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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, $10 \times 10^3$, $100 \times 10^3$, $1000 \times 10^3$ 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, $10 \times 10^3$, $100 \times 10^3$, $1000 \times 10^3$ 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 $\theta_1$ is the upper asymptote of the response range and corresponds to what is called "Top" in GraphPad Prism, parameter $\theta_2$ is the slope parameter, and parameter $\theta_3$ corresponds to the log of the $IC_{50}$. Note that there is no estimation

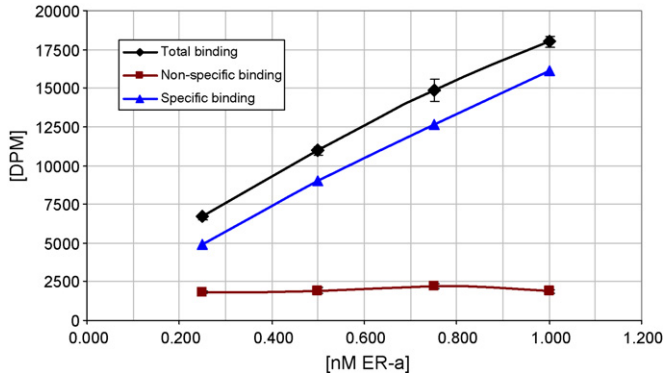


**Fig. 1.** Determination of the necessary receptor concentration–linear relationship between specific binding and receptor concentration. Under conditions corresponding to competitive binding increasing ERα concentrations were incubated with 1 nM [$^3$H]-17β-estradiol. Non-specific binding was assessed in parallel in the presence of 1 μM unlabeled 17β-estradiol. Following incubation overnight, unbound radiolabeled ligand was removed by absorption to dextran-bound charcoal (DBC) and sedimentation of DBC by centrifugation. ERα-bound radiolabel in the supernatant was determined by liquid scintillation counting. Specific binding was calculated as the difference of total and non-specific binding. For each condition six replicates were performed. Means ± S.D. are given.

of the lower asymptote of the response range. The "Bottom" was assumed to be 0 after correction for non-specific binding in case of full displacement of ligand from the receptor. In the context of receptor binding assays, the $IC_{50}$-value represents the concentration of a test compound displacing 50% of receptor bound ligand. $IC_{50}$-values and 95% confidence intervals were calculated using the `ED` function of package `drc`. This software program was also used to generate graphs of competitive binding experiments.

## 3. Results

### 3.1. Determination of the necessary amount of receptor

The model protocol requested an amount of receptor resulting in a specific binding corresponding to 20 ± 5% of the added ligand under conditions of competitive binding (i.e., 1 nM [$^3$H]-17β-estradiol). When increasing amounts of estrogen receptor-α were incubated with 1 nM [$^3$H]-17β-estradiol in the absence and presence of unlabeled 1 μM 17β-estradiol (to assess non-specific binding), a linear relationship between total and specific binding and receptor concentration was obtained. Non-specific binding was similar irrespective of the receptor concentration (Fig. 1). The optimal range used for the following studies was at a receptor concentration of 0.5 nM, concomitant specific binding corresponding to 23.1% of the added ligand (Table 1).

### 3.2. Saturation of estrogen receptor-α binding

To characterize the functionality of the receptor, saturation experiments were performed. Receptor was incubated with increasing concentrations of [$^3$H]-17β-estradiol, non-specific binding was assessed in the presence of 1000-fold molar excess of unlabeled 17β-estradiol. Non-specific binding increased linearly and moderately in relation to the [$^3$H]-17β-estradiol concentration, whereas total binding increased strongly and almost linearly up to 0.6 nM [$^3$H]-17β-estradiol. At higher concentrations of [$^3$H]-17β-estradiol total binding was still rising, but at a slower rate. Specific binding increased strongly and almost linearly up to 0.6 nM [$^3$H]-17β-estradiol, but at higher concentrations approached a plateau. These findings correspond well with a fully functional receptor showing saturable binding at higher ligand concentrations (Fig. 2). Results of the Scatchard analysis of the saturation experiments are given in Table 2. The observed mean Kd of 0.3734 nM is in the reported range for the human ERα (see also Section 4), and the Bmax of 0.3439 nM corresponds well to the added receptor concentration of 0.5 nM.

