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Assessing combined toxicity of estrogen receptor agonists in a primary culture of rainbow trout (*Oncorhynchus mykiss*) hepatocytes

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ABSTRACT

The presence of highly complex mixtures of chemicals in the environment challenges our ability to assess single chemical effects and the interaction that occurs with cellular receptor targets and regulation of endocrine processes. In this study concentration addition (CA) and independent action (IA) prediction models were used to assess the combined toxicity of mixtures of environmental relevant estrogen receptor (ER) agonists (hormones and anthropogenic pollutants) in a primary culture of rainbow trout (*Oncorhynchus mykiss*) hepatocytes using the ER-mediated production of vitellogenin (Vtg) as a biological marker (biomarker) for estrogenicity. Nine of the eleven tested chemicals induced the production of Vtg and the parameters from the fitted concentration–response curves were used to model four mixtures containing four (17β-estradiol, estrone, estriol and diethylstilbestrol), five (musk ketone, 4-*tert*-octylphenol, bisphenol A, o,p′-DDT and dibenzothiophene), seven (17β-estradiol, estrone, estriol, diethylstilbestrol, 4-*tert*-octylphenol, bisphenol A and o,p′-DDT) and nine compounds (17β-estradiol, estrone, estriol, diethylstilbestrol, musk ketone, 4-*tert*-octylphenol, bisphenol A, o,p′-DDT and dibenzothiophene). The CA and IA prediction model proved to be a good estimation for the combined effect of mixtures of ER agonists at low relative mixture concentration (e.g. relative to the maximum mixture concentrations used), but a deviation from the prediction models was observed when exposing hepatocytes to high relative mixture concentrations. The CA and IA prediction models' ability to predict the combined estrogenic effect of complex mixtures, especially in the low concentration–response range, is of ecological relevance since organisms in the environment generally encounter low concentrations of chemicals from a wide array of chemical groups that may not elicit estrogenic effects on their own.

1. Introduction

A large number of environmental pollutants cause activation of estrogen receptor (ER) and may interfere with normal endocrine functions potentially leading to endocrine disruption. Recent studies have shown that both natural and anthropogenic compounds from different chemical classes; alkylphenols, pesticides and musks are potential ER agonists (Bitsch et al., 2002; Leanos-Castaneda et al., 2002; Vazquez et al., 2009), and that some of the anthropogenic compounds such as diethylstilbestrol (DES), 4-*tert*-octylphenol (OP), bisphenol A (BPA), o,p′-DDT, and musk ketone (MK) may have the same mode of action (MoA) as the natural estrogenic hormones by inducing estrogenic effects through interaction and activation of the ER (Bitsch et al., 2002; Lutz and Kloas, 1999; Tollefsen et al., 2002). Interaction with the ER may in turn cause modulation of endocrine functions and consequently alter normal development, growth and reproduction, and potentially leading to endocrine disruption. This has been seen as alterations in testicular structure and function in male fish as a response to environmentally relevant concentrations of BPA and impaired male gonadal development (intersex) and altered female oogenesis in fathead minnow (*Pimephales promelas*) after chronic exposures to low concentrations of xenoestrogens such as ethynylestradiol (Crain et al., 2007; Kidd et al., 2007).

The concentrations of the most abundant ER agonists in effluents from sewage treatment works in European countries are usually in the ng/L range. Concentrations of 17β-estradiol (E2) and estrone (E1) range from the low ng/L to as much as 100 ng/L (Baronti et al., 2000; Desbrow et al., 1998), whereas the concentrations of E2 measured in surface water are generally in the low ng/L range (Belfroid et al., 1999). The level of BPA and OP detected in effluents from waste water treatment plants have been reported in the low ng/L range (Kuch and Ballschmiter, 2001), and the effluent also contains detectable concentrations of PAHs (Blytt and Storhaug, 2008). Although the concentrations of estrogenic compounds are gener-

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ally low, the combination of several compounds with similar MoA may potentially lead to effects through combined toxicity (Brian et al. 2005; Rajapakse et al., 2002). The result of combined toxicity have been seen in wild or transplanted organisms exposed to effluents containing complex mixtures of estrogenic compounds where estrogenic responses in male fish such as development of intersex and increased concentration of the female-specific protein vitellogenin have been reported (Jobling et al., 1998; Harries et al., 1997).

Presence of highly complex mixtures of chemicals in the environment challenges our ability to assess single chemical effects and the interaction that occurs between chemicals at the cellular receptor targets. Recent developments in modelling have enhanced our ability to assess combined toxicity through use of two different modelling approaches known as concentration addition (CA) and independent action (IA) based on the assumption that interactions occur by concentration additivity and independent action, respectively (Altenburger et al., 1996; Kortenkamp and Altenburger, 1999). Despite availability of predictive models, most risk assessments are still based on effect endpoints derived from single chemical toxicity assessment. Additionally, a major portion of the reported combined toxicity assessment studies are focused on acute toxicity or sub-lethal toxicity in recombinant and non-native *in vitro* test systems that in most cases lack the complexity of intact (native) cells (Olsen et al., 2005; Wang et al., 2010). The predictability of the effect of mixtures of estrogen agonists by CA have specifically been tested in a variety of assays, including the recombinant *in vitro* yeast estrogen screen (Silva et al., 2002), MCF-7 human breast cancer cells (the E-SCREEN) (Rajapakse et al., 2004) and *in vivo* vitellogenin (Vtg) induction in fish (Kunz and Fent, 2009; Thorpe et al., 2001). However, few studies have been conducted with native *in vitro* systems such as primary cell cultures or liver slices, where inherent cellular toxicity mechanisms and xenobiotic metabolism may mimic that occurring in cells *in vivo* (Castaño et al., 2003; Schmieder et al., 2004). Studies using primary cells may potentially support the interpretation of *in vitro* studies using recombinant cell systems and potentially assist in extrapolation of data to more environmentally realistic *in vivo* situations.

