Reproductive Toxicology 30 (2010) 2–8

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Assessment of a recombinant androgen receptor binding assay: Initial steps towards validation☆

Alexius Freyberger[a,*], Marc Weimer[b], Hoai-Son Tran[a], Hans-Jürgen Ahr[a]

[a] Bayer Schering Pharma AG, BSP-GDD-GED-GTOX Special Toxicology, Aprather Weg 18, D-42096 Wuppertal, Germany
[b] German Cancer Research Center, Biostatistics-C060, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany

ARTICLE INFO

Article history:
Received 10 August 2009
Received in revised form
29 September 2009
Accepted 1 October 2009
Available online 13 October 2009

Keywords:
Rat recombinant androgen receptor
Binding assay
Screening for endocrine active compounds

ABSTRACT

Despite more than a decade of research in the field of endocrine active compounds with affinity for the androgen receptor (AR), still no validated recombinant AR binding assay is available, although recombinant AR can be obtained from several sources. With funding from the European Union (EU)-sponsored 6th framework project, ReProTect, we developed a model protocol for such an assay based on a simple AR binding assay recently developed at our institution. Important features of the protocol were the use of a rat recombinant fusion protein to thioredoxin containing both the hinge region and ligand binding domain (LBD) of the rat AR (which is identical to the human AR-LBD) and performance in a 96-well plate format. Besides two reference compounds [dihydrotestosterone (DHT), androstenedione] ten test compounds with different affinities for the AR [levonorgestrel, progesterone, prochloraz, 17α-methyltestosterone, flutamide, norethynodrel, o,p′-DDT, dibutylphthalate, vinclozolin, linuron] were used to explore the performance of the assay. At least three independent experiments per compound were performed. The AR binding properties of reference and test compounds were well detected, in terms of the relative ranking of binding affinities, there was good agreement with published data obtained from experiments using recombinant AR preparations. Irrespective of the chemical nature of the compound, individual IC_{50}-values for a given compound varied by not more than a factor of 2.6. Our data demonstrate that the assay reliably ranked compounds with strong, weak, and no/marginal affinity for the AR with high accuracy. It avoids the manipulation and use of animals, as a recombinant protein is used and thus contributes to the 3R concept. On the whole, this assay is a promising candidate for further validation.

1. Introduction

Concerns have been raised whether natural and man-made chemicals might have the potential of interfering with the endocrine system and thus may affect wildlife and humans and/or their progeny. Initial studies focused on interactions with estrogen receptor-mediated signaling, more recently, interactions with other receptors such as the androgen receptor (AR) gained attention [1–5]. Both the recommendations to the US Environmental Protection Agency (EPA) of their Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) [6] and the OECD conceptual framework for the testing and assessment of endocrine disrupting chemicals [7] addressed receptor binding assays as important tools to study interactions with sex hormone receptors and these assays represent important components of the US EPA tier 1 screening battery and of level 2 of the conceptual frame work. Interestingly, despite more than one decade of research in the field of endocrine active compounds targeting the AR, and the availability of recombinant AR from several sources, still no validated androgen receptor binding assay using a recombinant AR protein and thus avoiding the use of animal tissues as a receptor source is available. With funding from the EU 6th framework project ReProTect, we made first steps towards such a validation. A simple AR binding assay developed at our institution [8] using a rat recombinant fusion protein to thioredoxin containing both the hinge region and ligand binding domain (LBD) of the rat AR (which is identical to the human AR-LBD), performed in a 96-well plate format, was optimised and performance of the assay protocol was evaluated. At least three independent experiments per compound were performed on different days, and dilutions of test compounds from deep-frozen stocks, solutions of radiolabeled ligand and receptor preparation were freshly prepared for each experiment. Besides the two reference compounds dihydrotestosterone (DHT) and androstenedione, ten test compounds (17α-methyltestosterone (17α-MT), levonorgestrel, norethynodrel, progesterone, prochloraz, flutamide, o,p′-DDT, lin-

☆ This work was in part presented at the spring meeting of the German Society for Clinical and Experimental Pharmacology and Toxicology in 2009.