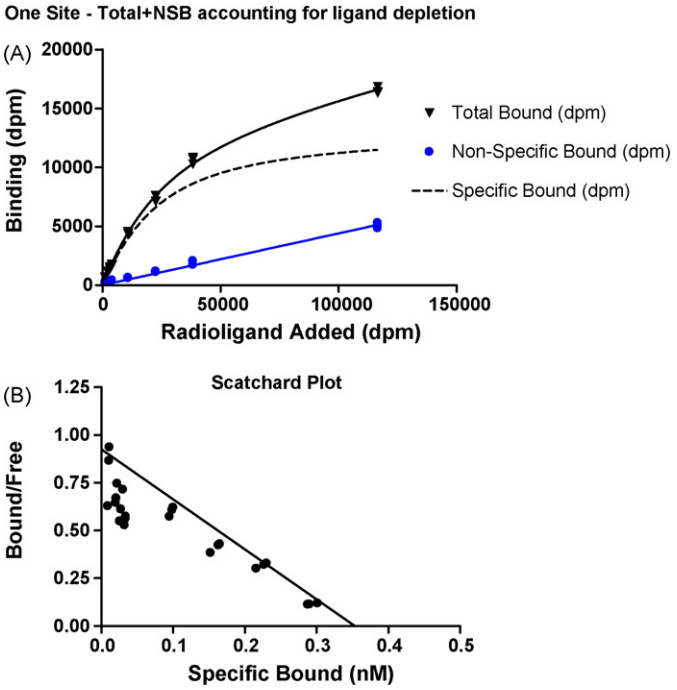


**Fig. 2.** (A) Saturation binding data of the human recombinant full length estrogen receptor-α utilizing [$^3$H]-17β-estradiol. ERα (nominally 0.5 nM) was incubated with increasing final concentrations (0.03, 0.06, 0.08, 0.10, 0.30, 0.60, 1.0, 3.0 nM) of [$^3$H]-17β-estradiol (1 nM = 39,072 dpm). Non-specific binding was determined in parallel in the presence of a 1000-fold molar excess of unlabeled 17β-estradiol. For each condition three replicates were performed. Following incubation overnight, unbound radiolabeled ligand was removed by absorption to dextran-bound charcoal (DBC) and sedimentation of DBC by centrifugation. Receptor-bound radiolabeled ligand in the supernatant was determined by liquid scintillation counting. Specific binding was calculated as the difference of total and non-specific binding Graphs were generated using the non-linear regression software program Prism version 5.02. The figure shows a representative experiment. (B) Scatchard analysis of the human recombinant full length estrogen receptor-α utilizing [$^3$H]-17β-estradiol. Data for total and non-specific binding shown in (A) were analysed by Prism version 5.02 taking into account ligand depletion. The figure shows a representative experiment.

**Table 1**
Determination of the necessary receptor concentration.

| Receptor concentration (nominal) [nM] | Mean specific binding (total incubation) [dpm] | Specific binding (relative to the nominally added ligand) [%] |
|---|---|---|
| 0.25 | 4,874 | 12.5 |
| 0.50 | 9,029 | 23.1 |
| 0.75 | 12,635 | 32.3 |
| 1.00 | 16,151 | 41.3 |

The assay was performed under conditions corresponding to competitive binding, i.e., in the presence of 1 nM [$^3$H]-17β-estradiol (nominally 39,072 dpm) and increasing ERα concentrations as indicated. Non-specific binding was assessed in parallel incubations containing 1 μM unlabeled 17β-estradiol. Following incubation overnight, unbound radiolabeled ligand was removed by absorption to dextran-bound charcoal (DBC) and sedimentation of DBC by centrifugation. Receptor-bound radiolabeled ligand in the supernatant was determined by liquid scintillation counting. Specific binding was calculated as the difference of total and non-specific binding. For each condition six replicates were performed.