Primary hepatocytes from fish such as rainbow trout (*Oncorhynchus mykiss*) represent a well-characterised high-throughput screening tool for single chemicals and mixtures (Tollefsen et al., 2008a,b,c). These native cells provide assessment of multiple MoA and has successfully characterised the potency of both ER agonists and antagonists *in vitro* through use of the estrogenic biomarker vitellogenin (Tollefsen et al., 2003, 2008c). Vitellogenin is the major precursor of the fish egg-yolk proteins, vitellins, and are synthesised in the liver of females in response to endogenous estrogens, and transported with the blood to the developing oocytes (Yaron, 1995). Estrogens bind to and activate ERs that consequently form homodimers that attach to the estrogen responsive element on the DNA and activates the transcription of the Vtg-specific genes and initiate the production of Vtg (Boelsterli, 2007). Vitellogenin-specific genes and the cellular machinery to produce Vtg proteins is also present in males but is normally silent due to low basal production of endogenous estrogen (Copeland et al., 1986). However, Vtg can be induced by exposure to both natural and anthropogenic estrogens and may therefore serve as a biological marker (biomarker) for ER agonists and antagonists (Harries et al., 1997; Tollefsen et al., 2003).

The current work present, to our knowledge, the first study to use the CA and IA prediction models to assess the combined effect of mixtures of estrogen agonists in a primary culture of rainbow trout hepatocytes. The CA and IA prediction models were used to assess the combined toxicity of mixtures of various ER agonists (hormones and organic pollutants) using the *in vitro* production of Vtg in the rainbow trout hepatocytes as a biomarker for estrogenicity. The combined effects of the mixtures were hypothesized to follow the principle of CA as the tested compounds were believed to act by the same MoA. Assessment of cytotoxicity by determination of membrane integrity and metabolic activity were included to assess whether deviations from predicted cellular Vtg responses were due to cytotoxicity. By constructing the mixture of ER agonists based on the CA model the suitability of the two prediction models to accurately model the experimental data could be determined.

2. Materials and methods

2.1. Chemicals

The test chemicals copper sulfate ($CuSO_4 \cdot 5H_2O$, cas 7758-99-8), 17β-estradiol (E2, ≥98%, cas 50-28-2), estrone (E1, ≥97,0%, cas 53-16-7), estriol (E3, ≥97%, cas 50-27-1), diethylstilbestrol (DES, ≥99%, cas 56-53-1), 17α-ethynylestradiol (EE2, ≥98%, cas 57-63-6), bisphenol A (BPA, 97%, cas 80-05-7), musk ketone (MK, ≥98%, cas 81-14-1), 4-*tert*-octylphenol (OP, 97%, cas 140-66-9), α-endosulfan (PESTANAL®, cas 959-98-8), dibenzothiophene (DBT, ≥98%, cas 132-65-0), and o,p′-DDT (Supelco, cas 789-02-6) were all from Sigma–Aldrich (St. Louis, MI, US). The brominated flame retardant BDE49 (98.8%) were obtained from Chiron AS (Trondheim, Norway). The test chemicals were all dissolved in dimethylsulfoxide (DMSO) and stored in the dark at −20 °C until use in the bioassay assessment.

2.2. Rainbow trout

Rainbow trout (size 200–500 g) were obtained from Haadem fish (Valdres, Norway) and kept in tanks at the Institute of Biology, University of Oslo (Norway) at a water temperature of ±6 °C, 100% oxygen saturation, pH 6,6 and a 12 h light/12 h dark cycle. The fish were fed daily with pellets (Skretting, Stavanger, Norway) corresponding to approximately 0.5% of total body mass.

2.3. Hepatocyte isolation, culturing and exposure

Fish were killed with a blow to the head and the sex was determined by visual inspection of the gonads. Only juvenile fish were subjected to liver perfusion according to the method described by Tollefsen et al. (2003). The liver was perfused in situ (5 ml/min, 10–15 min) with a calcium free buffer (NaCl 122 mM, KCl 4.8 mM, $MgSO_4$ 1.2 mM, Na_2HPO_4 11 mM, NaH_2HPO_4 3.3 mM, $NaHCO_3$ 3.7 mM, EGTA 26 μM, 0 °C) to remove the blood from the liver. The liver was then perfused with the same buffer (5 ml/min, 10–15 min, 37 °C) without EGTA and with added $CaCl_2$ (1.5 mM) and collagenase (0.3 mg/ml). The liver was transferred to a glass beaker, and dispersed in ice-cold calcium free buffer, supplemented with 0.1% w/v bovine serum albumin (BSA). The cell suspension was filtered first through a 250 μm nylon mesh, and then through a 100 μm nylon mesh before it was centrifuged three times at 500 rpm for 4, 3 and 3 min, respectively. Following the first centrifugation the supernatant was removed and the cells resuspended in ice-cold calcium free buffer supplemented with 0.1% w/v BSA. After the second and third centrifugation the cells were resuspended in serum-free L-15 medium containing L-glutamine (0.29 mg/ml), $NaHCO_3$ (4.5 mM), penicillin (100 Units/ml), streptomycin (100 μg/l) and amphotericin (0.25 μg/ml). The viability was assessed by counting live and dead cells on a bürckner chamber containing 2 parts trypan blue and one part cell suspension. Cells were diluted to 500 000 cells/ml and plated into 96 well primaria™ plates, 200 μl per well (Falcon, Becton Dickinson Labware, Oxnard, CA, USA) and kept at ambient atmosphere (15 °C). The selected incubation temperature has previously been used in several exposure experiments with primary rainbow trout hepatocytes (Gagne and Blaise,