SUPERINDUCTION OF THE CALUX BIOASSAY BY ENVIRONMENTAL EXTRACTS: CONSIDERATIONS IN SAMPLE ANALYSIS AND POTENCY DETERMINATION

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Introduction

Accurate evaluation of environmental samples for the presence of anthropogenic contaminants continues to be an important aspect of determining the human risk potential from exposure to these materials. While high resolution instrumental methods (i.e. GC/HRMS) are the gold standard for identification and quantitation of halogenated aromatic hydrocarbons (HAHs), such as polychlorinated dioxins, furans and biphenyls, numerous rapid and relatively inexpensive screening bioanalytical methods have been developed.[1] The Chemically Activated LUciferase eXpression (CALUX) cell bioassay is one technology that has been extensively used for the detection and relative quantitation of HAHs in a wide variety of environmental, biological and food matrices and is based on the ability of these chemicals to bind and activate the Ah receptor (AhR) and AhR signal transduction (i.e. gene expression).[1] The CALUX cell lines contain a stably transfected AhR-responsive firefly luciferase reporter gene that responds to dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin)) and related dioxin-like chemicals with the induction of firefly luciferase gene expression in a chemical-, dose-, time- and AhR-dependent manner. The CALUX bioassay provides an avenue to determine the relative quantity of AhR-active HAHs present in a given sample extract, relative to that of TCDD and this is typically accomplished by comparing the concentration-response curve for luciferase induction by the sample extract (containing a complex mixture of chemicals) directly to that obtained with increasing concentrations of the TCDD standard. Direct comparison between curves allows calculation of a Bioanalytical Equivalent (BEQ) (i.e., a luciferase induction equivalent) for the sample extract relative to that of TCDD dose curve. From a statistical perspective, comparison of the effective concentration at 50% induction (EC_{50}) of each induction curve (the point at which luciferase induction is 50% of maximal) provides the most accurate measurement. However, accurate determination of BEQs from direct comparisons of sample extract and TCDD standard curves requires that the sample extract concentration curves have similar characteristics to that of the TCDD standard curve (i.e. same slope and minimum and maximal induction activity), which would be expected since both utilize the AhR induction mechanism. However, given the nature of the biological CALUX cell system, it is not surprising that extracts containing complex mixtures of chemicals can produce CALUX induction curves that deviate from that of the TCDD standard and numerous methods for estimating sample extract potency from dose curves not meeting these criteria have been reported.[1,2] During our extensive analyses of environmental samples by CALUX, we have found numerous samples that induce CALUX luciferase gene expression to levels 2-3 times greater than that induced by a maximal inducing concentration of TCDD. This superinduction phenomenon complicates accurate estimation of the TCDD BEQs for a given sample extract and can result in overestimation of the potency if standard comparative analysis of induction curves are performed. Here we describe and characterize the CALUX superinduction phenomenon using sediment extracts and provide an method to more accurately calculate the BEQs for sample extracts that produce this response.

Methods

Sample Extract Preparation. Dry, crushed sediment, soil, and plant samples were acquired from our collaborators. Extracts were prepared in hexane or ethanol from 2 g of sample. For analysis of HAHs, sample extracts were cleaned up by filtering them through a sodium sulfate/celite column, followed by a 33% acid silica column and fractionated on a 1% X-CARB column.[3] To analyze crude sample extracts (without cleanup), extracts were only filtered through a sodium sulfate/celite column and the sample/column eluate collected. The final extract volume for each sample was ~4 ml of either hexane or ethanol. Prior to analysis, aliquots of each sample extract (crude or cleaned-up) were dried and resuspended in a small volume of DMSO or ethanol (ETOH) and tested in the CALUX bioassay as described.[3,4]

Cell Culture, Chemical Treatment and CALUX Analysis. Stably transfected CALUX cell lines, H1L6.1c3 (for AhR analysis) and BG1Luc4E2 (for xenoestrogen analysis) were grown as described. The inducing potency of

each sample was evaluated in a range finding study, followed by a nine-point dose curve.[4-6] Cells in a 96-well microplate format were incubated with standards or extracts for 24 hours and luciferase activity determined using a Anthos Lucy 2 or Berthold microplate luminometer as described.

