ICCVAM PROPOSED SUBSTANCES FOR THE VALIDATION OF *IN VITRO* ESTROGEN RECEPTOR (ER) AND ANDROGEN RECEPTOR (AR) BINDING AND TRANSCRIPTIONAL ACTIVATION (TA) ASSAYS

W S Stokes[1], B S Shane[1,2], C J Inhof[1,2], R R Tice[1,2], D Hattan[3] and M Wind[4]

[1]NTP Interagency Center for the Evaluation of Toxicological Methods (NICEATM), NIEHS, RTP, NC; [2]ILS, Inc., RTP, NC; [3]U.S. Food and Drug Administration, Washington, DC, [4]Consumer Products Safety Commission, Washington, DC.

Abstract

The U.S. EPA's proposed Endocrine Disruptor Screening Program (EDSP) includes a Tier 1 screening battery composed of *in vitro* and *in vivo* test methods designed to identify substances capable of interacting with the endocrine system. Prior to implementation of the EDSP, the component test methods must be adequately validated. An Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and NICEATM Expert Panel evaluated the validation status of *in vitro* ER and AR binding and TA (agonist/antagonist) assays that might be included in the Tier 1 battery. The Expert Panel determined that none of the *in vitro* assays had been adequately validated. To facilitate the necessary validation studies, a common list of 78 proposed substances was compiled that addressed the Expert Panel's recommendations. Substances were selected to ensure that reliability and accuracy of the *in vitro* assays would be adequately characterized across a broad range of chemical classes and responses. Selection criteria included quantity and quality of available data, potency, chemical class, selection for *in vivo* validation studies, and commercial availability. A minimum of 25% of the substances are known or expected to be negative in each of the different assay types. The use of a common substance list for validation will facilitate assessment of comparative assay performance, establishment of minimum performance criteria, and selection of acceptable *in vitro* test methods. Generation of both *in vivo* and *in vitro* data on many of these chemicals during validation studies will also aid the future development of more predictive *in vitro* endocrine disruptor assays. ILS staff supported by NIEHS Contract N01-ES-85424. The views expressed above do not necessarily represent the official positions of any federal agency.

ICCVAM Agencies

Agency for Toxic Substances and Disease Registry
Consumer Product Safety Commission
Department of Agriculture
Department of Defense
Department of Energy
Department of Interior
Department of Transportation
Environmental Protection Agency
Food and Drug Administration
National Cancer Institute
National Institute of Environmental Health Sciences
National Institutes of Health, Office of the Director
National Institute of Occupational Safety and Health
National Library of Medicine
Occupational Safety and Health Administration

Expert Panel Evaluation of Initial Draft Lists

I. Draft Lists of Substances Proposed by NICEATM in the Background Review Documents (BRDs)

- ❑ The BRDs submitted to the Expert Panel included a list of substances recommended for future validation studies for each of the six assay types.
- ❑ Selection of these substances was based on:
  - Availability of data demonstrating reproducible responses in multiple test methods
  - Achieving a range of responses from negative to weakly positive to strongly positive
  - Achieving distribution across a broad range of relevant chemical classes

Initial Draft Lists of Substances Proposed for Validation of *In Vitro* ER and AR Binding and TA Assays

In Vitro Assay Type	Number of Substances	Number of Positive Substances	Number of Negative Substances
ER Binding	**33**	**30 (91%)**	**3 (9%)**
ER TA Agonist	**31**	**26 (84%)**	**5 (16%)**
ER TA Antagonist	**21**	**17 (81%)**	**4 (19%)**
AR Binding	**31**	**28 (90%)**	**3 (10%)**
AR TA Agonist	**28**	**18 (64%)**	**10 (36%)**
AR TA Antagonist	**25**	**21 (84%)**	**4 (16%)**

II. Expert Panel Recommendations on Initial Draft Lists of Substances

- ❑ The Expert Panel agreed with the draft lists of proposed substances but also recommended:
  - For a specific receptor (ER or AR), the same substances should be tested in both binding and TA test methods.
  - To adequately evaluate test method specificity, at least 25% of the substances in the list must be negative for the endpoint being assessed.
  - A known positive control substance with a potency two orders of magnitude lower than the reference hormone.
  - Substances should be included for the validation of TA assays that might indirectly interfere with transcriptional activation. Substances of interest include:
    - Actinomycin D- inhibits RNA synthesis
    - Cycloheximide- inhibits protein synthesis
    - Sodium azide - cytotoxic
    - 12-*O*-tetradecanoylphorbol-13-acetate – ligand independent activation
  - Substances from under-represented chemical classes should be included. Examples are:
    - Phthalates
    - Polycyclic aromatic hydrocarbons [PAHs]
    - Polychlorinated biphenyls
  - A central repository to distribute substances of known purity for validation studies.

