ICCVAM Revised Recommended Substance List for the Evaluation of *In Vitro* Test Methods for Identifying Potential Endocrine Disruptors (EDs)

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Abstract

NICEATM recently re-assessed the commercial availability and cost for the 78 substances recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for use in *in vitro* estrogen receptor (ER) and androgen receptor (AR) binding and transcriptional activation (TA) validation studies. A minimum of 44 substances are recommended for AR binding and TA assays, while a minimum of 53 substances are recommended for ER binding and TA assays. This re-assessment indicated that three substances (anastrazole, CGS 18320B, fadrozole) are not commercially available, one substance has restricted commercial availability (ICI 182,780), and the cost to conduct a validation study with six other substances (actinomycin D, hydroxyflutamide, 4-hydroxytamoxifen, methyltrienolone, 12-O-tetradecanoylphorbol-13-acetate [TPA], zearalenone) was considered to be relatively expensive (>$2000/substance/lab). ICCVAM subsequently replaced the four original substances that are not commercially available or have restricted availability with ones having similar ER and AR activity profiles (4-hydroxyandrostenedione, chrysin, dicofol, raloxifene HCl). Suitable replacements (19-nortestosterone and resveratrol) were identified to replace two of the six expensive substances, methyltrienolone and zearalenone, respectively. Because of their unique activity profiles and/or chemical/physical properties, suitable replacements for the other four expensive substances could not be identified. The revised list of reference substances has now been published (ICCVAM 2006), and is being used for validation of *in vitro* ER TA methods by NICEATM, the European Centre for the Validation of Alternative Methods (ECVAM), and the Japanese Center for the Validation of Alternative Methods (JaCVAM). Supported by NIEHS Contract N01-ES-85424.

Introduction

ICCVAM recommended 78 substances for the validation of *in vitro* ER and AR binding and TA test methods (ICCVAM 2003). A number of selection criteria were considered by ICCVAM, including available data provided in Background Review Documents (BRDs) on ER and AR binding and TA test methods (ICCVAM 2002 a, b, c, d), and recommendations from the ICCVAM Endocrine Disruptor Expert Review Panel Final Report (ICCVAM 2002e). To allow for a direct comparison between results obtained from *in vitro* and *in vivo* endocrine disruptor (ED) test methods, the list also includes substances proposed for *in vivo* ED test method validation studies by the U.S. Environmental Protection Agency (EPA) and the Organisation for Economic Co-operation and Development (OECD) Test Guidelines Programme. These factors and considerations are discussed in detail in the report: *ICCVAM Evaluation of the In Vitro Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays* (ICCVAM 2003).

Two practical criteria for selecting reference substances for validation studies are that the substances should be: 1) commercially available, and 2) to the extent possible, reasonably priced. Subsequent to the publication of the original reference substance list, NICEATM re-assessed their commercial availability and price. Based on the information obtained, ICCVAM in consultation with the ICCVAM Endocrine Disrupter Working Group, has revised the recommended list, (ICCVAM 2006, available: http://iccvam.niehs.nih.gov/methods/endodocs/EDAddendFinal.pdf).

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NIEHS National Institute of Environmental Health Sciences

U.S. Department of Health and Human Services National Institutes of Health

ICCVAM The Interagency Coordinating Committee on the Validation of Alternative Methods

NICEATM The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

More information on ICCVAM and NICEATM can be accessed at http://iccvam.niehs.nih.gov/

