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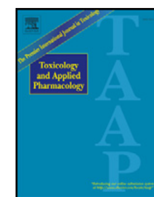

Additive effects of levonorgestrel and ethinylestradiol on brain aromatase (*cyp19a1b*) in zebrafish specific *in vitro* and *in vivo* bioassays

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ABSTRACT

Estrogens and progestins are widely used in combination in human medicine and both are present in aquatic environment. Despite the joint exposure of aquatic wildlife to estrogens and progestins, very little information is available on their combined effects. In the present study we investigated the effect of ethinylestradiol (EE2) and Levonorgestrel (LNG), alone and in mixtures, on the expression of the brain specific ER-regulated *cyp19a1b* gene. For that purpose, recently established zebrafish-derived tools were used: (i) an *in vitro* transient reporter gene assay in a human glial cell line (U251-MG) co-transfected with zebrafish estrogen receptors (zfERs) and the luciferase gene under the control of the zebrafish *cyp19a1b* gene promoter and (ii) an *in vivo* bioassay using a transgenic zebrafish expressing GFP under the control of the zebrafish *cyp19a1b* gene promoter (*cyp19a1b*-GFP). Concentration-response relationships for single chemicals were modeled and used to design the mixture experiments following a ray design. The results from mixture experiments were analyzed to predict joint effects according to concentration addition and statistical approaches were used to characterize the potential interactions between the components of the mixtures (synergism/antagonism). We confirmed that some progestins could elicit estrogenic effects in fish brain. In mixtures, EE2 and LNG exerted additive estrogenic effects both *in vitro* and *in vivo*, suggesting that some environmental progestin could exert effects that will add to those of environmental (xeno-)estrogens. Moreover, our zebrafish specific assays are valuable tools that could be used in risk assessment for both single chemicals and their mixtures.

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1. Introduction

Endocrine disrupting chemicals (EDCs) have been extensively studied during the last decade due to their adverse effects on aquatic organism reproduction and development. To date, most attention on EDCs has been focused on compounds able to interact with the estrogen receptors (ERs). The occurrence, fate and effects of both natural and synthetic estrogens (estradiol (E2), estriol, estrone and ethinylestradiol (EE2)) and estrogen-like compounds are now well documented. As estrogens can be found in mixtures in the aquatic environment, a number of studies evaluated the effects of their combined exposure on estrogen signaling in aquatic organisms. Results from these studies demonstrate that binary or multi-component mixtures of ER agonists generally act in an additive manner on the expression of ER-regulated genes in both brain and liver (Thorpe et al., 2001; Rajapakse et al., 2004; Lin and Janz, 2006; Kortenkamp, 2007; Petersen and Tollefsen, 2011; Brion et al., 2012; Petersen et al., 2013). In some cases, some deviations from additivity can be observed, especially with infra-additive effects, both in *in vitro* and *in vivo* experiments (Rajapakse et al., 2004; Lin and Janz, 2006; Petersen and Tollefsen, 2011; Petersen et al., 2013).

Estrogens and progestins are widely used in combination in human medicine, especially in oral contraceptives and hormone replacement therapy (Zeilinger et al., 2009). Their extensive use and poor removal by sewage treatment plants have led to contamination of the aquatic environment (Besse and Garric, 2009; Liu et al., 2011). However, compared to estrogens, the occurrence, fate and effects of progestins were poorly studied. They are found in effluents and in surface waters (rivers, lakes, streams) and ground waters at concentrations up to tens of ng/L, but also in sediments from rivers at concentrations up to tens of ng/g (for review see (Besse and Garric, 2009; Liu et al., 2011; Fent, 2015)). Moreover, progestins are potent developmental and reproductive toxicants for aquatic organisms (for review see Zeilinger et al., 2009; Kumar et al., 2015). Among these progestins, levonorgestrel (LNG) is a synthetic progestin structurally related to testosterone (19-Nortestosterone derivative), used alone or in association with an estrogen such as ethinylestradiol for contraception purposes (emergency contraceptives or birth control pills). LNG has been detected in some effluents,

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sediments, ground water, tap water, but also in surface water of rivers at concentrations up to 38 ng/L (Vulliet et al., 2008; Besse and Garric, 2009; Al-Odaini et al., 2010; Liu et al., 2011; Vulliet and Cren-Olivé, 2011; Fent, 2015). LNG exerts biological activities that differ from the natural progestin (progesterone) since it has progestagenic and androgenic activities (Besse and Garric, 2009) and also estrogenic activities both *in vitro* and *in vivo* (Jeng et al., 1992; Brion et al., 2012; Zucchi et al., 2012; Creusot et al., 2014; Kroupova et al., 2014). However, despite the joint exposure of aquatic wildlife to estrogens and progestins, very little information is available on their combined toxicity (Runnalls et al., 2015; Säfholm et al., 2015).

This study aims at investigating the effects of single and combined exposure to EE2 and LNG on the expression of the zebrafish *cyp19a1b* gene both in *in vitro* and *in vivo* models. In zebrafish, the *cyp19a1b* gene encodes the brain form of aromatase (aromatase B) which is only expressed in radial glial cells that act as neuronal progenitors both in developing and adult brain (Pellegrini et al., 2007). The *cyp19a1b* gene is extremely sensitive to (xeno-)estrogens and this regulation is ER-dependent (Le Page et al., 2008; Brion et al., 2012). In the past few years, we developed both *in vitro* and *in vivo* bioassays, based on zebrafish *cyp19a1b* gene, that were used in this study: i) a human glial cell culture (U251-MG) co-transfected with zebrafish ER subtypes (zfERα, zfERβ1 and zfERβ2) and a luciferase gene under the control of the zebrafish *cyp19a1b* promoter (Le Page et al., 2006), ii) a transgenic zebrafish (*cyp19a1b*-GFP) line expressing GFP under the control of the zebrafish *cyp19a1b* promoter which is suitable to detect estrogenicity of chemicals alone and in mixtures (Brion et al., 2012; Petersen et al., 2013). These *in vivo* and *in vitro* bioassays were used to assess the estrogenic responses of EE2 and LNG in mixtures; responses that were modeled using the concentration-addition (CA) prediction model. Deviations from this no-interaction model were characterized in terms of synergism or antagonism, modeled using Jonker's interaction terms (Jonker et al., 2005) and their significance was tested. By this approach, the present study reports additive estrogenic effects of EE2 and LNG in mixtures on the expression of an estrogen-regulated gene in a glial cell context.

2. Material and methods

2.1. Chemicals

EE2 (purity ≥ 98%, CAS number: 57-63-6; reference: E4876) and LNG (purity ≥ 98%; CAS number: 797-63-7; reference: N2260) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

2.2. Zebrafish maintenance and breeding

Animal culture, handling and experimentation were approved by the INERIS life science ethics committee and in accordance with French ethical laws. The *cyp19a1b*-GFP transgenic zebrafish (Tong et al., 2009) were held at the Institut National de l'Environnement Industriel et des Risques (INERIS, Verneuil-en-Halatte, France). They were maintained in 3.5 L aquaria in a recirculation system (Zebtec, Tecniplast, Buguggiate, Italy) on a 14 h light: 10 h dark cycle at a temperature of 25.1 ± 1.0 °C. They were allowed to reproduce (ratio of 2 males for 1 female). Fertilized eggs were harvested and disinfected 5 min in water supplemented with 0.1% of commercial bleach (2.6% of sodium hypochlorite).

2.3. Zebrafish exposure to EDCs

Fertilized *cyp19a1b*-GFP transgenic zebrafish eggs were exposed to chemicals (alone or in mixtures) or to solvent control (DMSO, 0.02% v/v) according to (Brion et al., 2012) with minor modifications. Briefly, for each experimental condition, 20 embryos were exposed in 100 mL of water. Embryos were kept at 28 °C, under semi-static conditions. Exposures were performed from 0 days post fertilization (dpf) to 4 dpf without water renewal. At the end of the exposure period (96 h), non-transgenic zebrafish were removed and 4-dpf old transgenic zebrafish were processed for fluorescence measurement by image analysis. Experiments were performed in accordance with European Union regulations concerning the protection of experimental animals (Directive 2010/63/UE).

2.4. In vivo imaging

In vivo fluorescence imaging was performed according to (Brion et al., 2012). Each live *cyp19a1b*-GFP transgenic embryo was photographed once in dorsal view using a Zeiss AxioImager Z1 fluorescence microscope equipped with an AxioCam Mrm camera (Zeiss GmbH, Göttingen, Germany). Each photograph was acquired under the same exposure conditions (×10 objective, 134 ms of fluorescent light exposure, maximal light intensity). Fluorescence quantification was performed using Image J software (available at: http://rsb.info.nih.gov/ij/). For each picture, the integrated density (IntDen) was measured, *i.e.* the sum of the grey-values of all the pixels within the region of interest. All grey-values of 300 or less were defined as background values.

2.5. U251-MG cell bioassay

The ER-negative human glial cell line U251-MG (ECACC) culture, handling and the luciferase assay were performed according to (Le Page et al., 2006).

U251-MG cells were maintained at 37 °C in 5% CO2 atmosphere in phenol red–free Dulbecco's Modified Eagle's Medium (DMEM-F12, Sigma-Aldrich, St Louis, MO, USA) supplemented with 8% fetal calf serum (FCS), 2 mM L-glutamine, 20 U/mL penicillin, 20 μg/mL streptomycin and 50 ng/mL amphotericin B.

For transfection experiments, U251-MG cells were plated in 24-well plates at a density of 0.2×10^5 cells/mL. After 24 h, the medium was replaced with fresh phenol red-free DMEM containing 2% FCS. In each well, 25 ng of zfER expression vector (*i.e.* Topo-pcDNA3 expression vector containing the coding region of zfERα or zfERβ2 complementary DNA and the neomycin resistance gene (Menuet et al., 2002)), 25 ng of cytomegalovirus-β-galactosidase control plasmid and 150 ng of luciferase reporter construct (*i.e.* proximal promoter region of the zebrafish cyp19a1b gene coupled to the luciferase reporter gene (Menuet et al., 2005)) were transfected using JetPEI™ reagent, as indicated by the manufacturer (Polyplus-transfection, France). After one night, medium was replaced with fresh DMEM-F12 containing 2% charcoal/dextran FCS with xeno-estrogens or vehicle (DMSO, 0.1% v/v). The luciferase activities were assayed after 48 h using the luciferase assay system (Promega). β-galactosidase activity was used to normalize transfection efficiency in all experiments. Results were expressed as fold induction relative to the solvent.

2.6. Data normalization

In the *in vivo* assay with *cyp19a1b*-GFP transgenic zebrafish, induction of GFP fluorescence was measured as IntDen and normalized by dividing by the geometric mean of the IntDen in the DMSO control group. In the preliminary single chemical experiments used for the design of mixture experiments, the concentration-response relationships were obtained in separate experiments. For that reason, the log-inductions were further normalized by the logarithm of the geometric mean of the positive controls (EE2; 0.05 nM), which corresponds to a maximum response level.

In the *in vitro* assay with U251-MG cell cultures, the data were normalized by dividing by the geometric mean of the corresponding solvent control group. EE2 and levonorgestrel as single chemicals were tested on the same plate therefore no additional normalization was required.

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