

7.0 IN VITRO AR TA TEST METHOD RELIABILITY ASSESSMENT

7.1 Introduction

The ICCVAM Submission Guidelines (ICCVAM, 1999) recommend that an assessment of test method reliability¹ be performed. This assessment includes an evaluation of the rationale for selecting the substances used to evaluate intra- and inter-laboratory reproducibility, the extent to which the substances tested represent the range of possible test outcomes, and a quantitative statistical analysis of intra- and inter-laboratory reproducibility. In addition, measures of central tendency and variation for historical negative and positive control data and an assessment of the historical control variability need to be conducted. However, no formal validation studies to assess the reliability of *in vitro* AR TA assays have been conducted and the limited nature of the current database for these assays precludes a formal analysis.

7.2 Assessment of *In Vitro* AR TA Assay Reliability

Although many of the reports indicated that the substances tested in *in vitro* AR TA assays were tested in triplicate or quadruplicate within an experiment and that at least replicate assays were conducted, associated error terms were not always provided and/or could not be estimated or calculated. Also, data analysis and presentation varied considerably among investigators assessing the *in vitro* AR agonist and antagonist activity of test substances. These two factors, combined with the great variability in assay protocols, the few substances tested multiple times within and across laboratories (and assays), and the lack of any validation studies made a formal assessment of assay reliability impractical.

In the only cross-assay evaluation located, Gray and colleagues (Hartig et al., 2002; Wilson et al., 2002) commented on the variability for DHT-induced luciferase activity in assays using stably transfected MDA-MB-453-kb2 cells and those using transiently transfected CV-1 cells. The interassay coefficient of variation for the MDA-MB-453-kb2 cell assays was 52.7% across 28 replicate plates, while that for CV-1 cells was 145% across eight replicates. The increased

¹ Reliability is a measure of the degree to which a test can be performed reproducibly within and among laboratories over time, where reproducibility is the variability between single test results obtained in a single laboratory (intralaboratory reproducibility) or in different laboratories (interlaboratory reproducibility) using the same protocol.

variability for the CV-1 cell-based assays was attributed to the variability in transfection efficiencies from replicate to replicate, a source of variation that does not exist when stably transfected cells are used.

For *in vitro* AR TA studies conducted to assess agonism activity, quantitative data in the form of EC₅₀ values were reported for only 24 substance/assay combinations (**Table 7-1**), three of which were for the reference androgen DHT. Of these 24 substance/assay combinations,

Table 7-1 Available EC₅₀ Values for Substances Tested for Agonism Activity in *In Vitro* AR TA Assays

Substance	Assay	No. Times Tested	EC ₅₀ Values (μM) ^a		
4-Androstenedione	CHO	2	0.00065* ⁶	0.0024* ⁶	
Cortisol	CHO	1	0.0427 ⁶		
<i>p,p'</i> -DDE	Yeast	2	350 ³	8820 ¹	
5 -DHT	PALM	1	0.00004 ⁸		
5 -DHT	CHO	1	0.00015 ⁶	0.00015 ⁶	
5 -DHT	Yeast	1	0.0035 ¹	0.0024 ⁵	0.002 ²
17 -Estradiol	Yeast	1	0.0861 ¹		
Estrone	CHO	1	0.0551 ⁶		
Hydroxyflutamide	Yeast	2	8.21* ¹	82.0* ³	
11-Ketotestosterone	CHO	2	0.0015* ⁶	0.0058* ⁶	
Levonorgestrel	CHO	2	0.00037* ⁶	0.0016* ⁶	
Methyltestosterone	CHO	2	0.000027* ⁶	0.00014* ⁶	
Mibolerone	PALM	1	0.00003 ⁸		
Mifepristone	CHO	1	0.0136 ⁶		
Mifepristone	Yeast	1	2100 ⁵		
Norethisterone	CHO	2	0.0037* ⁶	0.0072* ⁶	
Norgestrel	CHO	2	0.00040* ⁶	0.0010* ⁶	
19-Nortestosterone	CHO	2	0.000092* ⁶	0.00022* ⁶	
<i>p</i> -Nonylphenol	Yeast	1	2 ²		
Progesterone	Yeast	2	0.0089 ¹	5.2 ⁵	
Testosterone	CHO	2	0.000053* ⁶	0.00011* ⁶	
Testosterone	PALM	1	0.0002 ⁸		
Testosterone	Yeast	3	0.0047 ¹	0.012* ⁵	0.0099 ^{4*}
Toxaphene	PC-3	1	10 ⁷		

DDE = 1,1 Dichloro-bis[4-chlorophenyl]ethylene; DHT = 5 -Dihydrotestosterone.

