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Prediction of the combined effects of multiple estrogenic chemicals on MCF-7 human breast cancer cells and a preliminary molecular exploration of the estrogenic proliferative effects and related gene expression

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ABSTRACT

The environmental risks of environmental estrogens (EEs) are often assessed *via* the same mode of action in the concentration addition (CA) model, neglecting the complex combined mechanisms at the genetic level. In this study, the cell proliferation effects of estrone, 17α-ethinylestradiol, 17β-estradiol, estriol, diethylstilbestrol, estradiol valerate, bisphenol A, 4-tert-octylphenol and 4-nonylphenol were determined individually using the CCK-8 method, and the proliferation effects of a multicomponent mixture of estrogenic chemicals mixed at equipotent concentrations using a fixed-ratio design were studied using estrogen-sensitive MCF-7 cells. Furthermore, transcription factors related to cell proliferation were analyzed using RT-PCR assays to explore the potential molecular mechanisms related to the estrogenic proliferative effects. The results showed that the estrogenic chemicals act together in an additive mode, and the combined proliferative effects could be predicted more accurately by the response addition model than the CA model with regard to their adverse outcomes. Furthermore, different signaling pathways were involved depending on the different mixtures. The RT-PCR analyses showed that different estrogens have distinct avidities and preferences for different estrogen receptors at the gene level. Furthermore, the results indicated that estrogenic mixtures increased ERα, PIK3CA, GPER, and PTEN levels and reduced Akt1 level to display combined estrogenicity. These findings indicated that the potential combined environmental risks were greater than those found in some specific assessment procedures based on a similar mode of action due to the diversity of environmental pollutions and their multiple unknown modes of action. Thus, more efforts are needed for mode-of-action-driven analyses at the molecular level. Furthermore, to more accurately predict and assess the individual responses *in vivo* from the cellular effects *in vitro*, more parameters and correction factors should be taken into consideration in the addition model.

1. Introduction

The water ecological system is the ultimate sink of many environmental pollutants. With the development of new analytical methods, an increasing number of environmental pollutants identified as endocrine disrupting chemicals (EDCs) (Sumpter, 2005) have been detected at low levels (Petrovic et al., 2004). EDCs interfere with the function of the endocrine system by blocking or mimicking the normal effect of hormones and affecting their synthesis or metabolism in wildlife and humans (Diamanti-Kandarakis et al., 2009). Steroid estrogens (both natural and synthetic), also known as environmental estrogens (EEs), have raised concerns because of their non-negligible estrogenic (Peng et al., 2006) and adverse toxic effects on aquatic organisms (Zha et al., 2007). EEs are ubiquitous and concomitant in our environment because of their seemingly endless number of users and origins in residential, industrial and agricultural fields (Boyd et al., 2003; Desbrow et al., 1998). Previous observations have indicated that estrogenic chemicals could promote cell proliferation by binding to estrogen receptors (ERs) (Razandi et al., 1999), and also activate membrane receptor signaling pathways and second messengers (Aronica et al., 1994; Bunone et al., 1996; Chan et al., 2010; Filardo et al., 2000), inducing the expression of intracellular signaling enzymes and gene transcription. Organisms are constantly exposed to a variety of environmental pollutants, which result in complex molecular mechanisms of toxicity; these exposures may increase the combined toxicity of EEs and cause serious harm (Mu and Leblanc, 2004; Payne et al., 2001; Rajapakse et al., 2004). The

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increasing amounts of EEs coexisting in the aquatic environment are believed to pose great environmental risks even at a low concentration (ng/L) (Hotchkiss et al., 2008; Vandenberg et al., 2012).

The concentration-response curve of the biological effects of EEs is generally S-shaped, but the combined effects cannot be determined by the simple additive mode because it is unable to reflect whether components act in an additive, antagonistic, or synergistic manner with one another (Daston et al., 2003). Thus, the choice of the dose addition model appears to be appropriate since most studies have been based on an endpoint model, and many analyses of EDCs have utilized the dose addition or concentration addition approach in joint toxicity studies (Charles et al., 2002a, 2002b; Crofton et al., 2005; Rajapakse et al., 2004). The concentration addition (CA) model and response addition (RA) model are the two classical concepts that allow calculating the expected mixture toxicity based on the toxicities of the individual compounds and their concentrations in the mixture. Furthermore, synergism or antagonism of the components in a mixture can be identified if the observed toxicity of the mixture deviates from the prediction by the CA or RA model (Liu et al., 2013). The CA model assumes that all the components have a similar mode of action (MoA) competitively and independently and can rationally interpret the sham combination, thus causing the same outcome (Loewe, 1928). The RA model assumes that the mixed components act on different subsystems (tissues, cells, molecular receptors) of the exposed organism and affect the end point under observation independently (Bliss, 1939). Generally, the observed combined effects of multi-component mixtures are almost perfectly depicted by the predictions from the CA model for mixtures with similar modes of action and the RA model for those with dissimilar modes of action. However, the biological effects of many EEs are weak given the actual individual environmental concentrations, but the combined effects are significant in the presence of other pollutants (Silva et al., 2002), because one component, which by itself cannot induce the established effect but can modify the responses of interest, is provoked by another component in the mixture. Since the structures of estrogen receptors are very similar in different organisms, EEs can disturb the endocrine system of many species in the ecosystem by binding to estrogen receptors (Brzozowski et al., 1997). The use of the term “additivity” in mixture toxicology causes much confusion, partly because it is not always synonymous with additivity in the mathematical sense at a relatively low concentration (Kortenkamp, 2007). Therefore, how to predict and assess the combined effects of low concentration estrogenic chemicals in environmental media and human tissues has been a difficult and attractive project in the environmental field and requires more attention to clarify the possible health risks (Backhaus and Faust, 2012; Spurgeon et al., 2010; Vacchi et al., 2013).

