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Development and pre-validation of an *in vitro* transactivation assay for detection of (anti)androgenic potential compounds using 22Rv1/MMTV cells

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ABSTRACT

The endocrine-disrupting effects of androgenic signaling play crucial roles in several androgen-related diseases. In attempting to develop an *in vitro* cell line to be used in androgen receptor (AR)-mediated reporter gene assays, we developed a stable 22Rv1/MMTV cell line, which is a human prostate cancer cell line that endogenously expresses functional AR, to evaluate AR-mediated transcriptional activation (TA). Using 22Rv1/MMTV cells, we established and optimized a test protocol for the AR-TA assay and validated the proposed assay using 20 compounds recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). All the performance parameters for agonist and antagonist assays were 91–100% comparable between the 22Rv1/MMTV assay and the ICCVAM report. In conclusion, the AR-TA assay using 22Rv1/MMTV cells might be a quick and relatively inexpensive method for screening large numbers of chemicals for their potential to activate or inhibit AR-mediated gene transcription.

1. Introduction

A number of environmental chemicals interfere with the endocrine system and have adverse effects on the reproductive, developmental, neurological, and immunological systems of mammals. Over the last decade, it has been found that endocrine-disrupting chemicals (EDCs) exert adverse effects at low doses [1–3]. Exposure to endocrine disruptors has life-long effects and even consequences for the next generation [4]. Some EDCs mimic the biological activity of hormones by binding to hormonal receptors, leading to unexpected responses [2,5,6]. To address the rising global concern about EDCs, there is a need to screen chemicals with hormone-like activities. Worldwide organizations including those in Europe, the US, Japan, and Korea have developed new guidelines for the detection of endocrine activities.

The collaboration of groups studying endocrine disruptor testing and assessment was initiated in 1997 by the Organization for Economic Co-operation and Development (OECD). Cooperation between the international bodies working on the test guidelines is managed by the Endocrine Disruptors Testing and Assessment (EDTA) task force and the Validation Management Group for Non Animal Testing (VMG-NA), which has standardized the test guidelines. The communication adopted by the European Commission entitled 'Community Strategy for Endocrine Disruptors [COM (1999) 706]' contains the methodology for assessing hazard risk. In addition, the US Environmental Protection Agency recommended transcriptional activation (TA) as a Tier 1 screening tool to identify the interactions between chemicals and hormonal systems [19].

The OECD approved the 'OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupters' in 2002, and a revised version was published in 2012. This document describes five levels of testing and assessment requirements for endocrine disruptors. The *in vitro* assays that provide data on the selected endocrine mechanisms are outlined in level 2 of the framework.

Abbreviations: AR, androgen receptor; CSF, charcoal-dextran stripped FBS; DHT, 5α-dihydrotestosterone; EDCs, endocrine disrupting chemicals; EDTA, the Endocrine Disruptors Testing and Assessment; ER, estrogen receptor; GR, glucocorticoid receptor; ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; MMTV, mouse mammary tumor virus; OECD, Organization for Economic Co-operation and Development; PC, positive control; PgR, progesterone receptor; R1881, Methyltrienolone; RT-PCR, reverse transcription-polymerase chain reaction; SDs, standard deviations; TA, transcriptional activation; VC, vehicle control; Vmax, maximum values; Vmin, minimum value.

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Table 1
Chemicals used for pre-validation of 22Rv1/MMTV AR-TA.

No.	Chemical	Supplier	CAS number	Purity (%)	M.W.	Test Range (Log M)
1	Testosterone	TCI	58-22-0	≥98	288.4	−6 ~ −12
2	5α-dihydrotestosterone	Wako	521-18-6	≥95	290.4	−6 ~ −12
3	Methyltestosterone	TCI	58-18-4	≥98 (LC)	302.5	−6 ~ −12
4	Methyltrienolone	Dongguk Univ.	965-93-5	≥99	284.4	−6 ~ −12
5	Flutamide	Sigma	13311-84-7	–	276.2	−3 ~ −9
6	Hydroxyflutamide	Sigma	52806-53-8	≥98 (HPLC)	292.2	−4 ~ −10
7	Diethylhexyl phtalate	Aldrich	117-81-7	99	390.6	−3 ~ −9
8	Di(*n*-butyl) phtalate	Supelco	84-74-2	99.5	278.4	−3 ~ −9
9	Bisphenol A	Aldrich	80-05-07	≥99	228.3	−3 ~ −9
10	*p*-*n*-nonylphenol	Aldrich	84852-15-3	–	220.4	−3 ~ −9
11	4-*tert*-octylphenol	Aldrich	140-66-9	97	206.3	−3 ~ −9
12	o,p'-DDT	Supelco	789-02-6	99.2	354.5	−4 ~ −10
13	Vincozolin	Chem service	50471-44-8	99	286.1	−3 ~ −9
14	Ketoconazole	Sigma	65277-42-1	≥98 (TLC)	531.4	−5 ~ −11
15	Linuron	Chem service	330-55-2	99	249.1	−3 ~ −9
16	Methoxychlor	Supelco	72-43-5	99.9	346.0	−3 ~ −9
17	Diethystilbestrol	Sigma	56-53-1	≥99	268.4	−4 ~ −10
18	17β-Estradiol	Wako	50-28-2	97.0 ~ 103	272.4	−3 ~ −9
19	Atrazine	Supelco	1912-24-9	99.9	215.7	−3 ~ −9
20	Progesterone	Sigma	57-83-0	–	314.5	−5 ~ −11