ICCVAM Proposed List of Substances for Validation

I. Candidate Substances

122 candidate substances were identified including:

- 85 substances recommended in the four BRDs for future validation studies (SOT 2003, posters 763 and 1071)
- 44 substances scheduled for testing in *in vivo* endocrine disruptor (ED) assays by EPA and the Organisation for Economic Cooperation and Development (OECD)
  - 22 of these substances were included in the BRDs
- 38 substances scheduled for testing in *in vitro* ED assays by EPA
  - 29 of these substances were included in the BRDs
- 6 additional substances recommended by the Expert Panel

II. Selection of Final 78 Substances

List of 122 substances reduced to 78 substances based on following:

- methyl parathion and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin excluded as being highly toxic
- 4-chloro-4'-biphenylol, 2',4',6'-trichloro-4-biphenylyol and Arochlor 1254 excluded due to hazardous waste disposal concerns
- letrozole excluded as its *in vivo* testing was questionable and no *in vitro* data
- testosterone propionate excluded as it is readily hydrolyzed *in vivo* to testosterone, which is included
- tamoxifen citrate excluded and tamoxifen included because the latter had been tested more extensively
- excluded some substances not scheduled for *in vitro* testing by the EPA
- excluded some substances not scheduled for *in vivo* testing by EPA and OECD

Purpose and Advantages of the List of 78 Substances

Purpose of List

To ensure that the comparative reproducibility and accuracy of *in vitro* ER and AR binding and TA assays are adequately characterized across a broad range of chemical classes and responses.

Advantages

Inclusion of many substances proposed for validation of other Tier 1 and Tier 2 *in vivo* test methods will:

- help characterize the usefulness of Tier 1 screening battery
- help prioritize substances for Tier 2 testing
- facilitate development of more predictive *in vitro* endocrine disruptor assays and *in vitro* test batteries

Anticipated Responses of Proposed Substances

Distribution of Anticipated Responses of Proposed Test Substances in *In Vitro* ER and AR Binding and TA Assays

A. *In Vitro* ER Assays

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

B. *In Vitro* AR Assays

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

[a]Represents substances: 1) for which ER binding or TA data are available, which indicate a positive response in the respective test method (i.e., substances tested in more than one study that were positive in ≥ 50% of the studies); 2) that were positive in < 50% of reported studies; 3) that were positive but tested in only one study; and 4) 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.

[b]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.

ICCVAM Proposed Substances for Validation of *In Vitro* ER and AR Binding and Transcriptional Activation Assays

