ICCVAM/NICEATM EXPERT PANEL RECOMMENDATIONS FOR THE STANDARDIZATION AND VALIDATION OF *IN VITRO* ESTROGEN RECEPTOR (ER) AND ANDROGEN RECEPTOR (AR) TRANSCRIPTIONAL ACTIVATION (TA) ASSAYS.

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Abstract

A number of published studies have shown that a variety of natural and anthropogenic substances can interact with the endocrine system. As a result, legislation was enacted requiring the U.S. EPA to develop a screening and testing program to identify these substances. Within the Tier 1 battery of screening test methods, *in vitro* ER and AR TA assays are proposed to identify estrogenic or androgenic agonists or antagonists. The *in vitro* results will be considered with data from other Tier 1 *in vitro* assays and Tier 1 *in vivo* assays in a weight-of-evidence evaluation of the need for testing in the more definitive Tier 2 *in vivo* assays. A comprehensive literature review indicated that there are no adequately validated *in vitro* ER or AR TA assays. After considering the available data, an ICCVAM/ NICEATM-sponsored Expert Panel developed recommendations for future development, standardization and validation efforts. The Expert Panel recommended that, for ER TA assays, the focus should be on a mammalian cell line transfected with a human ER, and with a luciferase reporter construct containing multiple vitellogenin estrogen response elements. A pre-validation study should be performed to determine whether transiently or stably transfected cell lines would be the most useful for a general screening method. For AR TA assays, the recommended focus should be on the use of a mammalian cell line containing an endogenous human AR that is transduced with an adenovirus containing a luciferase reporter gene. Recommendations were also developed for minimum procedural standards and substances to use for validation studies. These recommendations should facilitate standardization and validation of protocols for ER and AR TA assays.

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Introduction

Endocrine disrupting substances are defined as chemicals that interfere with the normal function of endogenous hormones, either during development or during the life of the organism (EPA, 1998). Concern regarding endocrine disruption stem from reports of reproductive and developmental abnormalities in animal populations exposed to high levels of certain pollutants in the environment (Ankley et al, 1998). In addition, human health consequences, including increases in the incidence of birth defects, cancers in hormonally-receptive tissues, and decreased fertility, have been attributed to the exposure of humans to endocrine disruptors (Carlsen et al 1992; Toppari et al, 1996). Prompted by these concerns, the U.S. Congress directed the U.S. Environmental Protection Agency (EPA) in 1996 to validate and implement a screening and testing program to evaluate the potential of these substances to cause hormone-related health effects (Public Law [P.L.] 104-170). In response to advice from the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), EPA proposed the Endocrine Disruptor Screening Program (EDSP; EPA, 1998) (**Figure 1**). The EDSP consists of a Tier 1 screening battery of *in vitro* and *in vivo* assays which are designed to identify substances that can interact with hormone receptors in the cell or organism resulting in transcriptional activation of genes involved in endocrine functions. The Tier 2 of the EDSP is a battery of *in vivo* tests that provides detailed information on concentration response relationships and specific abnormal effects. Included among the proposed Tier 1 *in vitro* assays are estrogen receptor (ER) and androgen receptor (AR) binding and transcriptional activation (TA) assays.

In 2000, EPA, requested that ICCVAM[1] evaluate the validation[2] status of the available *in vitro* ER and AR binding and TA assays. In conducting this evaluation, ICCVAM directed NICEATM to prepare background review documents (BRDs) on each test method approach, summarizing the available information and data on these test methods. ICCVAM organized an independent Expert Panel to review the BRDs and to develop recommendations regarding future test method development and validation efforts, including:

- methods that should undergo further evaluation in validation studies, and their relative priority
- proposed minimum procedural standards for each type of assay
- adequacy of available test method protocols for validation studies
- appropriateness of the test substances proposed for future validation studies

This poster presents a summary of the results of the Expert Panel review of *in vitro* ER and AR TA assays. Please refer to poster #763 entitled "ICCVAM/NICEATM Expert Panel Recommendations for the standardization and validation of in vitro estrogen receptor (ER) and androgen receptor (AR) binding assays" for corresponding information about the ER and AR binding assays.

For more information about the Expert Panel review, please refer to NIH Publication 03-4503, titled *ICCVAM Evaluation of In Vitro Test Methods for Detecting Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays.* This report, as well as additional information about ICCVAM, are available on the ICCVAM website (http://iccvam.niehs.nih.gov).

[1]ICCVAM and its support center, NICEATM, coordinate evaluations of the scientific validity of new, revised, and alternative toxicological test methods proposed for specific regulatory uses. [2]Validation is the process by which the reliability and relevance of a test method are established for a specific purpose. Reliability is the extent to which a test can be performed reproducibly within and among laboratories and relevance is the extent to which a test method can correctly predict or measure the biological effect of interest (ICCVAM, 1997).

