DISRUPTION OF OXIDATIVE PHOSPHORYLATION (OXPHOS) BY HYDROXYLATED POLYBROMINATED DIPHENYL ETHERS (OH-PBDEs)

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Introduction

In last years naturally produced hydroxylated polybrominated diphenyl ethers (OH-PBDEs) have been reported in several wildlife species from the Baltic Sea (e.g. in mussels, fish, birds and seals), for references see **Table 1**. OH-PBDEs are not known to be produced commercially or used in any industrial processes. Instead they are likely to originate from naturally production in the marine environment or formed via metabolic transformation of polybrominated diphenyl ethers (PBDEs)[1,2,3]. In the Baltic Sea both red, brown and green filamentous macro algae and cyanobacteria are considered to be primary producers of OH-PBDEs [4,5,6]. These algae species have benefitted by increased eutrophication of the Baltic Sea during the 20th century, which has favoured fast-growing and highly productive algae species [7]. Today, filter feeding bivalves in the Baltic Sea (e.g. blue mussels) are known to be highly exposed to naturally produced OH-PBDEs during the summer months [8]. Blue mussels are an important food source for several mussel feeding fish (e.g. flounder) and sea duck species (e.g. Long-tailed duck, Common Eider, Steller's Eider, Common Scoter and Velvet Scoter). During the last two decades these sea duck species have shown dramatic decline in population in the Baltic Sea [9]. Furthermore have increasing number of wild birds in the Baltic Sea area been reported to be dying from an idiopathic paralytic disease which has been linked to thiamine (vitamin B_1) deficiency [10]. Thiamine is an essential vitamin and its phosphate derivatives are involved in several cellular processes (e.g. carbohydrate and lipid metabolism) [11]. A low hatchability of herring egg [12] and reduced fat content and wasting syndrome have also been reported in marine species such as herring [13], guillemot [14] and grey seal [15] in the Baltic Sea. The underlying cause/s for these adverse effects is unknown, but within this project we hypothesize that exposure to halogenated phenolic compounds (e.g. OH-PBDEs) may contribute these health effects by affecting the energy metabolism via disruption of oxidative phosphorylation (OXPHOS).

OXPHOS is the main pathway for production of adenosine triphosphate (ATP) in eukaryotic cells and involves the electron transport chain (ETC) coupled with the ATP synthase. Since disruption of OXPHOS decrease the ATP production in the cell, exposure over a prolonged period of time may lead to altered energy metabolism followed by body weight loss [16,17,18]. In fish, exposure to certain environmental pollutants like POPs has been suggested to reduce the available levels of the phosphorylated form of thiamine, as a result of an increased energy metabolism [19]. In 2007, van Boxtel et al. reported 6-OH-BDE 47 to be a potent disrupter of mitochondrial oxidative phosphorylation (OXPHOS) and to be acutely toxic in developing and adult Zebrafish [20]. Our aim with this study was to determine if OH-PBDEs present in the Baltic marine environment are able to disrupt OXPHOS *in vitro*. For this purpose we used a well established method [21], measuring changes in the inner mitochondrial membrane potential and mitochondrial respiration in isolated rat liver mitochondrial with help of oxygen- and tetraphenyl phosphonium (TPP^+) sensitive electrodes. The mitochondrial tests were complemented with studies on whole cells using a novel *in vitro* bioassay with TMRM. TMRM is a fluorescent cationic lipophilic rhodamine dye, which accumulates within the mitochondrial matrix in proportion to the negative membrane potential of the mitochondria. Although, TMRM has been used in many studies, most of them are limited to work on isolated mitochondria [22,23]. However, it has been demonstrated that this assay can be applicable for screening of cell lines as well [24]. In addition to the OH-PBDEs identified in the Baltic marine environment, both *ortho*-, *meta*- and *para*-substituted OH-PBDEs were included in this study, trying to cover as many structural varieties as possible.