Results and Discussion

CALUX is an AhR-based cell bioassay system that responds to TCDD and related TCDD-like HAHs with the induction of firefly luciferase reporter gene expression. Exposure of these cells to TCDD or extracts containing these chemicals or other AhR agonists results in a typical receptor-dependent sigmoidal concentration (dose)-response curve, from which the relative inducing potency (i.e. BEQ) of the test extract or chemical can be calculated when compared to the TCDD standard curve. Determination of the relative potency of sample extracts or chemicals that produce a full concentration-response curve in the CALUX bioassay with a maximal activity and slope comparable to that produced by TCDD are simple to calculate by direct comparison of the EC_{50}s of the curves (Figure 1; compare TCDD (point 1) and sample C (point 4)) and many linear curve fitting analyses such as the 4-parameter Hill plot or probit analysis have been used.[1,2] However, sample extracts or chemicals that fail to produce a full concentration-response curve comparable to that of TCDD, or the concentration response curve plateaus at a level significantly lower than that produced by TCDD (typified by partial agonists) or those whose slope of the concentration-response curve deviates significantly from that produced by TCDD are commonly encountered and determination of the relative potency of these sample extracts or chemicals are more complicated.[1,2] Several approaches have been described to attempt to determine the relative potency of these types of samples and unlike the full response curve results, these latter relative potency value estimates are less certain. During our extensive extract screening analysis of environmental, biological and food samples, as well as pure chemicals, each of these types of concentration response curves have been observed and we have evaluated and selected the mathematical approaches that lead to the most accurate and justifiable estimate of their relative potency. Our screening results also allowed identification of numerous sample extracts that when tested in the CALUX bioassay resulted in a concentration-response curve whose maximal luciferase activity was significantly greater than that of the TCDD standard curve. Determination of the relative potency of these "superinducing" extracts is particularly problematic given that there exist no published methodology to use to calculate/estimate potency of samples that induce more than the maximal activity of the most potent AhR agonist (TCDD). To examine this CALUX "superinduction" phenomenon in greater detail and to determine an appropriate method to calculate relative potencies for these extracts, we selected sediment sample extracts that induced CALUX luciferase activity to varying degrees. These included an extract that induced CALUX luciferase activity to the same maximal activity as that of TCDD (Figure 1, sample C) and two sample extracts that could "superinduce" CALUX luciferase reporter gene activity (one that produced a full concentration-response curve (Figure 1, sample B) and one that produced a submaximal concentration-response curve (Figure 1, sample A)). Sediment sample A and B induced CALUX luciferase gene expression to a level ~2- and 3-times greater than a maximal inducing concentration of TCDD, respectively. A common approach to estimate the relative potency for an unknown sample extract is to directly compare the luciferase activity at the EC_{50} for TCDD to the identical luciferase activity induced by the sample extract and using the concentration of TCDD at the EC50 to estimate the relative concentration of TCDD bioanalytical equivalents that must be present in the sample extract in order to generate the comparable induction response. While this is an appropriate approach for sample extracts whose induction curves are parallel to and reach the same maximal as the TCDD standard curve, it is not necessarily appropriate for those samples whose maximal CALUX induction activity is less than or greater than the maximal activity induced by TCDD. For example, estimation of the relative potency of sample C (5.2 ng TCDD equivalents/g sediment) using the TCDD standard curve would be appropriate since it meets all established criteria (Figure 1 [compare points 1 and 4] and Table 1). However, estimation of the relative potency of sample A (point 2) and B (point 3) using this same direct EC_{50} luciferase activity comparison approach results in 5.4 and 209 ng TCDD equivalents/g sediment, respectively. If we consider that the synergistic enhancement of the CALUX gene induction response is likely occurring across the entire concentration range, combined with the fact that both induction responses use the exact same receptor (i.e. the AhR), it is justified to normalize or scale the superinduction curves to a maximum of 100% relative to that of TCDD. For sample B, this is simple since a full concentration-response curve was obtained and recalculation of the relative potency of this extract using its actual EC_{50} (point 5 in Figure 1) to that of TCDD, results in a BEQ of 29.7 ng TCDD equivalents/g sediment, a

7-fold decrease in the estimated potency compared to the previous calculation (Table 1). In contrast, the superinduction response with sample A does not reach a maximal activity, so the concentration curve can't simply be scaled/normalized to that of TCDD. In this instance, we propose to use the highest induction response value for nonmaximal superinduction curves as the 100% response value and the concentration response curve scaled as described for sample B. While the EC_{50} value and BEQ generated using this approach would overestimate sample potency, since maximal activity was not obtained, the BEQ estimate resulting from this scaled determination would still be closer to the actual potency value. Using this approach, the relative potency (BEQ) for sample A was calculated to be 1.2 ng TCDD equivalents/g sediment, 4.5-fold less potent than that calculated using the direct TCDD EC_{50} comparison (Table 1). The observation of superinduction responses raises some concerns about the accuracy of relative potency estimations of any sample when the highest CALUX induction response obtained has not yet reached a maximal (i.e. it is still in the linear portion of the curve) and the absolute activity is less than that of the maximal activity of the TCDD standard. If this sample was actually the lower end of a superinduction response curve, the relative potency of the extract (BEQs) would be greatly overestimated as shown above. The superinduction phenomenon is not unique or an artifact of the AhR CALUX bioassay, as we have observed a similar superinduction responses using a stably transfected estrogen receptor (ER)-responsive luciferase reporter gene in human ovarian carcinoma cells (BG1) following exposure to crude solvent extracts of plants (data not shown). While it remains to be determined what accounts for the superinduction response to the sediment extracts in the CALUX cell line, we and others have observed that co-treatment of CALUX cells with TCDD and a chemical (phorbol ester) that stimulates protein kinase C activity will result in a synergistic increase in AhR-dependent gene expression (Figure 2).[6,7] One can envision that substances in the complex mixture of chemicals in an extract could stimulate the AhR (and ER) and protein kinase C, leading to enhanced transcriptional activation activity of the these receptors and/or basal transcription machinery. While the exact mechanism of superinduction is unknown, further investigation may allow identification of a step that can be incorporated into the standard CALUX bioassay protocol to significantly increase assay sensitivity and response. Such studies are underway.