Background

The current hypothesis for ER- and AR-mediated endocrine disruption is that certain xenobiotic substances can bind to the respective hormone receptors and the resulting complex initiates transcriptional activation of endocrine-responsive genes by specifically binding to hormone response elements upstream of the genes required for normal endocrine function (**Figure 2**). Besides acting as agonists, some substances act as antagonists because they compete for the endogenous steroid and thereby block the normal effects induced by the hormone.

In vitro ER and AR TA reporter gene assays are designed to measure transcriptional activation of a gene in cells that have been artificially manipulated by transfection of an expression vector for the receptor, and a vector for the reporter gene. Transcriptional activation is measured by induction of enzyme activity or by cell proliferation. Potential agonist or antagonist activity may be inferred for a substance by its ability to compete with the natural ligand for binding to the ER or AR. As opposed to receptor-binding assays, TA assays can differentiate between agonists and antagonists.

TA alone is not sufficient to indicate or predict subsequent cellular effects. For this reason, *in vitro* ER and AR TA assays will be used in conjunction with other *in vitro* assays as well as *in vivo* assays for Tier 1 screening. Results from all these assays will be used in a weight-of-evidence approach to select substances for Tier 2 testing.

Figure 1. Chronology of Events Leading to Expert Panel's Recommendations

Concern regarding Endocrine-disrupting substances

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1996 – Congress directs EPA to develop a screening program

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1998 – EDSP proposes Tier 1 screening battery - *in vitro* and *in vivo* assays and a Tier 2 testing battery - *in vivo* assays

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2000 – ICCVAM asked to validate status of Tier 1 *in vitro* assays - ER and AR binding and TA assays

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2001–2002 – NICEATM compiles relevant data into BRDs

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2002 – Expert Panel reviews BRDs and makes recommendations to ICCVAM

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2003 – ICCVAM recommendations will be submitted to EPA for consideration

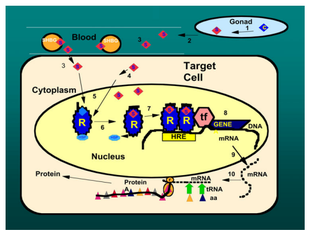

Figure 2. The numbers in the figure denote the sequence of events that occur in an organism leading to transcriptional activation of a hormone-responsive gene in the target tissue

Content of BRD's

- a description of the types of test methods used to measure the endpoints of interest;
- published and submitted data on substances evaluated in the test methods being considered;
- an evaluation of the reliability and performance of the test methods being considered;
- test method specific protocols provided by interested scientists;
- a prioritized list of test methods recommended for validation;
- proposed minimum procedural standards for the types of test methods being considered; and
- a list of substances proposed for future validation studies.

Specific Content of BRDs on TA Assays

Summary of *In Vitro* Estrogen Transcriptional Activation Assays in the BRD

- **95 Assays** (defined by cell line, ER, or specific endpoint)
  - **63 Mammalian cell TA assays**
    - 9 human cell lines
    - 3 non-human mammalian cell lines
    - Endogenous ER receptors or ERα and/or ERβ receptors transiently or stably transfected into human, mouse or rat cell lines.
    - Chloramphenicol acetyltransferase or luciferase reporter gene
  - **22 Yeast (*S. cerevisiae)* TA assays**
    - human ER receptors (hER, hERα, hERβ) mouse ER (mER), rainbow trout (rtER) receptors
    - β-galactosidase reporter gene
  - **10 Mammalian cell proliferation assays**
    - 4 human cell lines
- **698 substances were tested in one or more assays**

Summary of *In Vitro* Androgen Transcriptional Activation Assays in the BRD

17 Assays(defined by cell line, AR or specific endpoint)

- **15 Mammalian cell TA assays**
  - 6 human cell lines
  - 3 non-human vertebrate cell lines
  - AR (human, mouse, rainbow trout) receptors
  - Chloramphenicol acetyltransferase or luciferase reporter gene responses
- **1 Yeast (*S. cerevisiae)* assay**
  - AR (human) receptor
  - β-galactosidase reporter gene response
- **1 Mammalian cell proliferation assays**
  - 1 human cell line
- **147 substances were tested in one or more assays**

Cell Lines Used in ER TA Assays

Human cell lines

*** BG-1 (human ovarian carcinoma)
* HEC-1 (human endometrial cancer)
*/** HEK293 (human embryonal kidney)
*/** HeLa (human cervical cancer)
* HepG2 (human liver tumor)
* Ishikawa (human endometrial cancer)
* MCF-7 (2 strains) (human breast cancer)
* MDA-MB-231 (human breast cancer,)
*** T47D (human breast adenocarcinoma)

Rodent and Monkey cell lines

** CHO-1 Chinese hamster ovary)
* COS-1 (monkey kidney)
* ELT-3 (rat uterine leiomyoma)

Yeast

** 13 strains of *S. cerevisiae*

Cell proliferation

*** Ishikawa (human endometrial cancer)
*** MCF-7 (2 strains) (human breast cancer)
*** T47D (human breast adenocarcinoma)
*** ZR-75 (human breast cancer)