Materials and methods

Test compounds: Please note that all OH-PBDEs are here expressed as phenoxyphenols. 2,5-dibromo-4-(2,4-dibromophenoxy)phenol (4'-OH-BDE49), 2,6-dibromo-3-(2,4-dibromophenoxy)phenol (3-OH-BDE47), 4,6-dibromo-3-(2,4-dibromophenoxy)phenol (5-OH-BDE47), 3,5-dibromo-2-(2,4-dibromophenoxy)phenol (6-OH-BDE47), 3,6-dibromo-2-(2,4-dibromophenoxy)phenol (6'-OH-BDE49), 4,6-dibromo-2-(2,4-dibromophenoxy)phenol (2'-OH-BDE68), 3,4,5-tribromo-2-(2,4-dibromophenoxy)phenol (6-OH-BDE85), 3,4,6-tribromo-2-(2,4-dibromophenoxy)phenol (6-OH-BDE90), 3,5,6-tribromo-2-(2,4-dibromophenoxy)phenol (6-OH-BDE99), 4,5,6-tribromo-2-(2,4-dibromophenoxy)phenol (2-OH-BDE123), 3,4,5,6-tetrabromo-2-(2,4-dibromophenoxy)phenol (6-OH-BDE137), 4,6-dibromo-3-chloro-2-(2,4-dibromophenoxy)phenol (2'-OH-6'-Cl-BDE68), 3,5-dibromo-6-chloro-2-(2,4-dibromophenoxy)phenol (5-Cl-6-OH-BDE47) and 2,4-dibromo-1-(2,4-dibromophenoxy)-benzene (BDE-47) were synthesized in-house as described elsewhere [25,26]. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (≥98 % purity) and 3,5-di(tert-butyl)-4-hydroxybenzylidenemalononitrile (SF6847) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All test compounds were dissolved in dimethyl sulfoxide (DMSO). Tetramethylrhodamine methyl ester perchlorate (TMRM) (≥95% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Methods

The TPP assay

Measurement of the mitochondrial membrane potential and respiration was performed on isolated rat liver mitochondria. The experimental animals were sacrificed according to ethical procedures and the rat livers were immediately collected and kept in cold 0.9% $NaCl_{(aq)}$. The liver tissue was homogenized in isolation medium and the mitochondria were collected and prepared as described elsewhere [20]. The mitochondrial respiration and inner membrane potential were measured simultaneous by incubating the mitochondria in reaction medium in a closed and stirred acrylic glass vessel at 25 °C. The container was equipped with a Clarke-type oxygen electrode and a tetraphenylphosphonium ion (TPP^+) electrode coupled to a reference electrode. Coupling between respiration (O_2), membrane potential ($\Delta\psi$) and ATP synthesis in the isolated mitochondria was demonstrated by addition of succinate (1 mM) and ATP (10 mM) to initiate OXPHOS. Rotenon was added to block complex I (NADH-UQ reductase) of the ETC, which allows us to detect inhibitory effects on complex II (Succinate-Q oxidoreductase). All test compounds were added after the membrane potential was stable (i.e. when state-4 respiration had been reached) and each compound was measured at different concentrations.

The TMRM assay

The Zebrafish embryonic fibroplast (PAC2) cells used in the TMRM assay were cultured as described elsewhere [20]. The cells were plated in a 96-well black polystyrene plate with clear bottom at a density of 30,000 cells/well. After overnight incubation, the cells reached about 95-100% confluency. The TMRM solution was prepared by diluting TMRM in L-15 Leibovitz medium containing no phenol red. The TMRM solution (500 nM) was transferred to a 48 wells plate (1 ml/well) and the test compound was added. The cell culture media in the overnight cell culture was replaced with the same volume (100 µl) of test media and the cells were incubated at 28 °C for 45 min without CO_2 supplement. All exposure tests were carried out in triplicate and FCCP and BDE-47 were used as positive- and negative control respectively throughout the study, along with solvent blanks. After 45 minutes incubation, the exposure media was removed and the cells were washed with phosphate buffer saline (PBS). After washing, 150 µl PBS was added to each well and the TMRM fluorescence was measured immediately on the fluorescence reader (SpectraMax Gimini EM Fluorescence reader, Molecular Devices, CA, USA) at an excitation wavelength of 530 nm and emission wavelength of 580 nm using bottom read. The mean fluorescence was calculated from at least two independent experiments, each performed in triplicate.

Results and discussion

Results from the TPP and TMRM assay are presented **Table 1**. In the TPP assay using isolated mitochondria, all OH-PBDEs were found to disrupt OXPHOS either by protonophoric uncoupling and/or via inhibition of the ETC within the concentration range tested. The different modes of action were identified by protonophoric uncoupling causing a decrease in membrane potential and increase in respiration rate in a dose-dependent manner, whilst the inhibitory effect on the ETC, reduced the membrane potential and decreased the respiration rate. The classical disrupters of OXPHOS; FCCP and SF6847 which were included in this study as positive