The RA, as well as the CA model, assumes that there are no interactions between the components in a mixture, that is, they do not influence one another's uptake, distribution or metabolism in the exposed organisms. Therefore, the CA model is often considered as the standard additive model for the toxicity prediction of chemical mixture. This model was shown to be effective in toxicity analyses of EEs by a yeast two-hybrid assay (Silva et al., 2002; Yang et al., 2015), an estrogen receptor binding bioassay (Yang et al., 2014), and some human breast cancer cell proliferation assays (Rajapakse et al., 2004) *in vitro*. Meanwhile, RA model was used when any component in the mixture showed dissimilar modes of actions (Bliss, 1939). *In vitro* assays can be useful for a first screening of mixture toxicity of EEs, consequently allowing the assessment of toxic joint effects across a wider range of mixture effect levels and ratios. This study was designed to investigate the combined proliferation effects of multiple estrogenic chemicals individually and intuitively; to identify a reliable model for the assessment and prediction of environmental estrogenic risk, especially at environmentally relevant concentrations; and to explore the diverse estrogenic signaling pathways related to cell proliferation. In the present study, an E-screen assay was adopted for large-scale screening of suspected estrogenic chemicals (Colborn and Clement, 1992), and MCF-7 cells were used as target cells because of their widely acknowledged estrogen sensitivity (Soto and Sonnenschein, 1985). The CA and RA models were used to predict the combined action of an estrogen mixture. Based on these model, RT-PCR was used to explore the gene expression levels related to cell proliferation, to detect the related transcription factors that were activated by estrogenic signaling, to explain the molecular mechanisms of cell proliferation induced by estrogenic chemicals in MCF-7 cells, and to provide a theoretical basis for the environmental risk assessment of composite pollutants at environmentally relevant concentration by determining the specific transcription factors that were crucial for the estrogenic response of EEs.

2. Materials and methods

2.1. Chemicals

All chemicals, including estrone (E1, 99%), 17α-ethinylestradiol (EE2, 98%), 17β-estradiol (E2, 99%), estriol (E3, 98.5%), diethylstilbestrol (DES, 99%), estradiol valerate (EV, 98%), bisphenol A (BPA, 96%), 4-Tert-octylphenol (4-t-OP, 97%) and 4-nonylphenol (4-NP, a mixture of branched chain isomers), were purchased from J&K (Beijing, China), and dimethyl sulfoxide (DMSO, 99.5%) was purchased from Sigma Aldrich (St. Louis, MO, USA). Other chemicals were of analytical purity and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All the test chemicals were dissolved in DMSO and stored below − 20 °C.

2.2. Cell culture and reagents

The human breast cancer cell line MCF-7 was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai Institute of Cell Biology (Shanghai, China). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, HyClone, Shanghai, China) supplemented with 10% fetal bovine serum (PAN Biotech Ltd., Aidenbach, Germany), a 1% penicillin-streptomycin mixture (10,000 U/mL, HyClone, Logan, UT, USA), and 0.5% amphotericin B (25 μg/mL, Amresco, Solon, OH, USA) at 37 °C in a humidified incubator (Panasonic, Ehime-ken, Japan) with 5% CO_2. Cells were passaged every 4 days and used for experiments in exponential growth phase.

2.3. Cell proliferation assay

Cell proliferation assays were conducted using the method described by Flor et al. (2016) which was designed to test the estrogenicity of chemicals. MCF-7 cells were harvested by trypsinization, dispensed at 4000 cells/200 μL/well in tissue culture 96-well plates (Corning 3599) in hormone-free medium (phenol-red free DMEM with charcoal dextran treated serum) and cultured for 24 h to allow the cells to attach and eliminate endogenous hormones. Then, fresh hormone-free medium containing different concentrations of EEs was supplemented and co-incubated for 96 h. All the compounds were dissolved in DMSO and added to the medium at the indicated concentrations with a final concentration did not exceed 0.5% (*v/v*). After exposure to EEs for 96 h, the assay was stopped by removing the medium from the wells, the cells were carefully washed with phosphate buffered saline (PBS), and then, 10 μL of Cell Counting Kit-8 (Dojindo, Tokyo, Japan) stock solution was diluted by 100 μL hormone-free medium per well and added to cell culture medium. The plates were then incubated at 37 °C, 5% CO_2 for 2–3 h. Finally, absorbance of each well was determined with a microplate reader (M200, Tecan, Mannedorf Switzerland) at a 450 nm wavelength. Each 96-well plate had a positive control (1×10^{-10} mol/L E2) and a control (DMSO) in triplicate, which were used to keep the absorbance in the same scope. All experiments were repeated at least 3 times, and the results shown are representative of