* Corresponding author at: Bayer Schering Pharma AG, BSP-GDD-GED-GTOX Special Toxicology, Building 514, Aprather Weg 18, D-42096 Wuppertal, Germany. Tel.: +49 0202 36 8832; fax: +49 0202 36 4137.
E-mail address: alexius.freyberger@bayerhealthcare.com (A. Freyberger).

0890-6238/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.reprotox.2009.10.001

Table 1
Receptor binding characteristics in competition experiments in the absence of competitor.

Experiment	Solvent	Ligand concentration (% of nominal)	Plate	Binding (% of nominally added ligand)					
				Total (vehicle control)		Non-specific (excess DHT)		Specific (difference)	
				On plate	Mean	On plate	Mean	On plate	Mean
1	DMSO	104.9	A	33.67	33.67	1.83	1.83	31.83	31.83
2	DMSO	100.3	A	39.01	39.01	1.74	1.74	37.27	37.27
3	DMSO	100.6	A	31.50	31.50	1.37	1.37	30.13	30.13
4	Ethanol	101.4	A	21.55	21.80	3.79	3.32	17.77	18.48
			B	22.05		2.85		19.20	
5	Ethanol	97.7	A	42.27	40.94	1.94	1.85	40.33	39.10
			B	39.61		1.75		37.87	
6	Ethanol	104.6	A	59.11	56.28	2.60	2.41	56.51	53.87
			B	53.45		2.22		51.23	
8	Ethanol	101.0	A	45.44	44.70	2.04	1.88	43.41	42.82
			B	43.96		1.72		42.23	
9	Ethanol	100.0	A	42.63	40.49	2.56	2.38	40.07	38.11
			B	38.34		2.19		36.15	
10	Ethanol	98.3	A	35.11	36.97	2.16	2.11	32.95	34.86
			B	38.83		2.06		36.77	

Calculations are based on mean values from at least three technical replicates. For experimental details see Fig. 1.

uron, vinclozoin, dibutylphthalate) with different affinities for the AR selected and agreed upon in collaboration with the European Centre for the Validation of Alternative Methods (ECVAM) were studied, and results were subjected to an independent stringent statistical analysis. The outcome of this investigation is reported herein.

2. Materials and methods

2.1. Chemicals and receptor

Dihydrotestosterone (≥99.5%), androstenedione (99%), levonorgestrel (99%), progesterone (99%), norethynodrel (>99%), flutamide (99%), DL-dithiothreitol (DTT, >99%), human γ-globulin (99%) and dextran-coated charcoal, were supplied by Sigma (Taufkirchen, Germany). 17α-Methyltestosterone (97–103%) was delivered by Acros Organics (Geel, Belgium). Prochloraz (99.5%), linuron (99.7%), and vinclozolin (99.6%) from Riedel-de-Haen and o,p′-DDT (96.7%) from Supelco were obtained through Sigma. Dibutylphthalate (100%), DMSO (p.a.), ethanol (p.a.), anhydrous glycerol, sodium chloride, and trishydroxyaminomethan were purchased from E. Merck (Darmstadt, Germany). Ultima Flo AP™ and Ultima Gold scintillation cocktails were products of Canberra-Packard (Frankfurt, Germany). [17α-Methyl-^{3}H]-methyltrienolone (Metribolone, R1881) in ethanol with a specific activity of 2.579 TBq/mmol and a radiochemical purity of >97% was supplied by PerkinElmer (Rodgau-Jürgesheim, Germany). Androgen receptor (rat recombinant fusion protein to thioredoxin containing both the hinge region and ligand binding domain of the rat AR, the ligand binding domain being identical to that of the human AR, functional receptor concentration 3500 pmol/mL, specific activity 3933 pmol/mg) was obtained from PanVera (Madison, WI, USA) through MoBiTec (Göttingen, Germany). The receptor preparation was slowly thawed on ice, aliquots were placed in microvials, rapidly frozen in liquid nitrogen and kept deep-frozen at −80 °C. Aliquots were thawed only once.

