PROPOSED CLASSIFICATION SYSTEM FOR TIER-1 SCREENING OF ESTROGENIC EDCs USING THE LUMI-CELL® ER BIOASSAY

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Introduction

Concern worldwide has risen over the past several years due to the strong association between the exposure and bioaccumulation of endocrine disruptor chemicals (EDCs) and their adverse effects on human and wild life populations[1,2]. Some examples of the effects of EDCs are: decreased reproductive success and feminization of males in several wildlife species; increased hypospadias along with reductions in sperm counts in men; increase in the incidence of human breast and prostate cancers; and endometriosis[3-5]. Due to the detrimental effects of environmental exposure to EDCs, there is an obvious need to develop a classification system for these compounds.

A tier-1 screening classification system for EDCs would allow researchers and regulatory officials to determine which chemicals are more likely to result in detrimental effects due to exposure and prioritize research efforts. In this study, 50 compounds, which were on ICCVAM's list of compounds to validate an estrogen receptor transcriptional activation (ER TA) assay, were analyzed using the LUMI-CELL® ER bioassay for estrogen agonists. Twenty-five of these compounds were described as positive for ER agonistic effects across all Mammalian Cell Reporter Gene (MCRG) studies and the other 25 were described as negative for ER agonistic effects across all MCRG studies[9,10]. Dose response data on all of the compounds were graphed to determine if a logical classification scheme could be developed. Factors such as the association between estrogenic response and logical breaks in dose response of compounds, and potential of pharmacological concentrations being achieved in exposure scenarios were taken into account when the classification system was developed.

Methods and Materials

Compound Preparation and Analysis Procedure: A 10 mg/ml solution of each compound was prepared in DMSO. A range finding assay was preformed on each compound using six log dilutions. A serial dilution of the compound was analyzed in triplicate in the activity range as determined by the LUMI-CELL® ER bioassay range finding studies.

LUMI-CELL® ER Bioassay. The BG1Luc4E2 cell line was constructed as previously described by Rogers and Denison (2000). Briefly, BG1 cells were transfected with an estrogen-responsive luciferase reporter gene plasmid (pGudLuc7ere) and stable transfectants selected using G418 resistance[5]. For analysis, the BG1Luc4E2 cell clone resulting from this selection was grown in RPMI 1640 medium. The cells were transferred into flasks containing phenol red-free DMEM media (supplemented with 5% carbon stripped fetal calf serum and G418 sulfate solution), and incubated for four days before harvesting for BG1Luc4E$_2$ bioassay plating. The cells were then plated in 96 well plates and incubated at 37^{o}C for 24-48 hours prior to dosing. The media solution in each well was removed and two hundred micro liters of phenol red-free DMEM containing the indicated concentration of the desired chemical to be tested was added to each well. The plate was then incubated for 20 hours before analysis of luciferase activity.

Measurement of Luciferease Activity. After lysing the cells (Promega lysis buffer), the luciferase activity was measured in a Berthold Orion Microplate Luminometer, with automatic injection of 50 micro liters of luciferase enzyme reagent (Promega) into each well. The relative light units (RLUs) measured were compared to that induced

by the 17beta-estradiol (E2) standard after subtraction of the background activity. Each compound was tested at least three times on three different sets of plates and the EC50 value in Molar concentration was determined using the Microsoft Excel Forecast function.

Results and Discussion

There is a growing need for a reliable tier-1 screening classification system for estrogenic EDCs. The concern for EDCs arises from the detrimental effects on human and wildlife populations resulting from its bioaccumulation in the food chain. A classification system would help researchers and regulators determine which compounds pose the greatest potential threat to human and wildlife populations. Here we report a possible method for classification of xenoestrogenic EDCs based on dose response criteria using the LUMI-CELL® ER bioassay.

