Mirex	-[b]		

a. $n = 3$ for target chemicals except HCB. $n = 1$ for HCB.
b. no activity.

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