**Table 2**
Scatchard analysis of saturation experiments.

| Experiment No. | Kd [nM] | Bmax [nM] |
|---|---|---|
| 1 | 0.3822 | 0.3533 |
| 2 | 0.3851 | 0.2992 |
| 3 | 0.3530 | 0.3791 |
| Mean ± S.D. | 0.3734 ± 0.0178 | 0.3439 ± 0.0408 |

ERα was incubated 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. For each condition three replicates were performed. Following incubation overnight, unbound radiolabeled ligand was removed by absorption to dextran-bound charcoal (DBC) and sedimentation of DBC by centrifugation. Receptor-bound radiolabeled ligand in the supernatant was determined by liquid scintillation counting. Three independent experiments were run. Scatchard analysis was performed using Graph Pad Prism 5.02 taking into account ligand depletion.

### 3.3. Competition experiments

General binding characteristics in the absence of competitor are summarized in Table 3. In the presence of optimal ligand concentrations (99–101% of the nominal value) little variability for total, non-specific and specific bindings was observed across the experiments. Specific binding for vehicle controls corresponded to 22.6–29.8% of the theoretically added ligand, the corresponding coefficient of variation across the six experiments was 12.2%. Similar figures were obtained for specific binding for buffer controls (total binding in the absence of solvent minus non-specific binding), however, there seemed to be a trend to higher values for specific binding in DMSO controls compared to specific binding in buffer controls. Mean non-specific binding corresponded to 15% of total binding, accordingly, total binding mostly represented specific binding to the receptor.

Standard solvent was ethanol, and with the exception of 17β-estradiol and norethynodrel, all compounds were considered for testing up to 1 mM, an aim that could not always be achieved. A final concentration of 1 mM resulted in strong turbidity for dibutylphthalate, DES, and ethynylestradiol, and in slight turbidity for m-hexestrol and nonylphenol. Genistein and corticosterone could only be tested up to 100 μM (final conc.) due to their limited solubility in ethanol, whereas 100 μM *o,p′*-DDT still resulted in turbidity. In order to potentially improve their solubility, genistein, *o,p′*-DDT, and corticosterone together with the set of reference

**Table 3**
Receptor binding characteristics in competition experiments in the absence of competitor.

| Experiment | Solvent | Ligand concentration [% of nominal] | Binding [% of nominally added ligand] | | | | |
|---|---|---|---|---|---|---|---|
| | | | Total | | Non-specific | Specific | |
| | | | Buffer | Solvent | | Buffer | Solvent |
| A | Ethanol | 100.6 | 30.75 | 30.33 | 4.35 | 26.40 | 25.98 |
| B | Ethanol | 99.9 | 26.88 | 27.86 | 4.02 | 22.86 | 23.85 |
| D | Ethanol | 100.2 | 34.76 | 35.01 | 5.26 | 29.50 | 29.75 |
| E | DMSO | 99.1 | 24.21 | 26.33 | 3.71 | 20.50 | 22.62 |
| F | DMSO | 101.3 | 23.43 | 27.70 | 4.55 | 18.88 | 23.15 |
| G | DMSO | 99.3 | 29.18 | 33.77 | 4.34 | 24.84 | 29.43 |

ERα was incubated with 1.0 nM of [$^3$H]-17β-estradiol in the presence or absence of 1% solvent as indicated. Non-specific binding was determined in parallel in the presence of 1 μM unlabeled 17β-estradiol. For each condition six replicates were performed. Following incubation overnight, unbound radiolabeled ligand was removed by absorption to dextran-bound charcoal (DBC) and sedimentation of DBC by centrifugation. Receptor-bound radiolabeled ligand in the supernatant was determined by liquid scintillation counting. Three independent experiments were run for each solvent. Specific binding was calculated as the difference of total and non-specific binding. Corresponding mean values are given.