After observing the all of the data from the 50 compounds tested for this study, which are depicted in Figures 1 and 2, there were several observations made which lead to the first part of the classification scheme. The first observation was that there were two major groupings of data, where the estrogen responsive element (ERE) was being activated. The first was a grouping of compounds being activated around the 10^{-5}M to10^{-6}M concentrations, and the second was a grouping around the 10^{-9}M to 10^{-11}M concentration. There was also a region from 10^{-7}M to 10^{-9}M showing little or no compounds with activity at these concentrations. This same spread in activity has been observed by other researchers such as; Sonneveld et. al. 2005, Kojima et. al., 2003, and Legler et. al., 2002. These observations lead to the first set of criteria in the classification of estrogenic EDCs. This rule classifies any compound with an EC50 of less than $1x10^{-8}$M as a "Strong" inducer of estrogenic responses and any compound with an EC50 of greater than $1x10^{-8}$M as a "Weak" inducer of estrogenic responses.

The second part of the classification scheme deals with the midpoint Relative Light Unit (RLU) value for 17β-estradiol (E2). E2 is the standard used in the LUMI-CELL® ER agonist bioassay. The second part of the classification scheme states that any compound with a V-Max greater than or equal to the midpoint RLU for E2 would be classified as a Class I compound. Any compound with a V-Max above background, but less than the midpoint RLU for E2 would be classified as a Class II Compound. V-Max is being defined as the maximal RLU response of the compound.

The Third part of the classification scheme states that any compound with a V-Max RLU less than the 3 times the standard deviation of the background solvent (DMSO in this case) would be classified as a Class III compound.

Figure 1 depicts 25 compounds tested using the LUMI-CELL® ER bioassay. These compounds were described as positive for ER agonistic effects across all Mammalian Cell Reporter Gene (MCRG) studies[9,10]. Figure 2 depicts another 25 compounds, which were described as negative for ER agonistic effects across all MCRG studies[9,10]. The boxes represent the classification of the compounds (i.e. Strong, Weak, or Class I, Class II or Class III), or the criteria for each of these classifications. We suggest this classification system as a means of priority setting for potential estrogenic chemical entities that should be investigated for their ability to alter hormonal function in animal models. This system of *in vitro* classification should help limit the number of animals that are used for analysis of endocrine disrupting activity and provide initial dose response data on concentrations to test in animal models therefore limiting the number of animals necessary for further scientific investigations. The LUMI-CELL® ER in vitro testing method may actually be useful in replacing the use of animal models, in some instances, since it has been shown that the response in this system correlates highly with classical estrogen mediated responses such as uterine weight and cellular proliferation in uterine tissues[2].

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Figure 1. Classification scheme for Tier-1 *In Vitro* Screening of Estrogenic EDCs compounds previously classified as Positive for ER agonistic activity across all MCRG studies[9,10].

