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Assessment of a robust model protocol with accelerated throughput for a human recombinant full length estrogen receptor-α binding assay: Protocol optimization and intralaboratory assay performance as initial steps towards validation

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ABSTRACT

Despite about two decades of research in the field of endocrine active compounds, still no validated human recombinant (hr) estrogen receptor-α (ERα) binding assay is available, although hr-ERα is available from several sources. In a joint effort, US EPA and Bayer Schering Pharma with funding from the EU-sponsored 6th framework project, ReProTect, developed a model protocol for such a binding assay. Important features of this assay are the use of a full length hr-ERα and performance in a 96-well plate format. A full length hr-ERα was chosen, as it was considered to provide the most accurate and human-relevant results, whereas truncated receptors could perform differently. Besides three reference compounds [17β-estradiol, norethynodrel, dibutylphthalate] nine test compounds with different affinities for the ERα [diethylstilbestrol (DES), ethynylestradiol, meso-hexestrol, equol, genistein, *o,p′*-DDT, nonylphenol, n-butylparaben, and corticosterone] were used to explore the performance of the assay. 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. The ERα binding properties of reference and test compounds were well detected. As expected dibutylphthalate and corticosterone were non-binders in this assay. In terms of the relative ranking of binding affinities, there was good agreement with published data obtained from experiments using a human recombinant ERα ligand binding domain. Irrespective of the chemical nature of the compound, individual IC_{50}-values for a given compound varied by not more than a factor of 2.5. Our data demonstrate that the assay was robust and reliably ranked compounds with strong, weak, and no affinity for the ERα with high accuracy. It avoids the manipulation and use of animals, i.e., the preparation of uterine cytosol as receptor source from ovariectomized rats, as a recombinant protein is used and thus contributes to the 3R concept (reduce, replace, and refine). Furthermore, in contrast to other assays, this assay could be adjusted to an intermediate/high throughput format. On the whole, this assay is a promising candidate for further validation.

1. Introduction

There is concern that man-made and natural compounds may interfere with the endocrine system and thus may affect wildlife and humans and/or their progeny. Initial studies focused on interactions with estrogen receptor (ER)-mediated signaling and date back to the 1980s [see 1]. These studies have intensified over the years and have been used to characterize a number of chemicals and natural products as compounds with weak estrogenic properties. More recently, interactions with other receptors such as the androgen receptor have gained attention [2–4]. Both the recommendations to the US Environmental Protection Agency (EPA) by their Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) [5] and the OECD conceptual framework for the testing and assessment of endocrine disrupting chemicals [6] recognized receptor binding assays as important tools to study interactions with sex hormone receptors. These assays, therefore, represent important components of the US EPA tier 1 screening battery and level 2 of the OECD's conceptual framework. Interestingly, despite two decades of research in the field of endocrine active compounds and the availability of human recombinant ER from several sources, no validated human recombinant ER bind-

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ing assay is available. In a joint effort, US EPA and Bayer Schering Pharma (BSP) (with funding from the European Union-sponsored 6th framework project, ReProTect), developed a model protocol. Additional advisement to this activity was given from an international expert group working on the validation of recombinant ER and AR binding assays under the auspices of the OECD's Validation Management Group for Non-Animal Testing (VMG-NA). Important features of the protocol for competitive binding studies were the use of (a) a commercially available full length human recombinant (hr) estrogen receptor-α (ERα), (b) [^{3}H]-β-estradiol (1 nM) as ligand, (c) an amount of ERα resulting in a specific binding corresponding to approximately 20 ± 5% relative to the added [^{3}H]-ligand, and (d) a 96-well plate format that allows feasibility for up to nine chemicals to be tested at a time (not including the reference chemicals). A full length hr-ERα was chosen, as it was considered to provide the most accurate and human-relevant results, whereas as truncated receptor could perform in a different manner. A commercially available full length hr-ERα was considered helpful for people not experienced in the isolation of receptors, not having the equipment to grow (human) cells prior to isolation, or that do not want to use animals for the preparation of receptors. A set of reference (17β-estradiol, a strong binder; norethynodrel, a weak binder; and dibutylphthalate, a non-binder) and test compounds (diethylstilbestrol (DES), ethynylestradiol, meso-hexestrol, equol, genistein, *o*,*p*′-DDT, nonylphenol, n-butylparaben, and corticosterone) that had been agreed upon by a Chemical Advisory Board (CAB) jointly appointed by the hr-ERα binding assay validation working group of the OECD's VMG-NA (see above) was used to explore the performance of the assay by evaluating a variety of chemical classes and differing affinities for the receptor. Results were subjected to an independent statistical analysis. The outcome of this effort is reported herein.