Development and Purpose of the Original ICCVAM Recommended Reference Substances

In February 2002, four draft Background Review Documents (BRDs) were published that documented available data for ER and AR binding and TA test methods for detecting endocrine disruptors (ICCVAM 2002 a, b, c, d). An Expert Panel (Panel) met in June 2002 and developed recommendations on the adequacy and appropriateness of the substances proposed in the draft BRDs for use in future validation studies. In late 2002, ICCVAM reviewed the Panel's recommendations and used them to develop a list of 78 recommended reference substances to be used for the validation of *in vitro* ER and AR binding and TA test methods. The rationale for using 78 substances is to ensure that the comparative performance of *in vitro* ER and AR binding and TA test methods are adequately characterized across a broad range of chemical classes and responses using a common set of substances. To meet the Panel's recommendation that at least 25% of the substances proposed for validation studies should be negative for binding or TA for the respective receptor, an assumption was made that substances positive in ER binding or TA test methods would likely be negative in the corresponding AR-based test methods and vice versa, and that such substances could serve as presumptive negatives in the alternative receptor-based test methods. This approach would also minimize the total number of different substances that would be needed to validate the ER and AR test methods. **Table 1** contains the expected responses for all substances recommended for *in vitro* ER-based test methods while **Table 2** provides similar information for 53 of these substances that ICCVAM identified as a priority for validation. **Table 3** contains the expected responses for all substances recommended for *in vitro* AR-based test methods while **Table 4** provides similar information for 44 of these substances that ICCVAM identified as a priority for validation. The minimum lists include most of the available confirmed positive substances and the recommended ≥25% negative substances for ER and AR binding test methods.

Table 1 Distribution of Anticipated Responses of the 78 Recommended Test Substances for Validation of *In Vitro* ER Binding and TA Assays[a]

Expected Response	ER Binding	ER TA	
		Agonist	Antagonist
Positive[b] and Presumed Positive[c]	41 (53%)	35 (45%)	11 (14%)
Negative[d] and Presumed Negative[e]	37 (47%)	43 (55%)	67 (86%)
Total	78	78	78

[a]Based on information provided in Sections 3.0 through 6.0 of the ICCVAM ED Test Method Evaluation Report (NIH Publication No: 03-4503).

[b]Represents substances for which available quantitative ER binding or TA data indicated a positive response in the respective test method.

[c]Represents substances that have no relevant quantitative receptor binding or TA data available for the respective test method but which are presumed positive based on their known mechanism of action or their responses in other endocrine disruptor screening test methods (e.g., methyl testosterone, an ER TA agonist, is presumed positive in ER binding assays).

[d]Represents substances that tested negative for ER binding or ER TA in multiple studies, when tested up to the limit dose of 1 mM.

[e]Represents substances which are presumed negative based on the available data, their known mechanism of action, or their responses in other endocrine disruptor screening test methods (e.g., crysin, a known aromatase inhibitor, is presumed negative in ER binding and TA assays).

Table 2 Distribution of Anticipated Responses of the 53 Recommended Minimum Test Substances for Validation of *In Vitro* ER Binding and TA Assays[a]

Expected Response	ER Binding	ER TA	
		Agonist	Antagonist
Positive[b] and Presumed Positive[c]	40 (75%)	34 (64%)	11 (21%)
Negative[d] and Presumed Negative[e]	13 (25%)	19 (36%)	42 (79%)
Total	53	53	53

[a, b, c, d, e]See table 1

Table 3 Distribution of Anticipated Responses of the 78 Recommended Test Substances for Validation of *In Vitro* AR Binding and TA Assays[a]

Expected Response	AR Binding	AR TA	
		Agonist	Antagonist
Positive[b] and Presumed Positive[c]	34 (44%)	22 (28%)	21 (27%)
Negative[d]	44 (56%)	56 (72%)	57 (73%)
Total	78	78	78

[a]Based on information provided in Sections 3.0 through 6.0 of the ICCVAM ED Test Method Evaluation Report (NIH Publication No: 03-4503).

[b]Represents substances for which receptor binding or TA data are available, which indicate a positive response in the respective test method.

[c]Represents substances that have no relevant receptor binding or TA data available for the respective test method but which are presumed positive based on their known mechanism of action or their responses in other endocrine disruptor screening test methods (e.g., ketoconazole, an AR agonist, is presumed positive in AR binding assays).

[d]Represents substances that tested negative but had not been tested in multiple AR binding or in multiple AR TA studies up to the limit dose of 1 mM); or that have no relevant receptor binding or TA data available for the test method of interest but which are presumed negative based on their known mechanism of action or their responses in other endocrine disruptor screening assays (e.g., crysin, a known aromatase inhibitor, is presumed negative in AR binding and TA assays).

