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Transcriptome alterations in zebrafish embryos after exposure to environmental estrogens and anti-androgens can reveal endocrine disruption

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ABSTRACT

Exposure to environmental chemicals known as endocrine disruptors (EDs) is in many cases associated with an unpredictable hazard for wildlife and human health. The identification of endocrine disruptive properties of chemicals certain to enter the aquatic environment relies on toxicity tests with fish, assessing adverse effects on reproduction and sexual development. The demand for quick, reliable ED assays favored the use of fish embryos as alternative test organisms. We investigated the application of a transcriptomics-based assay for estrogenic and anti-androgenic chemicals with zebrafish embryos. Two reference compounds, 17α-ethinylestradiol and flutamide, were tested to evaluate the effects on development and the transcriptome after 48 h-exposures. Comparison of the transcriptome response with other estrogenic and anti-androgenic compounds (genistein, bisphenol A, methylparaben, linuron, prochloraz, propanil) showed commonalities and differences in regulated pathways, enabling us to classify the estrogenic and anti-androgenic potencies. This demonstrates that different mechanism of ED can be assessed already in fish embryos.

1. Introduction

Endocrine disruption is of high environmental concern since it affects the reproductive health of aquatic vertebrates. For this reason, endocrine disruption has been in the focus of the hazard assessment of substances occurring in the environment. Since 2007, with the implementation of REACH, every environmental relevant substance produced by the industry has to be tested for its endocrine potential. To this end, regulatory authorities like the US EPA (United States Environmental Protection Agency) or the OECD (Organisation of Economic Co-operation and Development) proposed testing strategies, which also include in vitro and in vivo studies for fish [1,2]. Although promising efforts have been made to promote in vitro testing, the final decision for a substance to be classified as endocrine active relies on in vivo data from tests with adult animals. In order to reduce the number of animals used for, e.g., life-cycle studies, which are regarded as a standard for assessing reproduction related and thus considered endocrine disrupting effects for fish, alternative test guidelines for short-term screening assays were published by the OECD and the OPPTS (Office of Prevention, Pesticides, and Toxic Substances) in 2009. Although these test guidelines (TG) (i.e., TG OECD 229 and 230, OPPTS 890.135) comply with the 3R concept of animal testing (replacement, reduction and refinement) by reducing the number of animals [3] and minimizing the duration of suffering from several months to 21 days, neither of these tests replaces animals fully. Alternative testing methods to further reduce the number of animals and to replace current standard tests for endocrine disruption, are therefore of urgent need.

In this context, the zebrafish embryo emerged as a potential alternative model because according to the revised European directive on the protection of animals used for scientific purposes (Directive 2010/63/EU), fish embryos are excluded from protection. Zebrafish embryos represent whole organisms and offer many advantages including a rapid development (the majority of the organs develop within the first 48 h post fertilization (hpf) of the embryonic phase), small size, transparency and easy handling. However, with regard to endocrine disruption, a disadvantage is that any morphological effects occurring in the embryos due to exposure cannot be associated with endocrine or reproductive

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disruption. In juvenile and adult fish, endocrine disruption is conventionally demonstrated by impairment of sexual organs and diminished reproductive performance, thus characteristics which are not yet developed in an embryo. Yet, by adding gene expression analysis to the testing procedure, this problem can partially be overcome. Previous studies have shown that omics-based approaches, which aimed at elucidating molecular responses at transcriptome, proteome or metabolome level could provide significant information on the mechanistic effects of chemicals [4]. Moreover, it is being discussed whether molecular endpoints are suitable to even indicate long-term phenotypic or population-relevant effects [5,6]. For zebrafish, it has been reported that certain marker genes are affected by endocrine disruptors already in embryos [7,8]. With our study, we now wanted to obtain more in-depth knowledge on the mechanistic effects of endocrine disruptors in the developing embryo. To this end, we analyzed the transcriptome response of whole 48hpf zebrafish embryos after exposure to the two model endocrine disruptors 17α-ethinylestradiol (EE2), an estrogen, and flutamide, an anti-androgen. Our aim was to elicit whether the endocrine modes of action of these two compounds are distinguishable at gene expression level and whether a specific expression pattern may allow the identification of further estrogenic or anti-androgenic substances. We included in the analysis three additional compounds for each mode of action, which are regarded as either mostly estrogenic or anti-androgenic.

The synthetic estradiol analogue EE2 was used as a model estrogen due to its strong binding affinity to the estrogen receptor [9]. It is the main component of many oral contraceptive pills and is known to be released into the aquatic environment via waste water at concentrations harmful for aquatic vertebrates [10–13] and is therefore of environmental concern.