2. Materials and methods

2.1. Estrogen receptor-α

Full length human recombinant estrogen receptor-α was obtained from Invitrogen Corporation (Carlsbad, CA, USA) (order no. P2187, lot no. 34216I). Functional receptor concentration according to the manufacturer was 2088 pmol/mL with a specific activity of 8700 pmol/mg. The receptor preparation was slowly thawed on ice, and aliquots (20 pmol receptor) were placed in microvials, rapidly frozen in liquid nitrogen and kept deep-frozen at −80 °C. Aliquots were thawed only once.

2.2. Chemicals

17ß-Estradiol (CAS 50-28-2) 98%, 17α-ethynylestradiol (57-63-6) 98%, diethylstilbestrol (DES, 56-53-1) 99%, m-hexestrol (84-16-2) 98%, dibutylphthalate, DL-dithiothreitol (DTT) >99%, leupeptin semi-sulfate, bovine serum albumin (BSA), and dextran-coated charcoal was supplied by Sigma–Aldrich (Taufkirchen, Germany). Nonylphenol (84852-15-3) 96.9% and corticosterone (50-22-6) 98.5% were obtained from Fluka (Buchs, Switzerland) through Sigma–Aldrich. Racemic equol (531-95-3) 98.2% was delivered by Apin Chemicals (Abingdon, UK), 1,1,1-trichloro-2,2-bis(*o*,*p*′-chlorophenyl)ethane (*o*,*p*′-DDT, 789-02-6) 99.2% was purchased from Supelco (Bellefonte, PA, USA) through Sigma–Aldrich. Norethynodrel (68-23-5, United States Pharmacopial Convention) 100%, genistein (446-72-0, American Custom Chemical Corp.) 99.16%, and n-butylparaben (94-26-8, ChemService) 98.2% were kindly provided by U.S. EPA. Tris(hydroxymethyl)aminomethane p.a., anhydrous glycerol, ethanol p.a. and DMSO p.a. were supplied by E. Merck (Darmstadt, Germany). Ultima Flo AP™ and Ultima Gold scintillation cocktails were products of Canberra-Packard (Frankfurt, Germany). [^{3}H]-17β-estradiol (estradiol [2,4,6,7,16,17β-^{3}H(N)], specific activity: 4.07 TBq/mmol, 37 MBq/mL) in ethanol with a radiochemical purity of >97% was supplied by PerkinElmer (Rodgau-Jürgesheim, Germany).

2.3. Determination of receptor binding

Receptor binding experiments were performed in 96-well microtiter plates in at least triplicate incubations. Assay buffer (0.2 mM leupeptin, 2 mmol DTT, 10 g BSA and 100 mL glycerol made up to 1 L with 10 mM Tris–HCl; pH 7.5) was freshly prepared for each experiment prior to incubation. Stock solutions of reference compounds 17β-estradiol (10 mM), norethynodrel (10 mM), and dibutylphthalate (100 mM) and test compounds (100 mM) were prepared in ethanol, if 100 mM could not be achieved, DMSO was used, also for the determination of concurrent total and non-specific binding. Aliquots of stock solutions were kept deep-frozen at −80 °C, and each aliquot was used only once and discarded at the end of an individual experiment. Serial dilutions of stocks in solvent were made, then diluted into assay buffer 1:50 as the final step. [^{3}H]-ligand solution (4 nM if not otherwise indicated resulting in a final concentration of 1 nM) was prepared in assay buffer. Similarly ERα was dissolved in assay buffer. To study receptor binding, assay buffer (80 μL) containing test compound or solvent (2%) was mixed with 40 μL [^{3}H]-ligand solution for 10–15 min at 2–8 °C. Then, 40 μL of ERα solution was added and the whole mixture was incubated at 2–8 °C overnight under slight (appr. 250 rpm) continuous shaking. Following incubation overnight, 80 μL of a 5% dextran-coated charcoal suspension (the optimal percentage had been empirically determined) in cold assay buffer was added. After mixing in the cold at 2–8 °C for 10 min, charcoal was sedimented by centrifugation at 4000 rpm for 5 min in a cooling centrifuge at 4 °C. Finally, 50 μL aliquots of the clear supernatant containing the ERα-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 all studies, 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 6%. If the tolerance was exceeded, depending on the deviation, small volumes of buffer or small amounts of radiolabeled ligand were added to meet the limits.

