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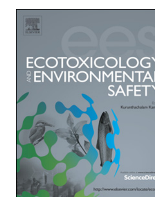

Combined action of estrogen receptor agonists and antagonists in two-hybrid recombinant yeast *in vitro*

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ABSTRACT

Estrogen receptor (ER) antagonistic chemicals in aquatic environments are believed to influence the binding of both endogenous and exogenous estrogens to ERs in aquatic organisms. Although the combined effects of estrogenic compounds have attracted much scientific concern, little work has been done on the influence of such antiestrogens on the biological effects of estrogens. This study focused on how the presence of different amounts of antagonists affects the results of ER agonist activity tests. To achieve this, three questions were stated and answered in sequence. A two-hybrid recombinant yeast assay mediated by ER was adopted, providing a single mode of action and single target of action for this study. Mixtures created by an ER agonist and three antagonists following the fixed-ratio principle were assessed. The concentration of 17β-estradiol causing maximum induction was set as the fixed dose of estrogen in the antagonist activity test (question 1). When the two classes of chemicals coexisted, antiestrogens, which as a whole behaved according to the concentration addition model (question 2), decreased the response of estrogen and compressed the concentration–response curves along the *y*-axis in the agonist activity test (question 3). This may cause the estradiol equivalent to be underestimated and potentially mask the action of estrogenic effects in toxicity evaluation of environmental samples.
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1. Introduction

Among the various endocrine disrupting chemicals (EDCs) in aquatic environments, the estrogen-disrupting chemicals are the topic of most EDC mixture researches (Sharpe and Irvine, 2004). They include the estrogen-mimicking chemicals that simulate the physiological behavior of natural hormones, antiestrogens that antagonize the estrogenic effect in a variety of ways, and chemicals with different actions in various tissues, such as the selective estrogen receptor modulators (SERMs). All of them are released to the environment via different sources and are presented simultaneously instead of separately. Thus, these chemicals not only have adverse effects on organisms at very low-level exposure (Myers et al., 2009), but also have the potential of additive or even synergistic effects when acting in combination (Kunz and Fent, 2006; Sun et al., 2009).

Estrogenic chemicals have been the focus of many studies for a long time. Most of these studies are based on two classic models, concentration addition (CA) and response addition (RA) (USEPA, 2000). The CA model assumes that all mixture components act on the same molecular site competitively and cause the same response (Loewe, 1928). In contrast, when all components behave independently and do not affect or alter the other components' toxicity, the RA model is the better choice (Bliss, 1939). In most scenarios of the mixture toxicity of estrogens, CA has been considered to describe the effects well, with the test organisms covering not only yeast and single cells, but also higher levels of complexity such as rodents and fish (Brian et al., 2005; Payne et al., 2001; Ramamoorthy et al., 1997; Silva et al., 2002). However, compared with the large number of studies on estrogenic chemicals, the joint effects of estrogens and antiestrogens have been less reported. This is probably because they belong to different classes of EDCs (Kortenkamp, 2007): estrogens exert their influence through their interactions with many enzymes and biomacromolecules, while antiestrogens inhibit the estrogenic effect by disrupting or even blocking these interactions.

Abbreviations: CA, concentration addition; DMSO, dimethyl sulfoxide; E2, 17β-estradiol; EC_{50}, half maximal effective concentration; EC_{submax}, sub-maximal effective concentration; EDCs, endocrine disrupting chemicals; ER, estrogen receptor; FUL, fulvestrant; HCH, γ-hexachlorocyclohexane; MoA, mode of action; OECD, Organization for Economic Co-operation and Development; OHT, 4-hydroxytamoxifen; OHT-EQ, tamoxifen equivalent; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; RA, response addition; REP, relative potency; SERM, selective estrogen receptor modulator; ToA, target of action; USEPA, U.S. Environmental Protection Agency; USDHHS, U.S. Department of Health and Human Services.

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Most studies on the combined effects of estrogen and antiestrogen mixtures have been conducted by exposing organisms to increasing concentrations of antiestrogen with a certain dose of estrogen (Petersen and Tollefsen, 2012; Sun et al., 2011). This kind of experimental design can be used to evaluate the efficacy of antiestrogenic drugs by describing how they decrease the response to a constant amount of an estrogen in organisms, but it cannot reflect the characteristics of the environmental samples. In a water sample where both estrogens and antiestrogens coexist, the doses of all chemicals change in proportion during the concentration and dilution processes. It is quite different from the former situation, thus we investigated this scenario.

The objective of this study was to evaluate the deviation of estrogenicity when antiestrogens are present in a sample by answering three questions: (1) for an untested antiestrogen, how do we choose the fixed concentration of the model estrogen for the antagonistic activity test? (2) For a combination of antiestrogens, which model should be applied to predict the combined effects well, CA or RA? (3) For a mixture containing x estrogens and y antiestrogens, does and if so how does the presence of antiestrogens affect the bioassay results of estrogens when the fixed-ratio design is adopted? Among the various modes of action (MoAs) of estrogen-disrupting chemicals, the estrogen receptor (ER) binding capacity plays a significant role, because it is the basis for many estrogen screening bioassays in real environmental applications. Therefore, the ER-mediated production of β-galactosidase in two-hybrid recombinant yeast was used here to offer a single MoA (binding to ER) and a single target of action (ToA, ER) (Li et al., 2008b; Sheeler et al., 2000). One estrogen and three antiestrogens – referred to as ER agonist and antagonists – were selected for mixture preparation, including an endogenous estrogen, two drugs for breast cancer therapy, and one ER antagonistic pesticide.