Table 4 Distribution of Anticipated Responses of the 44 Recommended Minimum Test Substances for Validation of *In Vitro* AR Binding and TA Assays[a]

Expected Response	AR Binding	AR TA	
		Agonist	Antagonist
Positive[b] and Presumed Positive[c]	33 (75%)	20 (45%)	20 (45%)
Negative[d]	11 (25%)	24 (55%)	24 (55%)
Total	44	44	44

[a, b, c, d]See table 3

Revised ICCVAM Reference Substance List for Validation of *In Vitro* Endocrine Disruptor Test Methods

In 2006, NICEATM re-assessed the commercial availability for the complete original list of 78 recommended substances. The re-assessment indicated that anastrozole, CGS18320B, and fadrozole are not commercially available and that the commercial availability of ICI 182,780 continues to be restricted to the purchase of 100 mg/year/institution. Of the remaining 74 commercially available substances on the original list, the cost to purchase 500 mg, the estimated amount needed per laboratory to conduct a validation study, for actinomycin D ($2,285), zearalenone ($2,760), hydroxyflutamide ($2,940), 4-hydroxytamoxifen ($5,270), TPA ($11,220), and methyltrienolone ($15,500) was > $2000 per substance.

Actinomycin D was retained as a reference substance despite its cost as it is the only RNA synthesis inhibitor (Gorski et al. 1975; Kersten and Kersten 1974; Villee et al. 1975) on the current list of 78 reference substances.

Hydroxyflutamide was retained as an ED reference substance because it was specifically recommended by the Panel and because its AR activity is well documented in the scientific literature.

TPA was retained as a reference substance because it is the only phorbol ester on the list of 78 recommended substances and because it has mitogenic activity that is not mediated via an ER-dependent pathway (Bamberger et al. 1998; Darne et al. 1998; Gagne et al. 1994; Martin et al. 1995; Whitman et al. 1989).

4-hydroxytamoxifen was retained as a reference substance because it is the active metabolite of tamoxifen and is therefore active in all cell based systems and because its activity is well documented in the scientific literature.

The replacements for the six substances that were not currently commercially available, were available only in limited quantities, or did not meet reasonable pricing criteria (with the exceptions noted above) were chosen based primarily on the similarity of their ER or AR binding or agonist TA activity profiles, or on similar concordance for antagonist TA activity across studies. Activity profiles for substances were either derived from quantitative ER and AR relative binding affinity (RBA) data, or from quantitative ER and AR TA agonist EC_{50} data (half maximal effective concentration) or antagonist IC_{50} data (concentration inhibiting reference estrogen or androgen response by 50%) . The replacements were preferentially selected from the original list of 122 substances considered by ICCVAM when finalizing the list of 78, and secondarily from substances proposed for test method validation studies by the EPA or OECD, or from further review of published literature. The six ED reference substances that were replaced and their replacements are provided in **Tables 5** and **6**.

Table 5 ED Reference Substances that are Not Commercially Available versus Their Replacement Substances

Status	Substance	Action	EPA/OECD *In Vivo* Testing[b]	ER Binding Activity[c]	ER Agonist Activity[d]	ER Antag.[e,f]	AR Binding Activity[c]	AR Agonist Activity[d]	AR Antag.[e]	Total Cost Per 500 mg
Original	Anastrozole	Aromatase Inhibitor	IM					–		Non Commercially Available
Replacement	4-OH Androstenedione	Aromatase Inhibitor	AROM				+++			$53
Original	CGS 18320B	Aromatase Inhibitor	407							Non Commercially Available
Replacement	Chrysin	Aromatase Inhibitor	AROM							$60
Original	Fadrozole	Aromatase Inhibitor	F-PA; FRS; IM							Non Commercially Available
Replacement	Dicofol	Aromatase Inhibitor	AROM							$88
Original	ICI 182,780	ER Antagonist	IM	+++	–	+++				Limited to 100 mg/yr
Replacement	Raloxifene HCl	ER Antagonist		+++	+	+++				$235

[a] Min. = Minimum

[b] 407 = 407 protocol of the Uterotrophic Assay, AROM = The EPA Placental Aromatase Assay; F-PA = Female Pubertal Assay; FRS = Fish Reproductive Screen; IM = The Intact Male Assay.

