A robotic MCF-7:WS8 cell proliferation assay to detect agonist and antagonist estrogenic activity

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ABSTRACT

Endocrine disrupting chemicals with estrogenic (EA) or anti-estrogenic (AEA) activity have been extensively reported to possibly have many adverse health effects. We have developed robotized assays using MCF-7:WS8 cell proliferation (or suppression) to detect EA (or AEA) of 78 test substances supplied by the National Toxicology Program's Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) for validation studies. We also assayed ICI 182,780, a strong estrogen antagonist. Chemicals to be assayed were initially examined for solubility and volatility to determine optimal assay conditions. For both EA and AEA determinations, a range-finder assay was conducted to determine the concentration range for testing, followed by a comprehensive assay. Test substances with potentially positive results from an EA comprehensive assay were subjected to an EA confirmation assay that evaluated the ability of ICI 182,780 to reverse chemically-induced MCF-7 cell proliferation. The AEA assays examined the ability of chemicals to decrease MCF-7 cell proliferation induced by non-saturating concentrations of 17β-estradiol (E2), relative to ICI or raloxifene (RAL), also a strong estrogen antagonist. To be classified as having AEA, a saturating concentration of E2 had to significantly reverse the decrease in cell proliferation produced by the test substance in non-saturating E2. We conclude that our robotized MCF-7 EA and AEA Assays have accuracy, sensitivity, and specificity values at least equivalent to validated test methods accepted by the US EPA and the Organisation for Economic Co-operation and Development (OECD).

INTRODUCTION

- Endocrine disrupting chemicals (EDCs) are substances in the environment that interfere with the normal function of hormones in the endocrine system.
- Public health concerns have been raised by studies reporting that animal populations exposed to EDCs have an increased incidence of reproductive and developmental abnormalities.
- In 2005, CertiChem Inc. (CCi) nominated their *in vitro* MCF-7 cell proliferation test method to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) as a screening assay to identify estrogen agonists and antagonists (CCi MCF-7 Assay)
- NICEATM coordinated an international validation study with its counterparts in Japan (JaCVAM) and Korea (KoCVAM) using laboratories sponsored by each validation organization
- Seventy eight reference substances recommended by ICCVAM for the validation of *in vitro* ER and AR binding and TA test methods (ICCVAM 2006) are being tested in the validation study
- However, this poster presents the data only from CCi as the leading validation organization:. Performance standards were developed by ICCVAM and OECD to allow test method developers to assess the performance of new methods against those that have been previously validated.. CCi data show that roboticized MCF-7 EA and AEA assays have accuracy, sensitivity, . and specificity values at least equivalent to values obtained for other test methods accepted by U.S. EPA and OECD.

MATERIALS AND METHODS

Summary of EA and Anti-EA Procedures

- MCF-7 cells - human breast adenocarcinoma cell line that endogenously expresses estrogen receptors (ERα and ERβ)
- Measures whether and to what extent a substance induces (ER agonist) or reduces (ER antagonist) estrogenic activity
- Automated liquid handling system (EpMotion 5070, Eppendorf)
- Cells treated for 6 days in estrogen free media (EFM) followed by a diphenylamine (DPA) assay to measure DNA content
- Range finder testing (8 concentrations at log serial dilutions)
- Comprehensive testing (12 concentrations at 2.5:1 or 5:1 serial dilutions)
- If potentially positive in comprehensive test (**Fig.1 &3**), an EA or AEA confirmation testing is conducted to confirm the effect on cell proliferation is ER mediated
  - If the substance-stimulated cell proliferation is significantly reduced by the presence f the antagonist ICI182,780 (**Fig. 2**), EA is then confirmed.
  - If the reduction of 2E-12M E2-induced cell proliferation is reversed by the presence of 2E-9M E2, AEA is then confirmed (**Fig. 4**). Otherwise, AEA is negative (**Fig. 5**)

RESULTS

Fig. 1: Example of EA positive dose-response curve

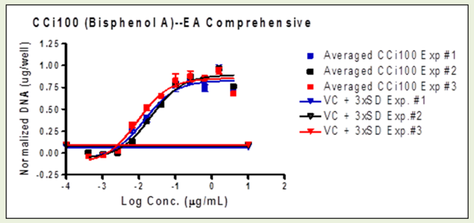


Fig. 2: Example of EA confirmation dose-response curve

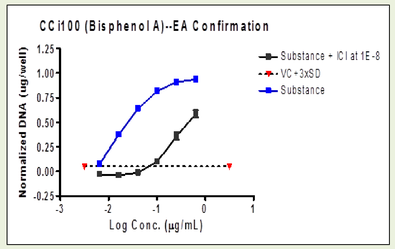
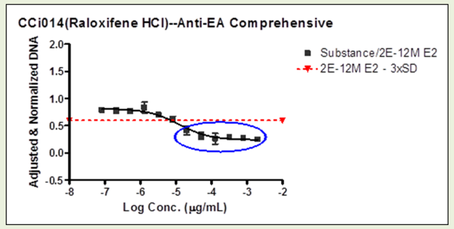
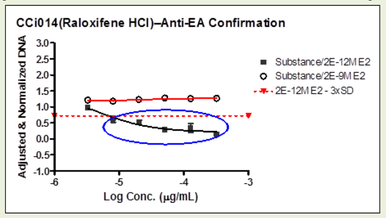