2. Materials and methods

2.1. Chemicals

17β-estradiol (E2, 97%), 4-hydroxy-tamoxifen (OHT, 98%), fulvestrant (FUL, 98%) and dimethyl sulfoxide (DMSO, 99.5%) were purchased from Sigma-Aldrich (St Louis, MO, USA). γ-hexachlorocyclohexane (HCH, 99.3%) was purchased from AccuStandard Incorporation (New Haven, CT, USA). All test chemicals were dissolved in DMSO to make the standard solutions and stored at $-20\,^{\circ}C$.

2.2. Experimental design

We selected one estrogen (E2) and three antiestrogens (OHT, FUL and HCH) to create the mixtures used in this study (see Table 1). All test chemicals dissolved well and were stable in the solvent, and their concentrations did not change during the 2-h exposure. Thus, we believe the actual and nominal concentrations should be in good agreement with each other.

A series of three experiments was designed according to questions 1–3: an antagonist activity test for single antiestrogens with varying E2 concentrations, an antagonist activity test for antiestrogenic mixtures, and an agonist activity test for estrogen plus antiestrogen mixtures. DMSO was used in the dilution process and served as a co-solvent in the individual chemical and the mixture tests. Its final concentration was set to 0.5% in exposure cultures, which does not cause any cytotoxicity in yeast cells.

First, according to the E2 concentration–response curve, three concentration levels were chosen as the fixed estrogen concentration for the antagonistic activity measurement: EC_{50}, EC_{submax} (which causes the sub-maximum response, also used as the positive control) and $2 \times EC_{submax}$. Seven decreasing concentrations of both OHT and FUL were obtained by double dilution, and their effects were separately assessed based on the different levels of E2.

Then, four mixtures of antiestrogens including three binary mixtures (M1–M3) and a tertiary mixture (M4) were designed using the equivalent effect principle (Table 2), which means that all concentrations of components in a mixture were set to cause equal effects based on the antiestrogenic activity of each chemical. The concentration–response sigmoid curve of every mixture was obtained by testing seven concentration gradients of the fixed-ratio combination with the co-exposure of E2 at the level of EC_{submax}.

Finally, mixtures containing both estrogens and antiestrogens were created, and the assay was conducted without the presence of E2 at a constant concentration. The relationships between estrogens and antiestrogens were arranged in the order of one-to-one and one-to-many. For one estrogen and one antiestrogen, three ratios – EC_{50}(E2):EC_{50}(OHT), $2 \times EC_{50}$(E2):EC_{50}(OHT) and EC_{50}(E2):$2 \times EC_{50}$(OHT) – were set, which were recorded as 1:1, 2:1 and 1:2 (group I). For multiple antiestrogens, the concept of tamoxifen equivalent (OHT-EQ) was introduced to describe the total antagonistic effect. The concentration ratio was arranged by imitating group I, where the combination of OHT and FUL (group II) was used to replace OHT alone. Every antiestrogen was designed to cause an even antagonistic effect and to keep the total OHT-EQ unchanged (details in Table 3).

2.3. Bioassay

The modern theory of ligand–receptor interaction was established based on the discovery of coactivators in 1990s (Hong et al., 1996). The ligand-binding domain of ER contributes to the dimerization interface with estrogens, and a conformational change of the ligand-receptor complex occurs. The complex recruits a coactivator(s) and finally leads to the transcription and translation of downstream target gene. This is the theoretical basis of the two-hybrid yeast assay, which we conducted according to Li et al. (2008a), and all samples were assayed in quadruplicate. After overnight growth at 30 °C with shaking at 130 rpm, the yeast optical density at 600 nm was adjusted to 0.75, to ensure that the strain was in the logarithmic phase. When determining the estrogenic activity, 5 μL of control or dilution of single or mixed stock solutions of test substances were mixed with 995 μL of synthetic dextrose medium containing approximately 5×10^3 yeast cells per mL. The antagonistic activity of samples was measured by co-incubation of yeast cells with the positive control (5×10^{-8} mol/L E2 in DMSO or 2.5×10^{-10} mol/L E2 in exposure culture), which can produce a sub-maximum induction response. Two hundred milliliters of control/test culture were added to each well of a 96-well plate (Corning, Tewksbury, MA, USA). Then, a 2-h exposure at 30 °C was started with vigorous orbital shaking (800 rpm) on a titer plate shaker (Heidolph TITRAMAX 1000, Hamburg, Germany), to prevent yeast cells from precipitating, which was followed by a measurement of the cultures' absorbance at 600 nm (TECAN GENios A-5002, Salzburg, Austria). To lyse yeast cells and release the endoenzyme, β-galactosidase, 150 μL of test culture were removed, and the remaining culture was carefully mixed with 120 μL of Z-buffer (16.1 g/L $Na_2HPO_4 \cdot 7H_2O$, 5.5 g/L $NaH_2PO_4 \cdot H_2O$, 0.75 g/L KCl, 0.246 g/L $MgSO_4 \cdot 7H_2O$) and 20 μL of chloroform at 30 °C and 1300 rpm for 10 min. Then, 40 μL of *o*-nitrophenyl-β-D-galactopyranoside (ONPG, 13.3 mmol/L in Z-buffer) were added to initiate the enzymatic reaction. After incubation at 30 °C for 60 min, the reaction was terminated by adding 100 μL of $Na_2CO_{3(aq)}$ (1 mol/L). To prevent the influence of

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