TCI: Tokyo Chemical Industry Co, LTD.
Dogguk Univ.: Dong-guk University, Korea.
Wako: Wako Pure Chemicals industries, Ltd.
Sigma: Sigma-Aldrich Co. LLC.
Aldrich: Sigma-Aldrich Co. LLC.
Supelco: Supelco, Sigma-Aldrich.
ChemService: Chem Service, Inc.

Table 2
Log [PC_{10}(M)], Log [PC_{50}(M)] and Vmax value from pre-validation study in 22Rv1/MMTV AR-TA agonist assay.

No.	Chemical	22Rv1/MMTV AR-TA agonist					
		PC: DHT (1 nM)			PC: R1881 (10 nM)		
		Log[PC_{10}(M)]	Log[PC_{50}(M)]	V_{max} (% of DHT)	Log[PC_{10}(M)]	Log[PC_{50}(M)]	V_{max} (% of R1881)
1	Testosterone	−11.22	−10.37	160.5	−10.82	−9.77	113.8
2	5α-dihydrotestosterone	−11.17	−9.92	156.3	−10.36	−9.07	121.9
3	Methyltestosterone	−11.29	−10.35	149.8	−10.95	−9.88	112.4
4	Methyltrienolone (R1881)	−9.69	−8.88	105.7	−10.90	−9.90	106.3
5	Flutamide	–	–	4.6	–	–	1.8
6	Hydroxyflutamide	−6.35	−5.29	49.6	−5.78	–	26.2
7	Diethylhexyl phtalate	–	–	0.7	–	–	−0.2
8	Di(*n*-butyl) phtalate	–	–	2.2	–	–	9.1
9	Bisphenol A	–	–	0.1	–	–	0.0
10	*p*-*n*-nonylphenol	–	–	−0.6	–	–	−0.1
11	4-*tert*-octylphenol	–	–	−0.6	–	–	−0.3
12	o,p'-DDT	–	–	−0.4	–	–	0.0
13	Vincozolin	–	–	6.7	–	–	2.8
14	Ketoconazole	–	–	−0.1	–	–	0.2
15	Linuron	−4.11	–	15.8	−3.51	–	14.6
16	Methoxychlor	–	–	−0.1	–	–	0.0
17	Diethystilbestrol	–	–	0.4	–	–	0.0
18	17β-Estradiol	−8.78	−7.26	134.8	−8.54	−6.71	105.5
19	Atrazine	–	–	5.6	–	–	6.1
20	Progesterone	−7.87	−6.17	102.5	−7.63	−5.74	81.2

The responses of nuclear receptors play important roles in TA [7,8]. Nuclear receptors like estrogen receptors (ERs) or androgen receptors (ARs) initiate the endocrine disruptor response. When ligands with hormonal activity bind to the nuclear receptors within cells, the ligand-receptor complex undergoes a conformational change that leads to a transcriptional response [9]. Therefore, the TA mediated by endocrine disruptors is a plausible starting point for hazard assessment. Considering the mechanisms by which hormones act, reporter gene assay responses to the nuclear receptor complex can be used to detect the hormonal activity of exogenous chemicals [10].

To detect chemicals that have androgenic and anti-androgenic activities, Japan and the European commission have developed the AR-Stably Transfected Transcriptional Activation assay using AR-EcoScreen™ [11], PALM, AR-CALUX, and U2-OS cell lines transfected with the cDNA of the human AR and a luciferase reporter gene that has undergone a validation study [12–14]. However, no androgen-dependent cell line has been created and proposed to the OECD. A system measuring hormone receptor-mediated transcription in a stable cell line is preferable to others as it will have high sensitivity, specificity and reproducibility so that agonistic and antagonistic actions can be understood [12]. Experimental models differ not only in species-specificity but also in their physiological complexity, the stoichiometric ratio between endogenous AR protein and other transcriptional regulators might reflect a more natural situation than the overexpression of AR in human or rodent cells [15]. Each plasmid for the establishment of a stable cell line has a specific antibiotic marker. For the maintenance of a stable cell