controls were found to disrupt OXPHOS both via protonophoric uncoupling as well as via inhibition of the ETC. This double mode of action was also noted with the OH-PBDEs tested, except 6-OH-BDE47, 5-OH-BDE47 and 6-OH-BDE90 which were found to disrupt OXPHOS solely by inhibition of the ETC or by protonophoric uncoupling within the concentration range tested. The result that 6-OH-BDE47 act as an inhibitor is in agreement with the result presented by van Boxtel et al. (2007) which showed that 6-OH-BDE47 disrupts OXPHOS by inhibiting complex II in the ETC in Zebrafish mitochondria [20]. In the TMRM assay using whole cells, all OH-PBDEs tested except 3-OH-BDE47, 2'-OH-BDE68, 6'-OH-BDE49 and 4'-OH-BDE49 were found to cause a decrease in TMRM fluorescence (i.e. alter the inner mitochondrial membrane potential (MMP)) in a dose-dependent manner. BDE-47 was included in both the TMRM and TPP assays and was not found to alter the membrane potential or the respiration rate in either experiment.

The potency for OXPHOS disruption differed with a factor of 100 among the OH-PBDEs tested with 6-OH-BDE47 and 6-OH-BDE85 being the most potent ones with effect concentrations in the same order of magnitude as obtained for FCCP and SF6847 in both assays. As presented in **Table 1.** both 6-OH-BDE47 and 6-OH-BDE85 have been reported in several marine species from the Baltic Sea. In Blue mussels collected in June 2008 in the Baltic Sea, the concentration of Σ_7OH-PBDEs has been reported to be up to 3500 ng/g lipid weight which correspond to 50 ng/g fresh weight [8]. Given that eiders can eat up to 2.5 kg whole mussels per day [27,28], their daily intake may be substantial during the summer months. At present, data on the concentration of OH-PBDEs in eiders living in the Baltic Sea are lacking. Within this project we have sampled blood from Long-tailed ducks and several fish species (e.g. herring and flounder) and we are currently developing an improved analytical method to analyze OH-PBDEs in blood plasma from these species. To our knowledge, plasma concentrations of OH-PBDEs in sea birds from the Baltic Sea have so far only been reported in White-tailed sea eagle nestlings [29]. The White-tailed sea eagle is a predator at the top of the marine food web and the mean concentration of 6-OH-BDE47 in White-tailed sea eagle nestlings (2.3 ng/g fresh weight, which correspond to 0.005 nmol/g fresh weight)[29] indicate that the margin of safety might be small (MoS =LOEC/Exposure = (0.02 nmol/mL)/(0.005 nmol/g f.w.) = 4) based on calculations using the lowest observed effect concentration (LOEC) for 6-OH-BDE47 obtained in the TPP assay presented here.

Although considering the uncertainties when comparing results from in vitro test with environmental levels, the high abundance of OH-PBDEs in the Baltic marine environment and their possible additive effects stress the fact that further studies are needed to assess the exposure situation of OH-PBDEs in the Baltic Sea.

Table 1. Test compounds included in this study, their mode of action and their occurrence in Baltic Sea wildlife presented with references. FCCP and SF6847 were used as positive controls in this study.

Compound	TPP assay		TMRM assay	Baltic Sea wildlife				
	Protonophoric uncoupling	Inhibition of ETC	Altered MMP	Algae	Mussel	Fish	Bird	Mammal
FCCP	+	+	+					
SF6847	+	+	+					
2'-OH-BDE68	+	+	-	4,5,6,30	4,6,8,30	31,32	29,33	
2-OH-BDE123	+	+	+	4,5,6,30	4,6,8,30			6
3-OH-BDE47	+	+	-					34
4'-OH-BDE49	+	+	-			31		34
5-OH-BDE47	+	-	+					
6-OH-BDE47	-	+	+	4,5,6,30	4,6,8,30	6,31,32	29,33	6,34
6'-OH-BDE49	+	+	-			31		
6-OH-BDE85	+	+	+	4,5,6,30	4,6,8,30			6
6-OH-BDE90	+	-	+	4,5,6,30	4,6,8,30	31,32	29	6,34
6-OH-BDE99	+	+	+	4,5,6,30	4,6,8,30	6,31,32	29	6
6-OH-BDE137	+	+	+	4,5,6,30	4,6,8,30	6	29	
2'-OH-6'-Cl-BDE68	+	+	+		4			6
5-Cl-6-OH-BDE47	+	+	+			31		

BDE47	-	-	-					

Positive sign (+): showed effect within the concentration range tested.
Negative sign (-): showed no effect within the concentration range tested

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