



# COMPOUNDS OF DIFFERENT CHEMICAL CLASSES ELICIT SUPRAMAXIMAL RESPONSES IN IN-VITRO ANDROGEN AND ESTROGEN RECEPTOR MEDIATED ACTIVITY ASSAYS.

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## ABSTRACT

In-vitro assays designed to identify estrogen and androgen activity were evaluated as an efficient high throughput method for screening a wide range of environmentally significant chemicals for endocrine activity. In the present study more than 160 chemicals were tested for androgen (AR) and estrogen receptor (ER) mediated activity using a cell-based transcriptional activation (TA) model consisting of MDA-kb2 and T47D-KBluc cell lines, respectively combined with competitive receptor binding assays. Receptor binding assays used a human recombinant receptor in combination with polarographic fluorescence detection. Chemicals that were positive in the TA assay were tested in the receptor binding assay as a confirmation of the chemical's ability to elicit response via ER or AR. Solubility and cytotoxicity assessments were also performed. Several of these compounds, which included cadmium chloride, genistein, benzoic acid, 2-hydroxy-, 4-(1,1-dimethylethyl)phenyl ester, benzyl salicylate, 1,3-benzenedicarboxylic acid, diphenyl ester produced a supramaximal response in both assays with response values ranging from 0.5 to 3-fold greater than that obtained from endogenous high affinity ligands, such as estradiol. This supramaximal response was most pronounced when the compound was co-incubated with the reference agonist (Estradiol or DHT). One exception to this occurred with genistein which caused a 2- to 3- fold increase both with and without agonist co-incubation. Supramaximal responses have been observed by others in reporter gene assays. Possible explanations for this phenomenon, depending on the chemical class, could be ligand-dependent differences in the ability of receptor to bind co-activators or interaction of ions with transcription factors. Further study in light of these observations could help elucidate the mechanism of this phenomenon in transactivation models.

## INTRODUCTION

CeeTox is a contract research company providing *in-vitro* toxicity and endocrine screening. In cooperation with the U.S. EPA, endocrine screening of over 160 compounds from different chemical classes was performed in human derived whole-cell transactivation models and in purified human recombinant receptor binding models. The test systems were designed to identify chemicals capable of binding estrogen (ER) or androgen (AR) receptors or inducing estrogen, androgen and glucocorticoid-receptor (GR) mediated gene expression (transactivation). The transactivation models utilized T47D-KBluc and MDA-kb2 cells (derived from human breast cancer cell line) transfected with an ER or AR promoter linked to a luciferase gene (Wilson, et al. 2002, 2004). Several compounds from different chemical classes reported here produced luciferase signal induction beyond the standard agonist maximum level and did not necessarily correlate with binding or show full reversibility with antagonist. Several possible reasons for this supramaximal induction are discussed in the conclusions section.

## METHODS

**Transactivation Assays:** T47D-KBluc and MDA-kb2 cells models were developed by K. Bobseine (Wilson, et al. 2002, 2004) and were generously provided by Drs. Earl Gray and Vicki Wilson at U.S. EPA, NHEERL, RTP, NC, USA. Cells were maintained and assayed as outlined in Wilson, et al. 2004. Compounds were prepared and diluted in DMSO. The final concentration of DMSO was held constant at 0.5%. Control groups were included on each 96 well plate: vehicle control, an Estradiol (E2) agonist maximal response control, and the antagonist ICI-182,780 (ICI) alone or with the test compound at each exposure concentration or with E2. Induction Antagonism experiment plates were co-exposed to a near maximal induction producing concentration of agonist with vehicle or test compound. Cell viability was determined in a separate plate using Propidium Iodide. Solubility was determined by measuring light scattering with a nephelometer.

## METHODS

**Binding Assays**  
Displacement of ligand was measured using InVitrogen/PanVera PolarScreen recombinant human receptor fluorescent polarization assays. Compound solubility was determined via nephelometry. GraphPad Prism was used for graphing and regression analysis.

## RESULTS

Plots represent average and standard error of at least 2 experiments each with an n of 6 wells. Dashed line shows standard agonist control maximum response.

Fig. 1. Standard Compounds : E2 & DHT

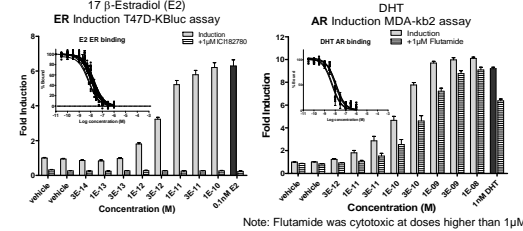


Fig. 2. Cadmium Chloride (CdCl)

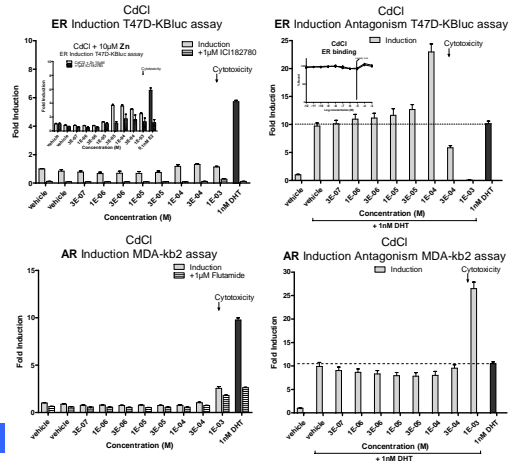
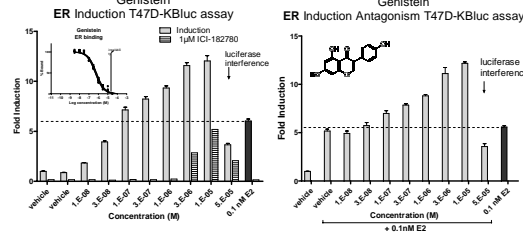