* indicates that the ER was transiently transfected into the cell line
** indicates that the ER was stably transfected into the cell line
*** indicates that the ER is endogenous in this cell line

Cell Lines Used in AR TA Assays

Human cell lines

** HeLa (human cervical cancer)
* HepG2 (human liver tumor)
*** MDA-MB-453 (human breast cancer)
*** MDA-MB-453-kb2 (human breast cancer)
** PALM (human prostate adenocarcinoma)
* PC3 (human prostate adenocarcinoma)

Rodent and Monkey cell lines

*/** CHO-1 Chinese hamster ovary)
* CV-1 (monkey kidney)

Fish cell line

* EPC (carp epithelioma papulosum)

Yeast

** *S. cerevisiae* (2 strains)

Cell proliferation

*** LNCaP-FGC (human prostatic adenocarcinoma)

* indicates that the AR was transiently transfected into the cell line
** indicates that the AR was stably transfected into the cell line
*** indicates that the AR is endogenous in this cell line

Number of Positive and Negative Substances Selected for Validation in ER and AR TA BRDs

	ER TA		AR TA	
	Agonism	**Antagonism**	**Agonism**	**Antagonism**
No. positives	25	16	18	19
No. negatives	6	4	10	4
TOTALS	31	20	28	23

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Expert Panel Recommendations

Development and Validation of *In Vitro* ER TA Assays

- **No specific ER TA assay can be recommended**
- **A comparative study is required to measure:**
  - responses of cell lines transiently or stably transfected with human ER and luciferase (Luc) reporter gene constructs
  - response of cell line with endogenous ER and transfected with the same reporter construct
  - response of a selected set of test substances in this comparative study.

Development and Validation of *In Vitro* AR TA Assays

- **No specific AR TA assay can be recommended at this time**
- **Development of an human cell line with**
  - an endogenous AR, that is transduced with an adenovirus containing a reporter vector; cell line cannot be transfected with recombinant human AR because of patent constraints.
  - minimal response levels to the glucocorticoid (GR) and progesterone (PR) receptors by
    - replacing MMTV response element
    - identifying a cell line with a low level of PR receptor or use of DHT (most cell lines require GR for normal function)
  - a Luc reporter gene construct

General Recommendations

- **Do not incorporate exogenous metabolic activation system in the TA assays at this time**
  - Evaluate the metabolic capacity of cell lines chosen for ER and AR TA assays
- **Appropriate pre-validation studies should be conducted to generate data for biostatisticians to develop appropriate statistical methods**

Selected Minimum Procedural Standards

Reference Estrogen and Androgen:

- Reference estrogen for ER TA agonism and antagonism assays should be 17β-estradiol.
- Concentration of 17β-estradiol used for antagonism assays should yield 70-80% of the maximal induction of the 17β-estradiol response in the agonism assay
- Reference androgens for AR TA assays are methyltrienolone (R1881), which has high specificity and is not metabolized, or 5-dihydrotestosterone (DHT) particularly if the cell line contains high PR levels.

Preparation of Test Substances:

- Test substances should be prepared in water, 95-100% ethanol, or dimethyl sulfoxide (DMSO) (listed in order of preference).

Concentration Range, Dose Spacing and Limit Concentration of Test Substances:

- Seven concentrations ranging from 1 nM to 1 mM spaced at log intervals
- Limit concentration should be1 mM unless precluded by solubility and toxicity

Solvent and Positive Controls:

- **Solvent controls**
  - include in each assay
  - at highest concentration and volume added to test substances
- **Suggested positive controls**
  - **ER TA agonism -** 17β-estradiol and a weak acting agonist (e.g., genistein)
  - **AR TA agonism –** Methyltrienolone (R1881)
  - **ER TA antagonism -** strong antagonist (e.g., ICI 182,780)
  - **AR TA antagonism -** hydroxyflutamide (10 μM or lower)

Within-test Replication:

- Triplicates at each concentration; repeat test if questionable or borderline response

Data Analysis:

- Agonism – The response (measured in light units) is compared to the solvent control. If possible an EC_{50} value is determined; if not, a trend analysis is performed.
- Antagonism – An IC_{50} which is the ability of a test substance to inhibit TA induced by the reference substance by 50% is calculated
- Develop appropriate statistical methods during assay development.

Assay Acceptance Criteria:

- The EC_{50} or IC_{50} response for the reference substance and the positive control, respectively, should be consistent with historical values for that test system.

Interpretation of Results:

- **Agonist –** Reporter gene expression is significantly higher than solvent control determined by a statistical test
- **Antagonist –** Reporter gene expression is decreased significantly compared to reference estrogen or androgen determined by a statistical test

Other Recommendations

- 78 substances, derived from a number of lists, is recommended by ICCVAM for validation of each binding and TA assay (see Poster #1072 for additional details).
- A centralized repository of substances of verified purity needs to be established for validation studies.

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