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Novel full logistic model for estimation of the estrogenic activity of chemical mixtures

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ABSTRACT

Estrogenic compounds as well as other biologically active substances are commonly present in the form of complex mixtures in the environment. There is still no satisfactory model that would be capable of predicting the toxic effects of mixtures containing partial receptor agonists and compounds with different parameters of their dose-response curves. Therefore, a novel Full Logistic Model (FLM) of prediction using all the parameters of dose-response curves has been suggested and compared with previously published approaches. We tested the receptor-binding activities of selected estrogens including full and partial agonists and their mixtures using yeast reporter gene assays and the human T47D cell line. Combination effects were modeled with FLM and predicted curves were compared with the data obtained experimentally. FLM yielded a good fit to the experimental data from both the receptor-binding assays and gave better predictions than the previously published approaches. FLM also provided satisfactory results regarding final partial agonistic dose-response curves with maximum influenced by the inhibitory effect of the partial agonist. FLM is not limited by any simplification like the toxic equivalency factor approach or generalized concentration addition and therefore it could be employed for mixtures containing chemicals with different parameters of their dose-response curves (maximum, minimum, inflex point or slope).

1. Introduction

Estrogenic compounds are widely detected in the environmental samples including waters from treatment plants, wastewaters sludge, surface waters, etc. These compounds in combination with other micropollutants can result in complex mixtures that can exhibit a greater effect than individual compound alone. More than 95% of toxicological research studies are focused on single chemicals and almost completely neglect mixtures (Kortenkamp, 2007). Prediction of mixture effects is a great challenge, because synergism or antagonism in a combination of two or more drugs may occur and no currently available mathematical model can predict and solve this problem fully.

Concentration addition (CA or synonymously dose addition) is a widely used toxicological and pharmacological concept for prediction of the mixture effects of chemicals with a similar mode of action when only the toxicities of the individual components are known (Scholze et al., 2014). The concept of CA (Eq. (1)) estimates mixture effects according to the original mathematical formulation described by Loewe (Loewe and Muischnek, 1926).

$$\sum_{i=1}^{n} \frac{d_i}{ED_i} = 1 \quad (1)$$

where d_i is the dose of compound i in the mixture that produces a toxic effect and ED_i is the dose of the individual components that on their own produce the same effect as the mixture. The sum in this equation is always considered to be equal to 1, i.e. the individual compounds in the mixture simply act additively with no synergistic or antagonistic (supra- or infra-additive) effects. The quotients d_i/ED_i are called toxic units for the individual compounds (Scholze et al., 2014).

At the present time, several methods are available for evaluating combined exposures that are derived from the concept of CA. The toxic equivalency factor (TEF) approach described by Safe (1998a,b) is the most studied and published method. This approach assumes that one compound in the mixture can be replaced by a proportional amount of another compound in terms

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of their potency. This could be described by Eq. (2).

$$C_{mix} = \sum_{i=1}^{n} c_i \cdot \frac{EC_{50s}}{EC_{50i}} \quad (2)$$

where C_{mix} is the hypothetical sum of the concentrations of the compounds present in the mixture, c_i is the known concentration of compound i in the mixture and EC_{50s}/EC_{50i} is the relative potency of compound i according to the standard, s. This ratio is also termed the toxic equivalency factor.

The TEF method assumes that all the individual agents are full agonists with parallel dose-response curves differing only in their potency. This causes serious limitations for the applicability of this model and can lead to insufficient quality of the prediction. It is important to note that the whole concept of CA requires information about the dose of each component of the mixture that produces an effect equal to the toxicity effect of the whole mixture. Thus, it is very difficult to calculate the whole curve for a mixture that contains partial agonists for all the CA models. Eq. (1) cannot be used for description of mixture effects that exceed the maximum effect of the least potent component, because that effective dose cannot be defined (Scholze et al., 2014). This is a serious problem, because many environmental toxicants including estrogens or dioxin-like compounds are partial agonists of their respective receptors (Silva et al., 2007, 2011).

Many research studies approximate the maximum effect of a mixture as being equal to the maximum for the most potent compound (Grund et al., 2011; Johnson et al., 2013; Kunz and Fent, 2006; Payne et al., 2000; Rajapakse et al., 2002). However, it is obvious from other studies that the maximum mixture effect can also be substantially influenced by partial agonists (Scholze et al., 2014; Kunz and Fent, 2006; Rajapakse et al., 2001). This phenomenon should also be considered in a general model for the prediction of mixture effects. This is a serious problem in toxicology and only a few articles have attempted to examine this in detail (Geary, 2013; Howard and Webster, 2009).