Fig. 3: Example of AEA positive dose-response curve



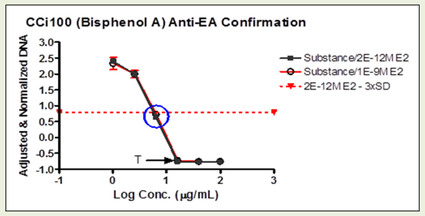
Blue circle: Data points are potentially AEA positive

Fig. 4: Example of AEA confirmation dose-response curve



Blue circle: Data points are confirmed to be AEA positive

Fig. 5: Example of AEA negative dose-response curves



Blue circle: Data points are not confirmed to be AEA positive

Table 1: Summary of CCi EA and AEA results for ICCVAM test substances used for Concordance analyses

Substance	ICCVAM Meta EC50	ICCVAM BG1 EC50	CCi MCF-7 EC50	CCi %Sup	ICCVAM Meta IC50	ICCVAM BG1 IC 50	CCi MCF-7 IC50	CCi %Rec
17ß-Estradiol	$8.7x10^{-11}$ S+	$3.4x10^{-12}$ +	$2.9x10^{-12}$ [2] S+	75, 63		-	-	
17α-Estradiol	$5.2x10^{-9}$ M+	$3.0x10^{-10}$ +	$1.6x10^{-10}$ [2] S+	59, 54		I	-	
17α-Ethinyl estradiol	$5.2x10^{-11}$ S+	$7.1x10^{-12}$ +	$8.3x10^{-13}$ S+	100, 79	-	-	-	
19-Nortestosterone	$2.0x10^{-7}$ +	$1.7x10^{-6}$ +	$5.4x10^{-8}$ M+	44, 44			-	
4-Cumylphenol	$3.2x10^{-7}$ W+	$3.0x10^{-7}$ +	$8.4x10^{-8}$ M+	101, 77			-	
4-Hydroxytamoxifen	I	-	-		$2.1x10^{-9}$ M+	$4.9x10^{-9}$ M+	$2.6x10^{-11}$ [2] S+	126, 125
4-*tert*-Octylphenol	$1.0x10^{-7}$ M+	$2.1x10^{-8}$ +	$1.9x10^{-8}$ M+	103, 101			-	
5α-Dihydrotestosterone	$1.3x10^{-7}$ M+	$9.0x10^{-8}$ I	$1.4x10^{-7}$ W+	39, 32	-	-	-	
Apigenin	$7.7x10^{-7}$ S+	$1.4x10^{-6}$ +	$4.4x10^{-7}$ [3] W+	61, 58	-	-	-	
Atrazine	-	-	[3] -		-	-	-	
Bicalutamide	-	-	-				-	
Bisphenol A	$5.0x10^{-7}$ W+	$4.0x10^{-7}$ +	$6.5x10^{-8}$ [3] M+	72, 66	-	-	-	
Bisphenol B	$9.2x10^{-8}$ M+	$2.4x10^{-7}$ +	$4.6x10^{-8}$ [3] M+	76, 58			-	
Butylbenzyl phthalate	M+	$2.7x10^{-6}$ +	$6.2x10^{-10}$ [4] S+	88, 80	-	-	-	
Chrysin	+	+	$6.0x10^{-8}$ [2] M+	57, 42	-	-	-	
Clomiphene citrate	+	I	[3] I				$5.0x10^{-9}$ [2] M+	160,156
Corticosterone	-	-	[3] -		-	-	-	
Coumestrol	$1.6x10^{-8}$ M+	$1.3x10^{-7}$ +	$1.8x10^{-9}$ M+	114, 99	-	-	-	
Daidzein	$4.9x10^{-7}$ W+	$6.8x10^{-7}$ +	$5.3x10^{-8}$ M+	100, 69	-	-	-	
Dicofol	$7.1x10^{-6}$ W+	$2.2x10^{-6}$ +	$6.6x10^{-7}$ W+	67, 60	-	-	-	
Diethylhexyl phthalate	I		$9.0x10^{-9}$ M+	44, 39	-	-	-	
Diethylstilbestrol	$6.6x10^{-11}$ S+	$2.1x10^{-11}$ +	$2.9x10^{-12}$ [2] S+	79, 65	-	-	-	
Di-*n*-butyl phthalate	W+		$5.2x10^{-9}$ M+	55, 52	-	-	-	
Estrone	$2.1x10^{-9}$ S+	$2.2x10^{-10}$ +	$2.7x10^{-11}$ S+	105 ,75	-	-	-	
Ethyl paraben	+	+	$3.9x10^{-6}$ W+	92, 92		-	-	
Fenarimol	$7.0x10^{-6}$ W+	$9.2x10^{-6}$ +	$6.0x10^{-8}$ M+	70, 64	+	-	-	
Flutamide	-	I	-				-	
Genistein	$6.8x10^{-8}$ W+	$3.0x10^{-7}$ +	$2.6x10^{-9}$ [3] M+	83, 79	-	-	-	
Hydroxyflutamide	-	-	-				$3.4x10^{-5}$ S+	109,101
Kaempferol	$1.6x10^{-7}$ W+	$2.6x10^{-7}$ +	$6.9x10^{-8}$ M+	105, 75	-	-	-	
Kepone	W+	+	$1.7x10^{-6}$ W+	49, 38	-	-	-	
L-Thyroxine	+	-	I			-	-	
Linuron	-	-	-				-	
meso-Hexestrol	$1.0x10^{-10}$ S+	$1.6x10^{-11}$ +	$1.4x10^{-12}$ [2] S+	67, 49		-	-	
Methyl testosterone	$1.6x10^{-8}$ M+	$6.5x10^{-7}$ +	$1.2x10^{-6}$ W+	36, 34			-	
Mifepristone	-	-	-		-	-	-	
Norethynodrel	$6.4x10^{-9}$ +	$1.3x10^{-7}$ +	$2.8x10^{-10}$ S+	59, 56	-	-	-	
o,p' - DDT	$1.7x10^{-6}$ W+	$4.2x10^{-7}$ +	$2.8x10^{-7}$ [3] W+	87, 79	-	-	-	
p-n-Nonylphenol	$3.6x10^{-7}$ M+	$2.5x10^{-6}$ +	$1.1x10^{-6}$ [3] W+	79, 78	-	-	-	
p,p' - Methoxychlor	$5.3x10^{-6}$ W+	$8.4x10^{-7}$ +	$3.4x10^{-6}$ W+	84, 78	-	-	-	
p,p' - DDE	W+	I	$2.3x10^{-5}$ W+	54, 54	-		-	
Phenobarbital	-	-	-				-	
Procymidone	-	I	-				-	
Progesterone	I		-		-	-	-	
Raloxifene HCl	-	-	-		$2.3x10^{-9}$ M+	$1.2x10^{-9}$ M+	$4.2x10^{-11}$ [34] S+	166,146
Resveratrol	M+	I	[3] =[a]	63, 44	-	-	-	
Spironolactone	-	-	-				-	
Tamoxifen	$5.3x10^{-7}$ +	$6.7x10^{-8}$ I	[3] I		$4.0x10^{-7}$ W+	$7.1x10^{-7}$ W+	$5.7x10^{-9}$ [3] M+	79, 78