^aEC₅₀ values in italics were estimated from a graphical representation of the data.

*Values obtained in the same laboratory for that substance/assay combination.

¹Gaido et al. (1997); ²Moffat et al. (2001); ³O'Connor et al. (1998); ⁴O'Connor et al. (1999); ⁵O'Connor et al. (2000); ⁶Otsuka Pharmaceutical Co. (2001); ⁷Schrader and Cooke (2000); ⁸Terouanne et al. (2000).

only nine sets of EC₅₀ values were for substances tested twice in the same laboratory using the same assay, and only two sets of EC₅₀ values were for the same substance tested using the same assay in more than one laboratory. For the same substance/assay/laboratory combination, the least difference in EC₅₀ values (about a 2-fold difference) was for norethisterone tested twice using CHO cells, while the greatest difference in EC₅₀ values (about 10-fold) was for hydroxyflutamide tested twice using yeast cells. There was about a 25-fold difference in EC₅₀ values for *p,p'*-DDE tested using yeast cells in two laboratories.

For *in vitro* AR TA studies conducted to assess antagonism activity, quantitative data in the form of IC₅₀ values were reported for 63 substance/assay combinations (**Table 7-2**), two of which were reference androgens. Of these 63 substance/assay combinations, only seven sets of IC₅₀ values were for substances tested at least twice (three of these substances were tested three times) in the same laboratory using the same assay, and only one set of IC₅₀ values were for the same substance tested using the same assay in more than one laboratory. For the same substance/assay/laboratory combination, there was no difference in IC₅₀ values for linuron tested twice using MDA-MB-453-kb2 cells, while the greatest difference in IC₅₀ values (about 4500-fold) was for cyproterone acetate tested three times using PALM cells. There was about a 10-fold difference in IC₅₀ values for flutamide tested in two laboratories using yeast cells.

Based on the inadequate database available, no conclusions can be made about the relative reliability of the 18 different *in vitro* AR TA assays considered in this BRD. However, these data do indicate the need for future validation studies to adequately evaluate this issue.

7.3 Conclusions and Recommendations

The *in vitro* AR TA assays that are the most useful as a screen for endocrine disruptors are those that are the most sensitive (i.e., have the greatest ability to detect weak acting AR agonists and antagonists (see **Section 6**), and the most reliable (i.e., exhibit the least variability within and across laboratories). Based on the available data, no valid assessment of assay reliability was possible.

Taking into account the available *in vitro* AR TA assay database, and the inability to adequately assess the reliability of the *in vitro* AR TA assays considered in this BRD, formal validation

studies should be conducted using appropriate substances covering the range of expected EC₅₀ values (for agonism) and IC₅₀ values (for antagonism). These substances should elicit a range of responses ranging from strong to weak to inactive to demonstrate the reliability characteristics of the *in vitro* AR TA assays considered as possible screening assays. A list of potential test substances for use in such validation efforts is provided in **Section 12**.

Table 7-2 Available IC₅₀ Values for Substances Tested for Antagonism Activity in *In Vitro* AR TA Assays