Substance	CASRN	*In Vitro* Data (NICEATM)[a]			Completed/ Anticipated *In Vivo* Testing	Chemical Class
		Binding[b]	Transcriptional Activation			
			Agonism[c]	Antagonism[d]		
Actinomycin D	50-76-0					Phenoxazone; Lactone; Peptide
Ammonium perchlorate	7790-98-9				y	Organic acid; Organic salt
Anastrazole	120511-73-1		AR-		y	Nitrile; Triazole
4-Androstenedione	63-05-8	ER+/AR+++	ER-/AR+++			Steroid, nonphenolic
Apigenin	520-36-5	ER+++	ER+++	ER#-	y	Flavanoid; Flavone; Phenol
Apomorphine	58-00-4				y	Hetenocycle; Quinoline
Atrazine	1912-24-9	ER+/AR+	ER-/AR-	ER-/AR-	y	Aromatic amine; Triazine; Arylamine
Bicalutamide	90357-06-5	AR+++	AR+	AR##		Anilide; Nitrile; Sulfone
Bisphenol A	80-05-7	ER++	ER+/AR-	ER-/AR#-	y	Diphenolalkane; Bisphenol; Phenol
Bisphenol B	77-40-7	ER++	ER++/AR-		y	Diphenolalkane; Bisphenol; Phenol
Butylbenzyl phthalate	85-68-7	ER±	ER++/AR-	ER-/AR-		Phthalate
2-*sec* -Butylphenol	89-72-5	ER+				Phenol
CGS 18320B	112808-99-8				y	Nitrile; Imidazole
Clomiphene citrate	50-41-9	ER++				Chlorinated triphenylethylene; Benzylidene; Stilbene
Corticosterone	50-22-6	ER-/AR+	ER-/AR-			Steroid, nonphenolic
Coumestrol	479-13-0	ER+++	ER++/AR-	ER-	y	Coumestan; Benzopyranone; Coumarin; Ketone
4-Cumylphenol	599-64-4		ER+/AR-			Phenol
Cycloheximide	66-81-9					Piperidine; Glutaramide
Cyproterone acetate	427-51-0	AR+++	ER-/AR+	AR##	y	Nitrile; Diphenyl ether; Organochlorine
Daidzein	486-66-8	ER++	ER+	ER-		Flavanoid; Isoflavone; Phenol
p,p' -DDE**	72-55-9	ER±/AR++	ER+/AR±	ER-/AR#-	y	Organochlorine; Diphenylalkene
o,p' -DDT**	789-02-6	ER++/AR+	ER+/AR-	ER#/AR#	y	Organochlorine; Diphenylalkene
Dexamethasone	50-02-2	ER-/AR-	ER-/AR+			Steroid, nonphenolic
Dibenzo[*a,h*]anthracene	53-70-3	ER-	ER-/AR+	ER##		Polycyclic aromatic hydrocarbon; Anthracene
Di-*n* -butyl phthalate	84-74-2	ER±	ER+/AR-	ER-	y	Phthalate
Diethylhexyl phthalate	117-81-7	ER-	AR-		y	Phthalate
Diethylstilbestrol	56-53-1	ER+++/AR++	ER+++/AR-	AR#	y	Stilbene; Benzylidene; Diphenylalkene
5α-Dihydrotestosterone***	521-18-6	ER++/AR+++	ER+/AR+++		y	Steroid, nonphenolic
17α-Estradiol	57-91-0	ER+++	ER++/AR-			Steroid, phenolic; Estrene
17β-Estradiol***	50-28-2	ER+++/AR++	ER+++/AR++	AR##	y	Steroid, phenolic; Estrene
Estrone	53-16-7	ER+++/AR++	ER+++/AR++			Steroid, phenolic; Estrene
17α-Ethinyl estradiol	57-63-6	ER+++/AR++	ER+++/AR-		y	Steroid, phenolic
Ethyl paraben	120-47-8					Paraben; Organic acid
Fadrozole	102676-47-1				y	Imidazole; Nitrile
Fenarimol	60168-88-9		ER+	ER#	y	Hetenocycle; Pyrimidine
Finasteride	98319-26-7		AR-	AR-	y	Steroid, nonphenolic; Androstene
Flavone	525-82-6	ER-	ER±	ER###	y	Flavanoid; Flavone
Fluoranthene	206-44-0	ER-	ER-	ER-/AR#		Polycyclic aromatic hydrocarbon; Fluorene
Fluoxymestrone	76-43-7	AR++	AR+	AR-		Steroid, nonphenolic