The amount of receptor as specified in the model protocol should result in a specific binding roughly corresponding to 20 ± 5% relative to the added labeled ligand under conditions of competitive binding, however, slight deviations would be acceptable if not occurring regularly. Furthermore, non-specific binding under these conditions should not exceed 35% of total binding. To determine the amount of receptor necessary experimentally, final concentrations of 0.25, 0.5, 0.75, and 1.0 nM ERα were incubated with 1 nM [^{3}H]-17β-estradiol, and non-specific binding was determined in parallel in the presence of 1 μM radioinert ligand. For each condition six replicates were performed.

Saturation of the receptor was studied by incubating the receptor with increasing final concentrations (0.03, 0.06, 0.08, 0.10, 0.30, 0.60, 1.0, 3.0 nM) of [^{3}H]-17β-estradiol. Non-specific binding was determined in parallel in the presence of a 1000-fold molar excess of unlabeled 17β-estradiol. All incubations were performed in triplicate.

Studies on competitive binding were performed using a ligand concentration of 1 nM [^{3}H]-17β-estradiol and had to be spread across several 96-well plates. On the first plate, ERα was incubated with the reference compounds 17β-estradiol (0.010, 0.10, 0.30, 1.0, 3.0, 10, 100, 1000 nM), norethynodrel (3.0, 30, 100, 300, 1000, 3000, 10,000, 30,000 nM), and dibutylphthalate (0.1, 1.0, 10, 100, 1000, 10×10^3, 100×10^3, 1000×10^3 nM); furthermore, vehicle controls (no competitor), buffer controls (no competitor, no solvent) and incubations for non-specific binding in the presence of 1 μM unlabeled 17β-estradiol were performed. On the other plates, the receptor was incubated with test compounds up to 1 mM (0.1, 1.0, 10, 100, 1000, 10×10^3, 100×10^3, 1000×10^3 nM), if possible.

In the course of this investigation, for each reference/test compound four independent competitive binding experiments (runs A–D) were performed. For solvent controls, buffer controls, and non-specific binding six replicates were performed each, incubations containing reference/test compounds were studied in triplicate. Run D is the repetition of run C which was invalidated. The statistical analysis for run C had consistently indicated a putative data error for all 12 compounds under investigation, and in the light of a ligand concentration of 1 nM the IC_{50}-value for estradiol of 0.08 nM was implausibly low.

2.4. Data handling

Using the data analysis program, Prism 5.02 (GraphPad Software, Inc., San Diego, CA), a user defined analysis titled "One site – Total + Non-Specific (NS) Binding, accounting for ligand depletion" was performed and entered into Prism templates for all saturation binding runs. More information on the equation used is available from the book by Motulsky and Christopoulos (2003), "Fitting models to biological data using linear and non-linear regression: a practical guide to curve fitting" GraphPad Software, Inc., San Diego, CA. Available online at: http://www.graphpad.com/manuals/prism4/RegressionBook.pdf—page 210.

For competitive binding experiments, prior to fitting a dose-response model and estimation of IC_{50}-values mean non-specific binding was subtracted from binding observed for solvent and buffer controls or in the presence of reference/test compounds to achieve the specific binding After subtraction specific binding in the presence of reference/test compounds was divided by the mean specific binding 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` [7,8]. 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