Substance	Assay	No. Times Tested	IC ₅₀ Values (μM) ^a		
Benzo[<i>a</i>]pyrene	CHO	1	3.9 ¹⁰		
Benz[<i>a</i>]anthracene	CHO	1	3.2 ¹⁰		
Bicalutamide	CHO	1	0.5 ¹⁰		
Bicalutamide	PC-3	1	0.5 ¹¹		
Bicalutamide	PALM	2	0.75 ^{9*}	18 ^{11*}	
2,2 Bis-(<i>p</i> -hydroxyphenyl)-1,1,1-trichloroethane	MDA-MB-453	1	0.1 ¹		
2,2 Bis-(<i>p</i> -hydroxyphenyl)-1,1,1-trichloroethane	MDA-MB-453-kb2	1	10 ¹¹		
Bisphenol A	PC-3	1	1 ¹²		
Butylated hydroxyanisole	PALM	1	7.6 ⁷		
Butylated hydroxytoluene	PALM	1	5.7 ⁷		
Chrysene	CHO	1	10.3 ¹⁰		
Cyproterone acetate	PALM	2	0.01 ^{11*}	45.0 ^{11*}	
Cyproterone acetate	PC-3	1	0.01 ¹¹		
Cyproterone acetate	CV-1	1	0.1 ²		
Cyproterone acetate	CHO	1	0.5 ¹⁰		
<i>o,p'</i> -DDE	PALM	1	1.5 ⁸		
<i>p,p'</i> -DDE	PALM	2	0.75 ^{8*}	15.2 ^{7*}	
<i>p,p'</i> -DDE	CHO	1	1 ¹⁰		
<i>p,p'</i> -DDE	MDA-MB-453-kb2	1	5 ¹¹		
3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	PALM	1	0.02 ⁸		
3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	MDA-MB-453	1	0.1 ¹		
3',5'-Dichloro-2-hydroxy-2-methylbut-3-enanilide	MDA-MB-453-kb2	1	0.2 ¹¹		
(4-[2,2-Dichloro-1-(4-hydroxyphenyl)vinyl]phenol)	MDA-MB-453	1	0.1 ¹		
(4-[2,2-Dichloro-1-(4-hydroxyphenyl)vinyl]phenol)	MDA-MB-453-kb2	1	5 ¹¹		
2-[[3,5-Dichlorophenyl)carbamoyl]oxy]-2-methyl-butenoic acid	MDA-MB-453	1	0.1 ¹		
2-[[3,5-Dichlorophenyl)carbamoyl]oxy]-2-methyl-butenoic acid	MDA-MB-453-kb2	1	0.2 ¹¹		
2-[[3,5-Dichlorophenyl)carbamoyl]oxy]-2-methyl-butenoic acid	PALM	1	0.5 ⁸		
Diethylstilbesterol	PALM	1	0.36 ⁷		
Dimethylbenz[<i>a</i>]anthracene	CHO	1	10.4 ¹⁰		
17 -Estradiol	MDA-MB-453-kb2	1	0.05 ¹¹		

Substance	Assay	No. Times Tested	IC ₅₀ Values (µM) ^a		
17 -Estradiol	CV-1	1	0.5 ²		
17 -Estradiol	CHO	1	1 ¹⁰		
Fluoranthene	CHO	1	4.6 ¹⁰		
Flutamide	Yeast	2	22 ⁵	220 ⁶	
2,2',3,4,4',5-Hexachlorobiphenyl	CHO	1	1 ¹⁰		
-Hexachlorocyclohexane	PALM	1	8.2 ⁷		
-Hexachlorocyclohexane	PALM	1	17.9 ⁷		
Hydroxyflutamide	CHO	1	0.01 ¹⁰		
Hydroxyflutamide	PALM	3	0.02 ^{11*}	0.1 ^{11*}	10 ^{11*}
Hydroxyflutamide	CV-1	1	0.1 ²		
Inocterone	PALM	1	30 ¹¹		
Kepone	PALM	1	6.9 ⁷		
Linuron	MDA-MB-453-kb2	2	5 ^{3*}	5 ^{11*}	
Linuron	CV-1	1	10 ⁴		
Methyltrienolone	CHO	1	0.0001 ^{10*}		
Mifepristone	PALM	1	0.05 ¹¹		
Neburon	MDA-MB-453-kb2	1	10 ¹¹		
Nilutamide	PALM	2	10 ^{11*}	0.3 ^{11*}	
Nilutamide	PC-3	1	0.15 ¹¹		
<i>p</i> -Nonylphenol	Yeast	1	0.001 ⁴		
Procymidone	CHO	1	5 ¹⁰		
Procymidone	MDA-MB-453-kb2	1	10 ¹¹		
Progesterone	CHO	1	0.1 ¹⁰		
Progesterone	CV-1	1	0.5 ²		
Promegestone	PALM	1	0.09 ¹¹		
RU2956	PALM	1	45 ¹¹		
RU56187	CV-1	1	0.0001 ²		
Spirolactone	PALM	1	0.09 ¹¹		
Spirolactone	CHO	1	0.5 ¹⁰		
2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin	PALM	1	6.5 ⁷		
Toxaphene	PALM	1	1935 ⁷		
Vinclozolin	MDA-MB-453-kb2	1	0.05 ¹¹		
Vinclozolin	CHO	1	0.5 ¹⁰		

DDE = 1,1 Dichloro-bis[4-chlorophenyl]ethylene.

^aIC₅₀ values in italics were estimated from a graphical representation of the data.

*Values obtained in the same laboratory for that substance/assay combination.

¹Hartig et al. (2002); ²Kemppainen et al. (1999); ³Lambright et al. (2000); ⁴Moffat et al. (2001);

⁵O'Connor et al. (1998); ⁶O'Connor et al. (1999); ⁷Schrader and Cooke (2000); ⁸Sultan et al. (2001);

⁹Terouanne et al. (2000); ¹⁰Vinggaard et al. (2000); ¹¹Wilson et al. (2002).