[c] +++ Indicates that the substance was strongly active as measured by the relative binding affinity (RBA) (RBA value was >1).

[d] + indicates that the substance was weakly active (half maximal effective concentration [EC_{50}] was >0.1 μM); - indicates that the substance was uniformly negative in all assays.

[e] Antag. is Antagonist

[f] +++ Indicates that the substance was strongly active (concentration inhibiting reference estrogen response by 50% [IC_{50}] was <0.001 μM).

Table 6 ED Reference Substances Where Total Cost Per Laboratory is in Excess of $2000 versus Their Replacement Substances

Status	Substance	Action	EPA/OECD *In Vivo* Testing[b]	ER Binding Activity[c]	ER Agonist Activity[d]	ER Antag.[e,f]	AR Binding Activity[c]	AR Agonist Activity[d]	AR Antag.[e]	Total Cost Per 500 mg
Original	Methyltrienolone	AR Agonist			–		+++	+++		$15,500
Replacement	19-Nortestosterone	AR Agonist		++	+/–		+++	+++		$90
Original	Zearalenone	ER Agonist		+++	++	+				$2,760
Replacement	Resveratrol	ER Agonist		+	++	+				$226

[a] Min. = Minimum

[b] Substances are not proposed for ED test method validation studies by the EPA or OECD.

[c] +++ Indicates that the substance was strongly active as measured by relative binding affinity (RBA) (RBA value was >1); ++ indicates that the substance was moderately active (RBA value was between 1 and 0.01); + indicates that the substance was weakly active (RBA value was < than 0.01).

[d] +++ Indicates that the substance was strongly active (half maximal effective dose [EC_{50}] value was <0.001 μM)++ indicates that the substance was moderately active (EC_{50} value was between 0.001 and 0.1 μM); +/- indicates that the substance was weakly active or negative in different assays; - indicates that the substance was uniformly negative in all assays.

[e] Antag. is Antagonist

[f] + Indicates that the substance was weakly active (concentration inhibiting reference estrogen response by 50% [IC_{50}] was >0.1 μM).

A *Federal Register* notice was published in March of 2006 requesting public comments on the proposed revisions to the ICCVAM recommended substances list. No public comments were received. In September of 2006, a *Federal Register* notice announcing the availability of the revised reference substances list (ICCVAM 2006) was published.

Summary

ICCVAM re-assessed the commercial availability and cost of the 78 original substances recommended for use for *in vitro* ER/AR binding and TA validation studies. This assessment indicated that replacements were desirable for 10 substances as follows:

- Anastrozole, CGS18320B, and fadrozole were not commercially available:
- Availability of ICI 182,780 was restricted
- Actinomycin D, hydroxyflutamide, 4-hydroxytamoxifen, methyltrienolone, TPA, zearalenone were considered to be relatively expensive (>$2000/substance/lab):

The primary criteria for identifying replacement substances were:

- Similar ER or AR binding or TA activity profiles
- Commercial availability and expense

Secondary criteria for identifying replacement substances were:

- On the original list of 122 ICCVAM ED candidate substances classified as "Substances Considered but not Included for Validation"
- The substance is proposed for test method validation studies by the EPA or OECD

After consideration, four of the relatively expensive substances (actinomycin D, hydroxyflutamide, 4-hydroxytamoxifen, TPA) were retained because of their unique properties. The six replacements were:

- 4-OH androstenedione for anastrozole
- Chrysin for CGS 18320B
- Dicofol for fadrozole
- Raloxifene for ICI 182,780
- 19-nortestosterone for methyltrienolone
- Resveratrol for zearalenone

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