We selected bisphenol A (BPA), genistein and methylparaben as further estrogenic compounds. BPA is an industrial compound used to produce polycarbonate and diverse other plastics [14]. It is known to be released to the environment and to cause endocrine effects in aquatic organisms [15,16]. Due to its binding affinity to estrogen receptors (ERs), BPA has been classified as a weak estrogen [17,18]. Genistein is a plant-derived isoflavone with structural similarity to estradiol, which has also been categorized as a weak estrogen [1]. Further, we chose methylparaben, which is widely used in many products for its preserving properties [19]. Methylparaben has been reported to bind to ERs and it has therefore been suggested to have estrogenic activity [20–22], but there is little data supporting its endocrine disruptive potency. Thus, we included this rather unknown endocrine disruptor to challenge our study approach and test whether an estrogenic mechanism becomes apparent in the fish embryos.

The non-steroidal compound flutamide was included as an anti-androgenic reference since it has been used for this purpose in various validations of endocrine disruption assays (Hershberger Assay, Fish Sexual Development Test OECD 234) [23,24]. Flutamide acts as anti-androgen by binding competitively to the androgen receptor (AR), resulting in the inhibition of testosterone uptake and signaling and is therefore used in prostate cancer therapy [25–28]. In fish, this can result in morphological effects similar to those of estrogens, but despite some similarities, the mechanism of action at transcription level shows differences [29]. Further, it has been shown that flutamide shares more similarities with DBT (dibenzothiophene), by not only blocking the androgen receptor, like vinclozolin, which is a more specific AR-antagonist. This indicates a more complex function of flutamide as expected. Despite abundant studies describing the phenotype of flutamide exposure [30,31], in general, only little information about the molecular mode of action of anti-androgens and specifically flutamide, exists [29,32]. In this context, our study provides supporting and complementary insight to the transcriptional effects of flutamide. While flutamide itself is of low environmental concern, many other anti-androgenic substances occur ubiquitously in the environment and may cause reproduction-relevant effects in aquatic vertebrates. In particular, many pesticides are known to have anti-androgenic activity and are thus suspected to contribute to the observed reproductive effects in male organisms [28,33–39]. Given their potentially high environmental concern, we chose three pesticides for our study, prochloraz, linuron and propanil, which have been reported to possess anti-androgenic properties. Prochloraz is a fungicide of the imidazole family used worldwide. Its action is based on the inhibition of steroidogenesis [40,41], but androgen receptor antagonizing effects have also been identified for prochloraz [42–44]. Linuron is a urea-based and propanil an amide herbicide which have both been reported to competitively bind the androgen receptor [45–47].

Together, in this manuscript, we demonstrate the transcriptome response to EE2 and flutamide to provide information on the mode of action of estrogenic and anti-androgenic disruption in fish embryos. Moreover, the transcriptome response to other estrogenic and anti-androgenic substances will be compared to the ones of the reference compounds to extract endocrine pathways and anticipate a mode-of-action specific categorization of the test compounds. With the results, we aim to show that fish embryos are suitable for the testing of endocrine disruptive chemicals.

2. Materials and methods

2.1. Fish maintenance and exposure

Adult zebrafish were maintained in 200 l glass aquaria under flow-through conditions, with a 14:10 h light:dark photoperiod. Tap water was charcoal- and particle-filtered, UV-sterilized, pre-heated to 26 ± 2 °C and aerated in a 400 l storage tank before entering the aquaria. Fish were kept in large spawning groups at a male to female ratio of approximately 2:1. The eggs were collected in metal-mesh-covered glass spawning trays and harvested from the aquaria 1 h after light onset. Before testing, the eggs were rinsed in 1:5 diluted ISO (International Organization for Standardization) medium, prepared according to the Annex 2 of the OECD Guideline 203 (ISO 6341-1982) [48]. All experimental procedures were conducted in accordance with local and national animal welfare regulations. The German Animal Welfare Act (TierSchG) of 18 May 2006 (BGBL. I S. 1206) allows the use of unhatched fish embryos in experimental procedures in all Federal States of Germany without permission. Since only embryos up to 2 days post-fertilization and prior to hatch were used in the current study, no approval by the ethics committee of the local federal state authority was required.

2.2. Chemicals

Chemicals were purchased from Sigma–Aldrich (Munich, Germany) (17α-ethinylestradiol E4876; flutamide F9397; bisphenol A 239658; linuron 36141; genistein G6649; propanil 45639; methylparaben 47889; prochloraz 45631). Stock solutions were prepared in acetone (ROTIPURAN®, purity ≥ 99.8%, p.a., Carl Roth, Karlsruhe, Germany).

2.3. Fish Embryo Toxicity Test (FET)

The fish embryo exposures were conducted in accordance with the procedures described in the first draft Test Guideline of the "Fish Embryo Toxicity (FET) Test" [49]. For prochloraz and bisphenol A, the test solutions were prepared by directly dissolving the chemicals in 200 ml pre-aerated ISO water. For all other substances, the chemicals were dissolved in acetone (100%) and dilutions for each concentration prepared in 2 ml of acetone. These 2 ml-acetone stocks were transferred to 250 ml screw-cap glass bottles