2.2. Determination of receptor binding

The assay was performed as described [8] with slight modifications. Receptor binding experiments were performed on 96-well microtiter plates generally in triplicate incubations. For total binding (= solvent control) and non-specific binding (binding in the presence of excess, i.e., 1 μM DHT) six replicates were performed. If an experiment had to be spread across several microtiter plates, on each plate total and non-specific binding was assessed. Assay buffer (800 mmol NaCl, 2 mmol DTT, 10 g γ-globulin and 100 mL glycerol made up to 1 L with 50 mM Tris–HCl pH 7.5) was freshly prepared for each experiment. Stock solutions of reference and test compounds corresponding to 100-fold the highest concentration tested were preferably prepared in ethanol, if ethanol was not suitable, DMSO was used. Aliquots of stock solutions were kept deep-frozen, each aliquot was used only once and discarded at the end of an individual experiment. Serial dilutions of stocks in solvent were made, the final step was 1:50 dilution into assay buffer. Radiolabeled ligand solution (8 nM ^{3}H-R1881) was prepared in assay buffer containing 4% ethanol. The actual amount of ligand was determined by liquid scintillation counting (LSC) in a β-counter (1900 TR Counter, Canberra-Packard, Frankfurt, Germany) with quench correction, the maximally tolerated deviation from the theoretical amount was ±5%. If the tolerance was exceeded, depending on the deviation, small volumes of buffer or radiolabeled ligand were added to meet the limits. AR was dissolved in assay buffer to achieve a nominal concentration of 8 nM. To study receptor binding, assay buffer (100 μL) containing test compound, excess DHT to assess non-specific binding or solvent (2%) only, was mixed with 50 μL ^{3}H-R1881 solution for 10–15 min at 2–8 °C. Then, AR solution in assay buffer was added and the whole mixture was incubated at 2–8 °C overnight under slight continuous shaking. Final concentration were 2 nM AR and 2 nM ^{3}H-R1881, for reference and test compounds concentrations were chosen to achieve as much as possible full concentration response curves or testing was performed up to the limit of solubility. The following concentrations were used:

- Dihydrotestosterone: 0.1, 0.3, 1, 3, 10, 30, 100, 300 nM
- Levonorgestrel and 17α-MT: 0.3, 1, 3, 10, 30, 100, 300, 1000 nM
- Norethynodrel and progesterone: 10, 30, 100, 300, 1000, 3000, 10,000, 30,000 nM
- Androstenedione: 30, 100, 300, 1000, 3000, 10,000, 30,000, 100,000 nM
- o,p′-DDT: 100, 300, 1000, 3000, 10,000, 30,000, 100,000 nM
- Prochloraz: 100, 300, 1000, 3000, 10,000, 30,000, 100,000, 300,000 nM
- Linuron: 300, 1000, 3000, 10,000, 30,000, 100,000 nM
- Flutamide: 300, 1000, 3000, 10,000, 30,000, 100,000, 300,000 nM
- Vinclozolin and DBP: 10, 100, 1000, 10,000, 100,000 nM

The next day, 50 μL of a 5% dextran-coated charcoal suspension in assay buffer was added. After mixing in the cold for 10 min, charcoal was sedimented by centrifugation at 4000 rpm for 5 min in a cooling centrifuge. Then, 50 μL aliquots of the clear supernatant containing the AR-ligand complex were placed in another 96-well plate and mixed with 200 μL Ultima Flo AP™ scintillation cocktail (Canberra-Packard, Frankfurt, Germany) and radioactivity was determined by LSC using a LSC microplate reader (1450 Microbeta™ Trilux, Wallac, Freiburg, Germany).

In the course of this investigation, run 7 (replaced by run 8) of the competitive binding experiments was invalidated. Inadvertantly, the shaker had been set at too high speed, and following the overnight incubation, material from various wells had spilled over the plates.

2.3. Data handling

For competitive binding experiments, prior to fitting a dose–response model and estimation of IC_{50}-values mean non-specific binding was subtracted from the response. After subtraction the response was divided by the mean of the solvent control. The three-parametric log-logistic function

$$f(x) = \frac{\theta_1}{1 + \exp(\theta_2(\log(x) - \theta_3))}$$

was fitted to the transformed data using the `drm` function of R-package `drc` [9]. Parameter θ_1 is the upper asymptote of the response range and corresponds to what is called "Top" in GraphPad Prism, parameter θ_2 is the slope parameter, and parameter θ_3 corresponds to the log of the IC_{50}. Note that there is no estimation