Fig. 3. Genistein



## RESULTS

Fig. 4. Benzoic acid, 2-hydroxy-, 4-(1,1-dimethylethyl)phenyl ester

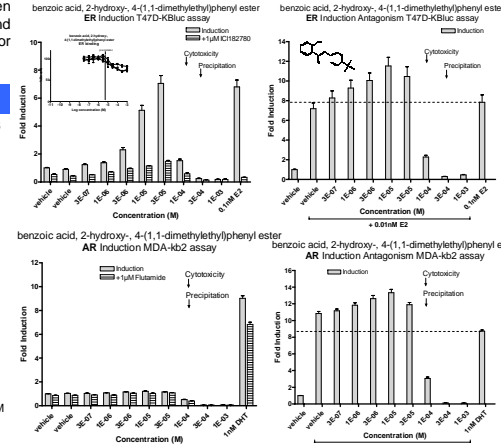


Fig. 5. Benzyl salicylate

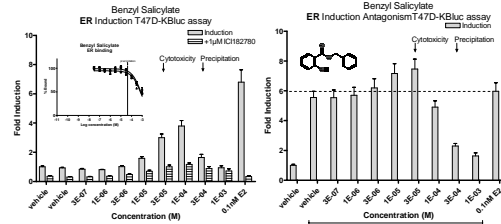
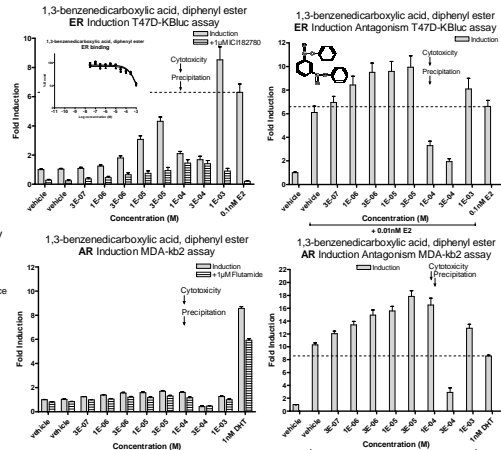


Fig. 6. 1,3-Benzenedicarboxylic acid, diphenyl ester



## RESULTS

Fig. 7. Hexafluorobisphenol A

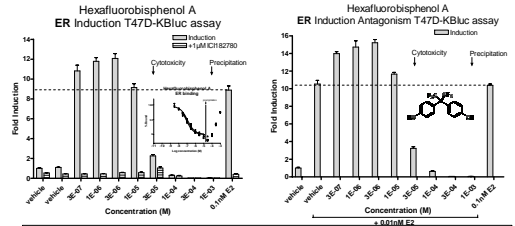


Table 1. Glucocorticoid and progesterone binding and transactivation activities EC50 in logM

Compound	ER $\alpha$ binding	ER T47D-KBluc	AR binding	AR MDA-KB2
Dexamethasone	-	Antag -7M	-4.5M	Ag -6.5M
Corticosterone	-4M	Antag: -7M	-5.5M	Ag -6M
Progesterone	> -4M	Antag: -9M	-7M	partial: -5M

## CONCLUSIONS

Screening of chemicals for endocrine receptor binding and transactivation using these models successfully identified strong and weak agonists and antagonists. Six compounds out of 160 tested in the T47D-KBluc and MDA-kb2 cell transactivation models produced supramaximal responses above positive control levels. Supramaximal responses have been observed by others in similar reporter gene assays (Sonneveld et al 2005, Legler et al., 1999).

These responses could be a result of one or more effects on the mechanisms involved in receptor mediated transactivation and luciferase signal production, which could vary depending on the chemical class. Several possible explanations for this phenomenon have been postulated:

- Ligand-dependent differences in the ability of receptor to bind co-activators (Routledge et al., 2000).
- Enhanced receptor or co-factor renewal, or increased luciferase stability. (Legler et al., 1999)
- Interaction of ions with transcription factors such as MTF-1 (Zhang, et al., 2003) in the case of Cadmium Chloride (CdCl) Fig. 2.
- Differential interaction with ER- $\alpha$  and ER- $\beta$  receptors has been reported with genistein (Escande et al 2006). Western blot analysis indicated that the T47D-KBluc cells contain both the  $\alpha$  and  $\beta$  ER isoforms (Power and Thompson 2003, Wilson 2004). Several of the compounds shown bear some structural resemblance to the ER- $\beta$  specific agonist DPN
- Both MDA-kb2 and T47D-KBluc cells contain endogenous glucocorticoid receptors which could affect co-factor recruiting properties (Wilson, et al. 2002, 2004).

Current investigations in our laboratories are designed to provide additional information on the mechanisms involved in a compound's modulation of transactivation in these models:

- To examine the possible ER- $\alpha$  and ER- $\beta$  components of induction, ER- $\alpha$  or ER- $\beta$  selective antagonists can be included.
- Differences in cofactor recruitment could be examined using recently developed TR-FRET assay models for co-activator interactions.
- Involvement of glucocorticoid and progesterone receptors could be examined by co-incubating glucocorticoid and progesterone receptor antagonists. (References can be provided separately).