Predicting the expected effects of a mixture of agents is an essential point of reference for the assessment of combination effects. At the present time, there is still no model that would be able to predict the whole dose-response curve of mixtures containing compounds with various potencies towards a receptor (EC_{50}), the slopes of their curves and their maximum observed effects.

Our study describes the development of a new predictive general model with verification using estrogenic compounds assayed with yeast recombinant and human cell line screens. This new model can be used to estimate the biological activity of mixtures comprising full and partial agonists and compounds with different slopes of their curves. This new approach assumes that every estrogenic compound acts towards the receptor according to a logistic function (Eq. (3)).

$$E = MAX + \frac{MIN - MAX}{1 + \left(\frac{c}{EC_{50}}\right)^{p}} \quad (3)$$

where E is the toxic effect, MIN and MAX represent the minimum and maximum of the curve, c is the concentration of the agent, EC_{50} is the concentration that gives half-maximal response represented by the inflection point and p is the slope parameter of the curve. All of these parameters and only these are the initial values for calculation of our model prediction. This logistic function is simply a different expression of the Hill function commonly used and recommended as regression in a dose-response relationship (Jenkinson et al., 1995). The equations for the Hill and logistic functions with minimum set to zero are equivalent. Our model also follows the principle of the CA model but does not use Eq. (1) as the initial basis for the derived equations. We call it the Full Logistic Model (FLM) since FLM uses all four parameters of the logistic curve without any simplification. The accuracy of predictions of mixture effects derived from this model were then compared with those calculated by the TEF and generalized concentration addition (GCA) approaches (Howard and Webster, 2009). The accuracy of the predictions was evaluated by comparison with the combination effects observed experimentally.

We employed the standardized yeast reporter gene assay to measure the effects of single compounds and their combinations including various ratios in the mixtures. This assay had the advantage that it monitors only activation or inactivation of the estrogen receptor without any other metabolic transformations or other adverse effects on the test organism. As a second test to verify our model, we employed the CXCL12 assay, which measured altered secretion of CXCL12/SDF1 by the T47D cell line. Secretion of this protein is regulated by activation of an estrogenic receptor and it was therefore an additional useful tool to measure the estrogenic response of our samples.

2. Materials and methods

2.1. Chemicals

Natural estrogen 17β-estradiol (E2; ≥98%) was used as a standard compound in all the calculations of mixtures. Other estrogenic compounds used in the tests were synthetic estrogen 17α-ethynylestradiol (EE2; ≥98%), bisphenol A (BPA; ≥99%) and the insecticide methoxychlor (MET; HPLC 98,7%). All the chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic).

2.2. Yeast assay

The yeast assay uses the genetically transformed strains of *Saccharomyces cerevisiae* (BMAEREluc/ERα and BMA64luc), described in detail in the article by Leskinen et al., (2005). Stock solutions of the estrogenic compounds were dissolved in 30% (v/w) dimethyl sulfoxide (DMSO; ≥99.9%, Sigma-Aldrich, Prague, Czech Republic). The stock solutions for the mixtures with selected ratios of concentrations of the estrogens were prepared using solutions of the individual compounds. Serial dilutions of each of the constituents were also performed in 30% DMSO. E2 was used as a positive control in each experiment. The stock solutions were diluted 11 times with media containing the respective yeast strain so that the concentration of DMSO in the medium did not exceed 3% (Ezechiáš et al., 2012). The growing yeast strain was incubated with the tested compounds at 30 °C for 2.5 h. The culture was then shaken briefly and D-luciferin was added to the medium and immediately measured using a Lumino-M90a luminometer (ZD Dolní Újezd, Czech Republic). All the measurements were performed in triplicate.

2.3. CXCL12 assay

The human T47D breast carcinoma cell line was kindly provided by Dr. Truksa, Academy of Sciences of the Czech Republic. The CXCL12/SDF1 test was used as a second estrogenic test to quantify the estrogenic activity of the compounds alone and of their mixtures. The altered secretion of the stromal cell-derived factor 1 (SDF-1) was measured by the enzyme-linked immunosorbent assay – ELISA. This assay was carried out with stock solutions of each estrogenic compound dissolved in ethanol. Serial dilutions of each constituent were also performed in ethanol. T47D cells were routinely maintained in RPMI medium (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine