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

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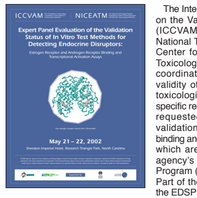
Abstract

A number of studies have suggested 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 substances with endocrine disrupting activity. Within the recommended Tier 1 battery of screening test methods, *in vitro* ligand binding assays are proposed to identify substances that might interact with the ER or AR. The *in vitro* results would be considered with data from other *in vitro* and *in vivo* Tier 1 assays in a weight-of-evidence evaluation for further testing in the more definitive *in vivo* Tier 2 assays. A comprehensive literature review prepared by NICEATM identified no adequately validated *in vitro* ER or AR binding assays. After considering the available data, an ICCVAM/NICEATM-sponsored Expert Panel developed recommendations for future test method standardization and validation efforts. For both types of binding assays, the Panel recommended that recombinant human receptor and high-throughput procedures be validated; however, patent issues with the human AR may make it necessary to use a recombinant receptor derived from a non-human primate. For ER binding assays, the Panel recommended the use of intact human ERα or β proteins, or the equivalent proteins from the rat. Recombinant receptors from wildlife should be used when screening for ecological effects. Recommendations were also provided for minimum procedural standards and substances for validation studies. These recommendations should facilitate standardization and validation of protocols for ER and AR binding assays. Supported by NIEHS Contract N01-ES-85424.

Introduction



The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and its support center, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Test Methods (NICEATM), coordinate evaluations of the scientific validity of new, revised, and alternative toxicological test methods proposed for specific regulatory uses. In April 2000, EPA requested that ICCVAM evaluate the validation status of *in vitro* ER and AR binding and transcriptional activation assays, which are proposed components of the agency's Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery. Part of the agency's mandate to develop the EDSP requires use of standardized test methods that are appropriately validated prior to their use in the testing program. In support of the ICCVAM evaluation, NICEATM conducted a comprehensive literature search for relevant peer-reviewed publications on the test methods, and summarized the available pertinent data, protocols, and other relevant information about the assays, in background review documents specific to each assay type. A preliminary assessment of this information by ICCVAM and EPA determined that there were no adequately validated test methods. Although none of the test methods were sufficiently standardized at that time, there was ample information to develop recommendations for future development and validation efforts. To facilitate standardization and validation of the test methods, ICCVAM and NICEATM convened an independent Expert Panel (Panel) in May 2002 to evaluate the available data and protocols for 14 *in vitro* ER binding methods, 11 *in vitro* AR binding methods, and a variety of *in vitro* ER and AR transcriptional activation methods. Based on the available information, the Panel made a number of recommendations on future test method development and validation efforts, including:

- The identification of test methods that should be the focus of future validation efforts, and their relative priority;
- Proposed minimum procedural standards for each type of test method;
- The adequacy of available test method protocols for validation studies; and
- Test substances proposed for future validation studies.

This poster presents a summary of the results of the Panel review of *in vitro* ER and AR binding assays, which was based on the information summarized in the NICEATM background review documents.

Please refer to SOT 2003 poster #1071 entitled "ICCVAM/NICEATM Expert Panel Recommendations for the Standardization and Validation of *In Vitro* ER and AR Transcriptional Activation Assays" for corresponding information about the transcriptional activation assays.

Background

In Vitro ER and AR Binding Assays

General Description of Competitive Binding Assays

Competitive binding assays measure the binding of a single concentration of radiolabeled reference estrogen or androgen in the presence of various concentrations of a competitor (the test substance). In a routine test, the concentration of competitor typically ranges over at least six orders of magnitude. Cells containing the receptor of interest, cytosolic fractions from cells containing the receptor, or purified/semi-purified receptor are treated with a saturating concentration of the radiolabeled reference substance[1]. Following this treatment, the cells, cytosol, or purified protein are challenged with the competitor and the amount of radioactive reference ligand remaining bound to the receptor is measured by scintillation counting.

Results from competitive ER and AR binding assays are generally reported as IC_{50} or relative binding affinity (RBA) values, where the IC_{50} is the inhibitory concentration of test substance that displaces 50% of the radiolabeled reference ligand from the receptor, and the RBA is calculated from the IC_{50} value, as shown below:

$$RBA = \frac{IC_{50} \text{ for reference ligand}}{IC_{50} \text{ for test substance}} \times 100$$

As a rule, the RBA of the reference ligand is set at 100. This allows the relative binding of test substances to be compared across test laboratories and assay types. The use of the RBA also minimizes differences in IC_{50} values that may be caused by differences in receptor concentrations from different preparations.

[1]For *in vitro* ER binding assays, the reference estrogen is typically 17β-estradiol. For *in vitro* AR binding assays, four different reference androgens have been used: two naturally occurring (5α-dihydrotestosterone and testosterone), and two synthetic (mibolerone and methyltrienolone).

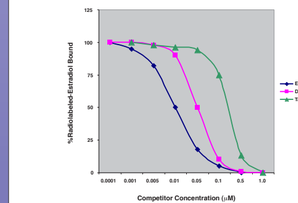


Figure 1. Representative Competitive Binding Curves (Estrogen Receptor)

Rationale for Inclusion of *In Vitro* ER and AR Binding Assays in the EDSP Tier 1 Screening Battery

- Suitable for large-scale screening
- Based on well-elucidated mechanisms of action
- Measure specific effects

Scientific Basis for Using *In Vitro* ER and AR Binding Assays as Screening Methods for Endocrine Disruption

The current hypothesis for ER- and AR-mediated endocrine disruption is that certain xenobiotic substances bind to the receptor and either mimic or block the action of the endogenous ligand (i.e., 17β-estradiol; 5α-dihydrotestosterone).

In vitro ER and AR binding assays are designed to identify substances that bind to the ER or AR and that might act as an ER or AR agonist or antagonist *in vivo*. Receptor binding assays detect both agonists and antagonists, but cannot distinguish between the two, and are therefore not sufficient to predict subsequent cellular effects, or to predict adverse effects in humans.

Expert Panel Review

Table 1. ***In Vitro*** **ER Binding Assays Reviewed by the Panel**

Method of Competitive Displacement	Source of Estrogen Receptor		Substances Reported in Publications	Primary Uses of the Assays
The majority of these test methods measure displacement of radiolabeled 17β-estradiol from the ER. The bound radiolabeled reference estrogen is measured by scintillation counting.	Cell cytosol Cytosol from animal tissues	MCF-7 cell cytosol Rat uterine cytosol Mouse uterine cytosol Rabbit uterine cytosol	434 substances were tested in one or more of 14 test methods. Only 2.4% of these substances had been tested in 10 or more assays. In contrast, 63.2% had been tested in one assay only.	In basic research studies to characterize the binding, kinetics of receptor binding, and binding processes. To characterize the binding of synthetic receptor agonists and antagonists (e.g., potential pharmaceutical agents). Limited use as a method for endocrine disruptor screening.
	Whole cells Semi-purified/purified recombinant receptor	Rat cells Human ERα Human ERβ Rat ERβ		
	Fusion proteins Glutathione-S-transferase fused with def domains of ER from various species	GST-human ERα GST-human ERβ GST-chicken ER GST-rainbow ER GST-rainbow trout ER		
One test method uses fluorescence polarization techniques to measure the competitive displacement of fluorescently-labeled estrogen from ER.	Purified recombinant receptor	Human ERα-FP		

Performance and Reliability Analysis

Data from the various *in vitro* ER binding assays reported in the literature were of limited value for an assessment of comparative performance and reliability because too few substances had been tested multiple times in different test methods. However, two-way and three-way analyses of variance (ANOVA) performed on selected IC_{50} and RBA data suggest that assays using purified/semi-purified recombinant receptor (human ERα or ERβ; rat ERβ) are more sensitive than assays using whole cells or cytosol.

Table 2. ***In Vitro*** **AR Binding Assays Reviewed by the Panel**

Method of Competitive Displacement	Source of Androgen Receptor		Substances Reported in Publications	Primary Uses of the Assays
All assays measure radioligand displacement of a radiolabeled reference androgen from the AR. The radiolabeled bound reference androgen is measured by scintillation counting.	Cytosol from human cell line containing endogenous AR Cytosol from mammalian cell line transfected with human AR Cytosol or nuclear fraction from animal tissues	MCF-7 cell cytosol LNCaP cell cytosol Cytosol from COS-1 cells + human AR Rat prostate cytosol Rat epididymal cytosol Rat epididymal cell nuclear fraction Rat uterine cytosol	108 substances were tested in one or more of 11 test methods.	In basic research studies to characterize the receptor, the kinetics of receptor binding, and binding processes. To characterize the binding of synthetic receptor agonists and antagonists (e.g., potential pharmaceutical agents). Limited use as a method for endocrine disruptor screening.
	Whole cells with endogenous AR Whole cells transfected with human AR	Human genital fibroblasts CHO cells + human AR		
	Whole cells transfected with human AR and MMTV-LUC Semi-purified recombinant receptor	COS-1 cells + rainbow trout AR Human AR		

Performance and Reliability Analysis

There were not enough *in vitro* AR data in the literature for an assessment of comparative test method performance and reliability.

Selected Panel Recommendations for Development and Validation of *In Vitro* ER and AR Binding Assays

ICCVAM asked the Panel to make recommendations for further development and validation of in vitro ER and AR binding assays based on a consideration of factors such as comparative performance, reliability, elimination of animal use, and use of receptors from the species of interest. Some key recommendations are summarized below.

ASSAY PRIORITY

In Vitro **ER Binding Assays**

- Highest priority should be given to methods using recombinant purified/semi-purified receptors (i.e., human or rat ERα and ERβ) for standardization and validation efforts.

In Vitro **AR Binding Assays**

- Highest priority should be given to methods using recombinant purified/semi-purified receptors for standardization and validation efforts.
- Recombinant human and rat AR were considered most suitable scientifically for further assay development; however, patents and an exclusive license agreement might restrict commercial use of the cDNA sequences for these receptors.
- Use of an AR sequence from a species closely related to humans was suggested.
- The status of the patents and the license agreement requires further investigation and clarification in order for the development of AR binding assays to proceed in commercial laboratories.

METHODOLOGY

In Vitro **ER and AR Binding Assays**

- The Panel recommended that a standardized preparation of recombinant receptor be used to further develop and validate the assays, not only for quality control purposes, but also so that valid comparisons among experiments and laboratories could be made. Purified or semi-purified receptor preparations are preferred because such preparations:
  - Are free of other receptors that could interfere with the assay
  - Minimize variability among experiments and laboratories
  - Can be readily adapted to high-throughput methods
- To screen for possible effects in wildlife, recombinant receptors from relevant species should be used.
- Consideration should be given to methods that do not use radioactive materials (e.g., fluorescence polarization).
- Inclusion of an exogenous metabolic activation system (MAS) in the binding assays should be deferred pending further evaluation.
  - The MAS methodology will depend on the specific test method validated.
  - Available information on the metabolism of the substances proposed for validation should be compiled, including the degree to which metabolism of the substances affects binding to the receptor.

Selected Minimum Procedural Standards for *In Vitro* ER and AR Binding Assays

To facilitate assay standardization, ICCVAM asked the Panel to evaluate proposed minimum procedural standards that should be incorporated into protocols for in vitro ER and AR binding assays. The most pertinent recommendations are summarized below.

Recommended Reference Estrogen and Androgen:

- Hexa-tritium labeled 17β-estradiol for *in vitro* ER binding assays.
- 5α-Dihydrotestosterone (DHT) for *in vitro* AR binding assays that use purified recombinant protein. DHT should not be used for cytosolic or cell-based AR binding assays because it is metabolized.
- Methyltrienolone (R1881) is recommended for animal tissue cytosol or cell-based assays because it is not metabolized. However, since R1881 binds to the progesterone receptor (PR), which is present in some cytosol and cell preparations, a substance that has a high affinity for the PR must be added to block R1881 from binding to that receptor.

Dissociation Constant (K_d) of the Reference Estrogen and Androgen: The K_d of the reference ligand should be determined in each experiment.

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

Concentration Range, Dose Spacing and Limit Concentration of Test Substances: Use seven test substance concentrations, ranging from 1 nM to 1 mM, spaced at log intervals, with a limit concentration of 1 mM. Consider solubility characteristics and possible denaturing effects of the test substance.

Solvent and Positive Controls: Solvent controls should be included in each assay; the solvent volume should be identical to that used in reaction mixtures containing the test substance. In addition, each assay should include a positive control substance with a binding affinity about two to three orders of magnitude lower than the reference ligand. The positive control should be tested at multiple concentrations.

Within-test Replicates: Triplicates are recommended at each concentration.

Data Analysis: Ligand titration array was recommended as an alternative to traditional approaches for determining IC_{50} and K_d values. However, a careful analysis of the resulting data is needed to identify the most appropriate non-linear regression statistical models for computing IC_{50} and K_d values.

Test Acceptance Criteria: The response (i.e., IC_{50}) for the reference ligand and the positive control should be consistent with historical values.

Interpretation of Results: Substances that competitively bind to the receptor but do not induce a 50% reduction in binding of the radiolabeled reference ligand (i.e., an IC_{50} has not been achieved) should be considered "equivocal"

Receptor Preparation: Sodium molybdate and a cocktail of protease inhibitors should be added to receptor preparations from cell and tissue extracts to prevent receptor degradation.

Separation of Bound from Free Radioligand: Dextran-coated charcoal is recommended.

Protocols for *In Vitro* ER & AR Binding Assays

To determine the adequacy of existing protocols, the Panel was asked to review two protocols from the U.S. EPA: one for an ER binding assay that uses rat uterine cytosol (RUC), and the other for an AR binding assay that uses rat prostate cytosol (RPC). In addition, the Panel reviewed several protocols submitted by non-EPA scientists who routinely use these assays.

Panel Recommendations:

- The U.S. EPA RUC protocol could serve as a template for other *in vitro* ER binding assays after the protocol is revised to include the recommended minimum procedural standards.
- The U.S. EPA RPC protocol requires additional information. None of the other AR binding protocols was sufficiently detailed or standardized to support recommendations for or against their use.
- All protocols developed to standardize and validate the assays should incorporate the minimum procedural standards endorsed by the Panel.

Proposed Test Substances for ER Binding Validation Studies

- **Background Review Document Recommendations:**
  - 33 substances with *in vitro* ER binding data
    - 3 (10%) negative substances
    - 5 substances at each of 6 orders of RBA values (from <0.001 to >10)
- **Panel Recommendations:**
  - Accepted the BRD list
  - The proportion of negative substances should be increased to at least 25% to enhance assessment of assay specificity
  - For each receptor, the same substances should be used to validate both binding and transcriptional activation assays

Proposed Test Substances for AR Binding Validation Studies

- **Background Review Document Recommendations:**
  - 31 substances with *in vitro* AR binding data
    - 3 (10%) negative substances
    - 4- 5 substances at each of 6 orders of RBA values (from <0.001 to >10)
- **Panel Recommendations:**
  - Accepted the BRD list
  - Bicalutamide, hydroxyflutamide, and finasteride should be added to the list
  - The proportion of negative substances should be increased to at least 25% to enhance assessment of assay specificity
  - For each receptor, the same substances should be used to validate both binding and transcriptional activation assays

Other Selected Panel Recommendations

Chemicals selected for validation of *in vivo* endocrine disruptor validation studies should be tested in the *in vitro* assays.

A central repository of coded chemicals with verified purity should be organized for future validation studies to ensure comparability of data.

Please refer to SOT 2003 poster 1072 entitled "ICCVAM Proposed Substances for the Validation of In Vitro Estrogen Receptor (ER) and Androgen Receptor (AR) Binding and Transcriptional Activation (TA) Assays " for more information about the substances recommended for validation studies.

Expert Panel Members

The following individuals served as members of the Expert Panel that evaluated In Vitro Endocrine Disruptor Screening Assays:

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References

EPA. 1998. Endocrine Disruptor Screening Program; Proposed Statement of Policy. 63 *FR* 71542-71568.
Available: http://www.epa.gov/scipoly/oscpendo/fr122898_1.pdf

ICCVAM. 1997. Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the *ad hoc* Interagency Coordinating Committee on the Validation of Alternative Methods. NIH Publication No. 97-3981. National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina.
Available: http://iccvam.niehs.nih.gov/docs/guidelines/validate.pdf

ICCVAM/NICEATM Expert Panel Final Report. 2002. "ICCVAM/NICEATM Expert Panel Evaluation of the Validation Status of *In Vitro* Test Methods for Detecting Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays." National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina.
Available: http://iccvam.niehs.nih.gov/methods/endodocs/final/panelrpt.pdf

NIEHS. 2002a. NICEATM Background Review Document (BRD) "Current Status of Test Methods for Detecting Endocrine Disruptors: *In Vitro* Estrogen Receptor Binding Assays." National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina.
Available: http://iccvam.niehs.nih.gov/methods/endocrine.htm

NIEHS. 2002b. NICEATM Background Review Document (BRD) "Current Status of Test Methods for Detecting Endocrine Disruptors: *In Vitro* Androgen Receptor Binding Assays." National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina.
Available: http://iccvam.niehs.nih.gov/methods/endocrine.htm

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