Substance	CASRN	*In Vitro* Data (NICEATM)[a]			Completed/ Anticipated *In Vivo* Testing	Chemical Class
		Binding[b]	Transcriptional Activation			
			Agonism[c]	Antagonism[d]		
Flutamide	13311-84-7	AR++	ER-/AR-	AR#	y	Amide; Anilide; Nitrobenzene
Genistein	446-72-0	ER++	ER+	ER#	y	Flavanoid; Isoflavone; Phenol
Haloperidol	52-86-8				y	Butyrophenone; Ketone; Piperazine
Hexestrol	84-16-2	ER+++	ER+++			Diphenolalkane; Bisphenol; Phenol
Hydroxyflutamide	52806-53-8	ER±/AR++	AR+	AR##		Amide; Anilide; Nitrobenzene
4-Hydroxytamoxifen	68047-06-3	ER+++	ER±/AR-	ER###		Triphenylethylene; Benzylidene; Stilbene; Phenol
ICI 182,780	129453-61-8	ER+++	ER-/AR-	ER###/AR-	y	Steroid, phenolic
Kaempferol	520-18-3	ER++	ER+	ER-		Flavanoid; Flavone; Phenol
Kepone	143-50-0	ER++/AR++	AR-	AR#-		Organochlorine; Chlorinated bridged cycloalkane
Ketoconazole	65277-42-1		AR±	AR-	y	Imidazole; Piperazine
Linuron	330-55-2	AR+	ER-/AR+	AR#	y	Urea
Medroxyprogesterone acetate	71-58-9	AR+++	AR+			Steroid, nonphenolic; Polycyclic hydrocarbon
p,p' -Methoxychlor	72-43-5	ER+/AR+	ER+/AR-	ER-/AR#	y	Organochlorine; Chlorinated hydrocarbon
Methyl testosterone	58-18-4	ER++/AR+++	ER+++/AR++	AR###	y	Steroid, nonphenolic; Androstene
Methyltrienolone***	965-93-5	AR+++	ER-/AR+	AR-		Steroid, nonphenolic; Estrene
Mifepristone	84371-65-3	AR+++	ER-/AR+++	AR###	y	Steroid, nonphenolic; Estrene
Morin	480-16-0	ER+				Flavanoid; Flavone; Phenol
Nilutamide	63612-50-0	AR+++	AR±	AR##		Heterocycle; Imidazole
p -Nonylphenol	104-40-5	ER++	ER++/AR±	ER#/AR###		Alkylphenol; Phenol
Norethynodrel	68-23-5	ER++				Steroid, nonphenolic; Pregnenolone
4 -*tert* -Octylphenol	140-66-9	ER++/AR-	ER++/AR-	AR-	y	Alkylphenol; Phenol
Oxazepam	604-75-1				y	Benzodiazepine
Phenobarbital	57-30-7	AR-	ER-/AR-		y	Heterocycle; Pyrimidine
Phenolphthalin	81-90-3	ER+				Triphenylmethane; Diphenylalkane carboxylic acid
Pimozide	2062-78-4				y	Piperidine; Benzimidazole
Procymidone	32809-16-8	AR+	ER-/AR-	AR#		Organochlorine; Cyclic imide
Progesterone	57-83-0	ER+/AR+++	ER±/AR+	ER-/AR#-	y	Steroid, nonphenolic; Pregnenedione
Propythiouracil	51-52-5	AR-			y	Pyrimidine; Uracil
Reserpine	50-55-5				y	Heterocycle; Yohimban
Sodium azide	26628-22-8					Organic salt; Azide
Spironolactone	52-01-7	AR+++	AR+	AR##		Steroid, nonphenolic; Pregnene lactone
Tamoxifen	10540-29-1	ER+++/AR-	ER+/AR-	ER###		Triphenylethylene; Benzylidene; Stilbene
Testosterone	58-22-0	ER±/AR+++	ER±/AR+++	AR-	y	Steroid, nonphenolic
12-*O* - Tetradecanoylphorbol-13-acetate	16561-29-8					Phorbol ester; Terpene
L-Thyroxine	51-48-9				y	Aromatic amino acid
17β-Trenbolone	10161-33-8	AR+++	ER-		y	Steroid, nonphenolic; Estrene
2,4,5-Trichlorophenoxyacetic acid	93-76-5	ER-	ER+			Organochlorine; Chlorinated aromatic hydrocarbon
Vinclozolin	50471-44-8	ER±/AR++	ER-/AR-	AR###	y	Organochlorine; Cyclic imide; Carbamate
Zearalenone	17924-92-4	ER+++	ER++/AR-	ER#-		Resorcylic acid lactone; Phenol

*Inclusion of a substance in this table does not mean that EPA, NICEATM, ICCVAM, or the Expert Panel has or will make a determination that any of the uses of the chemical will pose a significant risk. Further, these substances should not be interpreted to be "endocrine disruptors"; the substances listed are simply compounds that have been, or may prove to be useful in developing, standardizing or validating screening and testing methods.