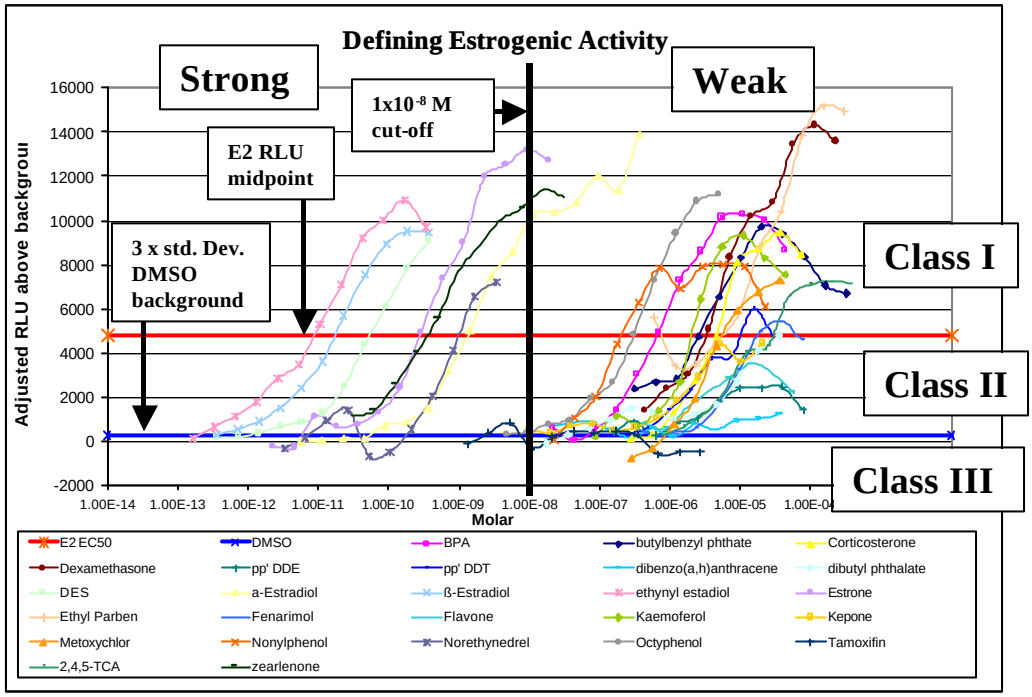
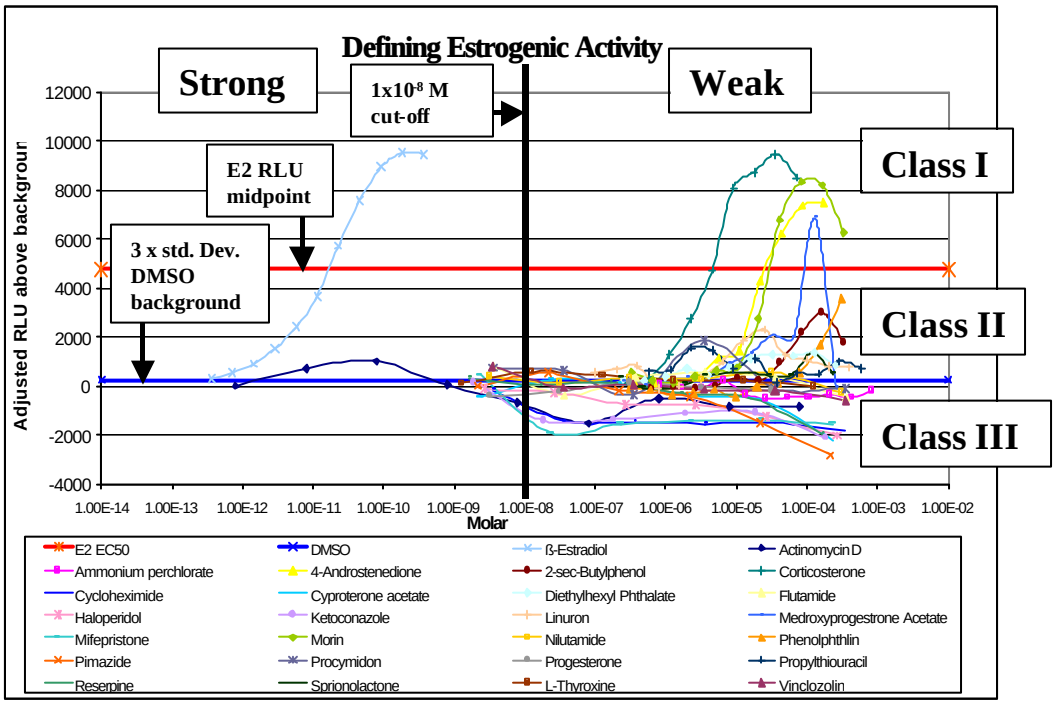


Figure 2. Classification scheme for Tier-1 *In Vitro* Screening of Estrogenic EDCs compounds previously classified as Negative for ER agonistic activity across all MCRG studies[9,10].



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