The 2nd and 6th columns give the published median values (EC50 andIC50) from the meta-analysis by ICCVAM (2011). and EA and AEA classification as follows: S+: test substance was strongly active (EC50/IC50 value < 1.0 × 10^{-9}M); M+: moderately active (EC50/IC50 value 1.0 × 10^{-9} to 1.0 × 10^{-7}M); W+: weakly active (EC50/IC50 value > 1.0 × 10^{-7}M); –: no detectable EA/AEA; I (inadequate): test substance positive in one assay but negative in one or more other assays; +: test substance positive in a single assay or in the majority of assays. The third and seventh columns give mean EC50 or IC50 molar values as determined by our MCF-7:WS8 EA or AEA assays, respectively.

CONCLUSIONS

- Robotized MCF-7 EA and AEA assays using estrogen-responsive MCF-7:WS8 cells to detect EA and AEA provide repeatable, reproducible, sensitive (as defined by EC50s or IC50s), and accurate results in high concordance with ICCVAM meta-analyses.
- Robotized MCF-7 EA and AEA assays have accuracy, sensitivity, and specificity values at least equivalent to validated test methods accepted by the U.S. EPA and the OECD.

Conflict of interest

CZY is employed by, and owns stock in, CertiChem (CCi). MAS, GJK and AWW are (or were) employed by CCi. GDB owns stock in, and is a consultant CEO of CCi. All authors had freedom to design, conduct, interpret, and publish research uncompromised by any controlling sponsor.

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