Empty cells indicate that no relevant data were identified and no validation tests are planned for that substance in that particular assay.

***p,p'* -DDE = 1,1-Dichloro-2,2-di(*p* -chlorophenyl)ethylene; *o,p'* -DDT = 1,1,1-Trichloro-2-(*o* -chlorophenyl)-2-(*p* -chlorophenyl)ethane; *p,p'* -DDT = 1,1,1-Trichloro-2,2-di(4-chlorophenyl)ethane

*** 17β-Estradiol is the recommended positive control substance for the ER binding TA assays; for AR binding, 5α-Dihydrotestosterone is the recommended positive control if a purified AR protein is used, while Methyltrienolone or Mibolerone is recommended if intact cells, or cytosol is used. For AR TA assays, either 5α-Dihydrotestosterone or Methyltrienolone is recommended as the positive control.

[a] *In vitro* data obtained from the literature or from reports submitted to NICEATM and summarized in four Background Review Documents available at http://iccvam.niehs.nih.gov/methods/endocrine.htm.

[b] +++ Indicates that the substance was relatively active as measured by the relative binding affinity (RBA) (RBA value was >1); + indicates that the substance was weakly active (RBA value was < than 0.01); - indicates that an IC_{50} value was not obtained and thus an RBA value could not be determined; ± indicates an equivocal response (i.e., in different studies, the substance was reported as positive and negative). The inhibitory concentration 50 (IC_{50} is the concentration of test substances that displaces 50% of the radiolabeled reference estrogen or androgen from the receptor.

[c] +++ Indicates that the substance was relatively active (EC_{50} value was <0.001); ++ indicates that the substance was moderately active (EC_{50} value was between 0.001 and 0.1); + indicates that the substance was weakly active (EC_{50} value was >0), or a positive response was reported without an EC_{50} value. The EC_{50} is the effective concentration that displaces 50% of the radiolabeled reference estrogen or androgen from the receptor.

[d] ### Indicates that the substance was uniformly positive in multiple assays; ## indicates that the substance was positive in the majority of assays in which it was tested; # indicates that the substance was positive in the single assay in which it was tested; #- indicates the substance was positive in one assay but was also negative in one or more assays; - indicates that the substance was uniformly negative in multiple assays.

Minimum Lists of Substances for Validation of *In Vitro* Endocrine Disruptor Assays

- ❑ ICCVAM developed minimum lists of substances that should be given priority during validation.
  - ***Justification:*** Because the purpose of these *in vitro* assays in the Tier 1 screening battery is to provide binding and transcriptional activation data that will be considered in a weight-of-evidence evaluation to prioritize substances for Tier 2 testing, characterizing the activity of all of the substances expected to be negative *in vitro* (e.g., thyroid disruptors, aromatase inhibitors) may not be essential.
- ❑ Minimum lists contain 53 substances for ER binding and TA assays and 45 substances for AR binding and TA assays, with similar distributions of substances across the ranges of responsiveness and chemical classes as contained in the list of 78 substances.
- ❑ Specific listing of minimum lists are included in report entitled, "ICCVAM Evaluation of *In Vitro* Test Methods For Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays." (NIH Publication No: 03-4503)

Minimum List of Substances for *In Vitro* ER Assays

Expected Response	ER Binding	ER TA	
		Agonist	Antagonist
Positive/ Presumed Positive	**40 (75%)**	**34 (64%)**	**11 (21%)**
Negative/ Presumed Negative	**13 (25%)**	**19 (36%)**	**42 (79%)**
Total	**53**	**53**	**53**

Minimum List of Substances for *In Vitro* AR Assays

Expected Response	AR Binding	AR TA	
		Agonist	Antagonist
Positive/ Presumed Positive	**33 (73%)**	**21 (47%)**	**20 (44%)**
Negative/ Presumed Negative	**12 (27%)**	**24 (53%)**	**25 (56%)**
Total	**45**	**45**	**45**

NIH Publication No: 03-4503

ICCVAM Evaluation of *In Vitro* Test Methods For Detecting Potential Endocrine Disruptors:

Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

ICCVAM/NICEATM WEBSITE
http://iccvam.niehs.nih.gov