Bioassay Analysis Of Mixtures Of Estrogenic And Dioxin-Like Compounds

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Abstract

Complex environmental mixtures contain compounds that act through different mechanisms of action. Thus, the interactions within such mixtures are difficult to characterize from instrumental analysis alone. Development of mechanism-based cell bioassays to detect specific classes of compounds has enhanced the ability to analyze such mixtures. However, little work has been done to evaluate interferences for these bioassays for compounds within mixtures that act through different mechanisms of action, especially mechanisms that can modulate another chemical's potency, efficacy, or time course for response. Moreover, sample extraction and clean-up procedures can potentially alter the ratio of interacting compounds, thus complicating a mass-balance type fractionation approach. In this study, two cell bioassays were utilized as bioanalytical tools to detect estrogenic and dioxin-like compounds in laboratory mixtures and environmental extracts. Total estrogenic and dioxin-like activities were detected by measuring luciferase activity in stably transfected cell lines which contain either an estrogen-responsive element (MCF-7 ERE-Luc cells) or a dioxin-responsive element (H4IIE DRE-Luc cells) linked to a luciferase reporter gene. Laboratory mixtures of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 17-β-estradiol (E_2) at different ratios displayed a dose-dependent inhibition of E_2-induced luciferase activity in MCF-7 ERE-Luc cells by TCDD at a dose as low as 30 fmoles/well. Conversely, E_2 at concentrations up to 75 fmoles/well had no effect on TCDD-induced luciferase activity in H4IIE DRE-Luc cells. Binary mixtures of weakly estrogenic chlorinated pesticides, including chlordane, dieldrin, endosulfan, and toxaphene were also assessed for interactions. Isobolographic analyses revealed that the binary mixture potencies were not statistically different from potencies of each individual compound, indicating that these compounds interact additively, but not synergistically. Taken together, these results demonstrate the utility of bioassays to: 1) characterize interactions within complex mixtures, and 2) be sensitive, rapid, and specific bioanalytical tools which can be used to complement conventional chemical analyses for the purpose of screening environmental samples for estrogenic and dioxin-like activity.

Introduction

The complexities of environmental mixtures require innovative methods and approaches to assess the potential for adverse effects. Many environmental mixtures contain compounds that modulate the responsiveness of multiple receptor-mediated pathways, such as the aryl hydrocarbon (Ah)

receptor, estrogen receptor, androgen receptor, epidermal growth factor receptor, *etc.* While *in vivo* tests are ideal for studying the overall adverse effects from exposure to a mixture, such tests are time-consuming and expensive making them inappropriate for screening or monitoring tools with large numbers of samples. Moreover, *in vivo* tests provide little information on mechanism of action. Likewise, analytical chemistry techniques suffer from the disadvantages of lack of standards and inability to predict interactive effects within mixtures on biological systems. One strategy is to have a tiered approach in which mechanism-based bioanalytical techniques can be used for an initial screen of samples. As positive "hits" are made with this Tier I approach, then samples can be fractionated and rerun through both bioassays and analytical chemistry methods in a toxicity identification and evaluation (TIE) approach.

The purpose of the current study was to evaluate the utility of two mechanism-based bioanalytical techniques to analyze mixtures in which there are compounds representing two different mechanisms of action. Given the current interest and legal mandates to screen for estrogenic compounds, we chose to study interactions between estrogenic and dioxin-like compounds. The goals were to characterize interactions among chemicals that act through the same mechanism of action (*e.g.*, mixtures of estrogen agonists) and interactions among chemicals that act through different mechanisms that may potentially interfere or cross-talk with other mechanisms (*e.g.*, E_2, TCDD, and epidermal growth factor).

Experimental Methods

Cell Culture

Rat hepatoma cells stably transfected with an AhR-controlled luciferase reporter gene construct (H4IIE-Luc; obtained from Dr. Jac Aarts, Univ. of Wageningen, The Netherlands)[1)] and human breast carcinoma cells stably transfected with an estrogen receptor-controlled luciferase reporter gene construct (MCF-7 ERE-Luc; obtained from Dr. Michel Pons, Institut National de la Sante et de la Recherche Medicale, Montpelier, France)[2)] were cultured in medium supplemented with either defined fetal bovine serum (FBS for routine culturing; Hyclone) or charcoal-stripped FBS (for treatment and exposure period; Hyclone). For the bioassay, 96-well culture ViewPlates (Packard Instruments) were seeded with 250 µl of cell suspension at a density of 6×10^4 cells/ml.

Treatment of Cells

After 24 hr, the medium was changed to a medium containing reduced estrogen (dextran-coated charcoal-stripped FBS; Hyclone), and the cells were dosed with either no treatment (blanks), solvent only, E_2, TCDD, or the various samples to be tested in a volume of 1.25 µl. Cells were dosed in triplicate with E_2 in ethanol (0 - 300 pM final concentration), TCDD in isooctane (0.1 - 30 pg/well) or test extracts dissolved in isooctane. Solvent controls for ethanol and isooctane were not significantly different from blanks. Cell viability and luciferase activity were measured three days after dosing. Cell viability was determined by using a Live/Dead™ kit (Molecular Probes) and quantified by using a Millipore Cytofluor 2300 fluorescence measurement system. Luciferase activity was measured by incubating cells with LucLite™ reagent (Packard Instruments) for five minutes at room temperature. Light production, a measure of luciferase activity, was determined with a Dynatech ML 3000 luminometer at 30^0 C. Confluency of wells was verified microscopically, which was found to make normalization to protein unnecessary;

therefore, luciferase activity is reported as either relative light units (RLU) or percent of solvent control.