References:

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Table 1. Relative potency comparison of sediment extracts. Results were calculated relative to the TCDD EC_{50} or scaled values from the superinduction curves. See text for details.

	EC50 Values		BEQ Values (ng TCDD Eqv. /g Sediment)	
Sample Curves	Direct TCDD EC50 Comparison	Superinduced Curve	Direct TCDD EC50 Comparison	Superinduced Curve
TCDD Standards (pg)	4.049 (1)[a]	-	-	-
Sample A Eq. (g)	0.75E-3 (3)	3.38E-3 (6)	5.39	1.20
Sample B Eq. (g)	0.019E-3 (2)	0.14E-3 (5)	208.93	29.66
Sample C Eq. (g)	0.76E-3 (4)	-	5.31	-

a. Numbers refer to calculated points in figure 1.

Figure 1. Concentration-response curves for TCDD and sediment extracts. The numbers indicate the EC50s of the induction curves relative to TCDD (points 1-4) or relative to the superinduction curve (points 5 and 6).

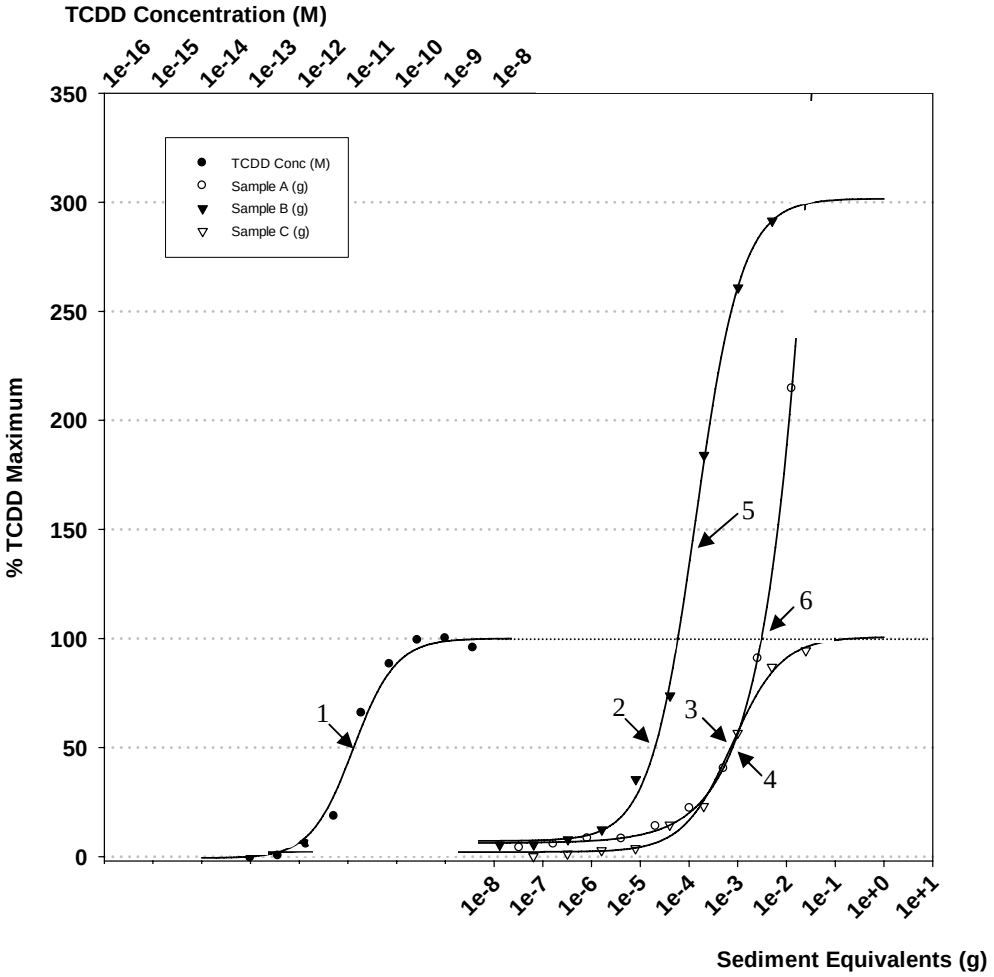

Figure 2. Stimulation of protein kinase C with a phorbol ester (PMA) synergistically increases TCDD-dependent induction of luciferase activity in the CALUX cell bioassay.