Assessment of the estrogenic potency of chemicals, alone or in combination, and complex environmental mixtures using a novel transgenic cy19a1b-GFP *in vivo* zebrafish assay (EASZY assay).

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1. Introduction

Concern about the effects of Endocrine Disrupting Chemicals (EDCs) to wildlife and human reproductive health has stimulated the development and implementation of screening assay for EDCs. One major challenge to ecotoxicology is to develop species-specific biological tools that allow rapid and cost-efficient assessment of EDCs for environmental hazard and risk assessment but also for monitoring the contamination of aquatic systems by EDCs. As aquatic organisms are often exposed to complex mixtures of EDCs rather to single compound, evaluating the impact of combined exposure using appropriate bio-assays has also become important. Assessing the effect of combined exposure to EDCs is also relevant in the perspective of using these assays for environmental monitoring purposes.

In this context, the use of zebrafish (*Danio rerio*) embryo assay allowing the detection and quantification of EDCs based on their mode of action appear as usefulness and relevant to address these issues. We recently developed a mechanism-based *in vivo* zebrafish assay called EASZY that allows the detection of Endocrine Active Substance, acting through zebrafish estrogen receptors (ER), using transgenic cyp19a1b-GFP Zebrafish embryos [1]. The Cyp19a1b gene is a strictly ER-regulated gene that codes for the brain aromatase which is mainly expressed in radial glial cells of the brain of fish.

The present work intends to review the use of the EASZY assay for screening estrogenic activity of chemicals, alone or in combination, and its relevance and interest for regulatory purposes. It also aims at investigating the potential use of the EASZY test to detect endocrine active substances in complex environmental matrices sampled from French river sites.

2. Material and Methods

2.1. Exposure to test substance alone

Newly fertilized zebrafish cyp19a1b-GFP eggs are exposed to test substance, alone or in combination for 96 hours. The fluorescence intensity of each fish is acquired using in vivo fluorescence imaging and the data expressed as fold induction above control. For each test substance, concentration-response relationships were measured and modelled.

2.2. Exposure to mixture of estrogenic compounds

These data were used to design the binary mixture experiments. The mixture experiments results were analyzed to predict joint effects according to concentration addition (CA) and independent action (IA) models. Two different statistical approaches (Jonker's dose-response surface models and Hewlett and Streibig's isobole-based models implemented by Sorensen) were used to assess deviations from the CA model and to characterize interactions between the components of the mixtures (synergism/antagonism).

2.3. Exposure to mixture environmental extracts

Surface waters from various rivers in France were sampled Polar Organic Chemical Integrative Sampler (POCIS) and extracted using Solid Phase Extraction (SPE) [2]. These organic extracts were tested in EASZY assay to detect the presence of compounds being able to bind to ERs and activate the GFP driven by the cyp19a1b gene.

3. Results and discussion

3.1. Estrogenic activities of chemicals using EASZY

The relevance of the EASZY assay for assessing the estrogenic potency of substance was evaluated by testing more than 50 different compounds belonging to various chemical classes. We found that in EASZY GFP is induced in a ER-specific manner by (i) compounds that bind directly to estrogen receptors as agonists (ii) compounds that require metabolic activation into estrogenic metabolites (iii) aromatizable androgens as well as some non aromatizable androgens. EASZY allows a rapid, specific and reliable quantification of estrogenic activity of chemical or its metabolites *in vivo* at very early critical developmental stages. Its sensitivity is outstanding and comparable to the most performing *in vitro* assays while taking into account the biodisponility and pharmaco-dynamics of chemicals. Because of its numerous attractive advantages in hazard assessment of chemicals; a proposal for validation of the EASZY assay was recently submitted and accepted at the OECD level.

3.2. Combined effects of estrogenic chemicals using EASZY.

Several binary mixtures of estrogenic compounds were studied and generally lead to additive effects. For instance, we showed that mixtures of agonist ligands with similar binding affinity and transactivation capacity of ERs acted in an additive manner on the expression of GFP expression and their combined effects being predicted by the CA model. However, some deviations from additive effects could be observed at the highest mixtures concentrations of some mixtures and antagonism was revealed in case of exposure to the binary mixture of oestradiol and genistein. Overall, our data highlights the interest of using the EASZY assay in combination with CA and IA models to assess combined effect of estrogenic compounds. To further assess the potential use of this assay for complex mixtures, several environmental samples were tested.

3.3. Screening of environmental samples using EASZY

All organic POCIS extracts collected from French rivers were tested *in vivo* using EASZY. POCIS-based bio-monitoring using EASZY resulted in the detection of estrogenic activities at some river sites, which suggest that estrogenic activities were due to polar to mid-polar compounds. Interestingly, the estogenic activities obtained *in vivo* confirmed those obtained *in vitro* using specific zebrafish-based ER bioassays. However, several POCIS extracts that were less active on *in vitro* bioassays were not able to show any GFP induction *in vivo*. This could be explained by limitation of extracts with highest concentration factor for *in vivo* testing, which can be resolved by redefining sampling procedure.

4. Conclusions

In conclusion, EASZY assay clearly emerges as a simple, fast and reliable *in vivo* assay for screening the capacity of chemical to activate ER-signalling *in vivo* at very early critical developmental stages. It is based on the use of an endogenous promoter and thus shows of a true physiological brain-specific response. The sensitivity of EASZY is outstanding while taking into account the bioavailability and the pharmacodynamics of chemicals, thus enhancing the relevance of EDCs screening for hazard assessment. Because EASZY is a small-scale assay, it is well-suited to assess complex environmental mixtures in complement to *in vitro* assays enhancing the relevance of the detection of fish-specific EDCs in aquatic environment.

5. References

[1] Brion F, Le Page Y, Piccini B, Cardoso O, Tong S-K, Chung B-C, Kah O. 2012. Screening Estrogenic Activities of Chemicals or Mixtures In Vivo Using Transgenic (cyp19a1b-GFP) Zebrafish Embryos. PLoS ONE 7(5): e36069.

[2] PhD theses. Creusot N. 2011. Contribution to EDA approach for identification of EDCs in the aquatic environment. Bordeaux, France. University of Bordeaux.

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