Data Analysis

Relative potencies were calculated through conversion of the data to probit values and then an EC_{50} was determined if a full dose-response curve was obtained. In cases in which an incomplete dose-response curve was obtained (see Figure 2), the concentration producing a response equivalent to 10% of the maximal response produced by the standard (*e.g.*, E_2 or TCDD) was calculated and used to determine relative potency. Isobolographic analysis of binary mixtures was performed to test for non-additivity. Statistical significance between samples and controls was determined by performing a two-tailed Student's *t*-test (at $p \leq 0.05$).

Results and Discussion

Interactions Between TCDD and E_2

The antiestrogenicity of TCDD has been well-documented[3]. In this study, the dose-dependence for TCDD's antiestrogenicity was evaluated for the full dose-response curve of 17-β-estradiol (E_2) in MCF-7 ERE-Luc cells (Figure 1). In general, parallel dose-response curves were obtained and there was a dose-dependent increase in the EC_{50} for E_2 (*i.e.*, a decrease in potency) with increasing concentrations of TCDD.

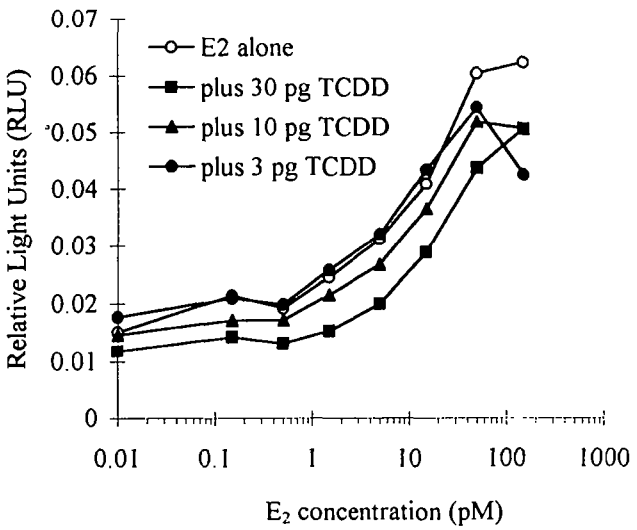

Figure 1. Dose-dependent inhibition of ERE-mediated luciferase activity by TCDD in MCF-7 ERE-Luc cells. Each point represents the mean of three replicates. Coefficients of variation (standard deviation/mean * 100) were generally 5-10 %.

Interactions Between Binary Mixtures of Chlorinated Insecticides

Recent controversy over the potential for mixtures of weakly estrogenic pesticides to act in a synergistic[4] or additive[5] manner prompted us to test such a question in MCF-7 ERE-Luc cells. The results (Figure 2) show that dieldrin, endosulfan, and a 1:1 mixture of endosulfan and dieldrin (E+D) all displayed very similar dose-response curves. Potencies for individual compounds and the binary mixture were not significantly different and were approximately a million-fold less potent than E_2. Thus, synergism was not observed in MCF-7 ERE-Luc cells. In addition, it is important to point out that the magnitude of response (efficacy) elicited by these chlorinated pesticides is considerably less than with E_2.

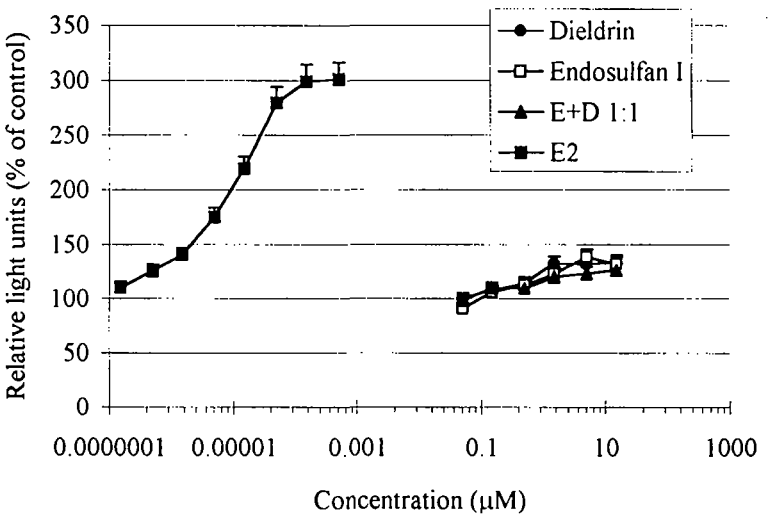

Figure 2. Estrogenic activities of chlorinated pesticides tested individually and as a binary mixture in comparison to E_2 in MCF-7 ERE-Luc cells.

Interactions Between Estrogenic and Dioxin-Like Compounds in Pulp and Paper Mill-Related Samples

A strategy was developed and tested for analysis of environmental samples containing both estrogenic and dioxin-like compounds. Pulp and paper mill-related samples from the area of Lake Saimaa, Finland were assessed for interactions in both the H4IIE DRE-Luc and MCF-7 ERE-Luc bioassays. These samples have been characterized elsewhere and found to contain both estrogenic and dioxin-like compounds (Koistinen *et. al.*, submitted)[6]. All extracts contained both dioxin-like activity (ranging up to 10 ng TCDD equivalents/g dry weight for sediments) and estrogen agonist activity (up to an equivalent of 10 nM E_2) when tested in the bioassays.

Strategies for bioassay analysis of such mixtures and considerations for interpretation of the results will be presented.

Taken together, these results demonstrate the utility of bioassays as reliable, sensitive, inexpensive, rapid, and specific bioanalytical tools which can be used to complement conventional chemical analyses for the purpose of screening environmental samples for estrogenic and dioxin-like activity.

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