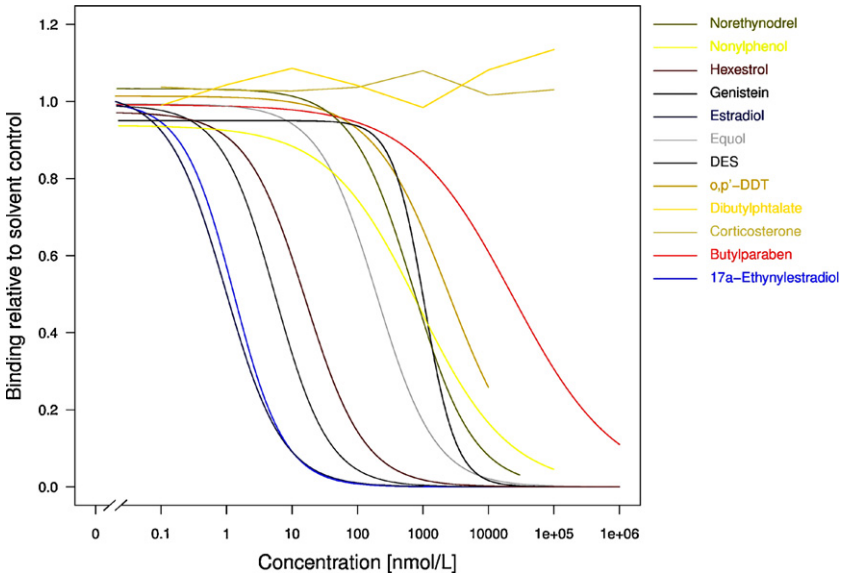


**Fig. 3.** Competitive binding to the human recombinant full length estrogen receptor-α of reference and test compounds using ethanol as solvent. Receptor (nominally 0.5 nM) was incubated with 1 nM [$^3$H]-17β-estradiol and increasing concentrations of compounds as indicated. Following incubation overnight, unbound radiolabeled ligand was removed by absorption to dextran-bound charcoal (DBC) and sedimentation of DBC by centrifugation. Receptor-bound radiolabeled ligand in the supernatant was determined by liquid scintillation counting. Binding was corrected for non-specific binding (NSB) and expressed relative to the corresponding solvent control. For the solvent control and for NSB in the presence of 1 μM 17β-estradiol six replicates were performed, any other condition was studied in triplicate. Data correspond to run D. For the sake of clarity, only the curve fit, but no individual data points are given.

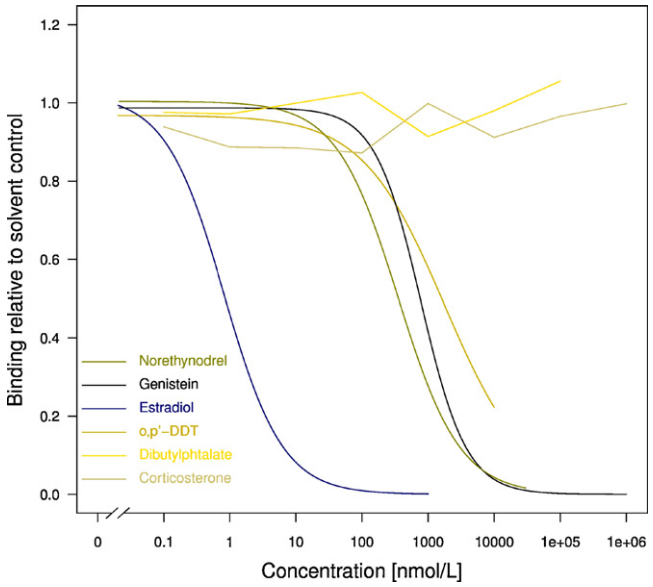


**Fig. 4.** Competitive binding to the human recombinant full length estrogen receptor-α of reference and test compounds using DMSO as solvent. Receptor (nominally 0.5 nM) was incubated with 1 nM [$^3$H]-17β-estradiol and increasing concentrations of compounds as indicated. Following incubation overnight, unbound radiolabeled ligand was removed by absorption to dextran-bound charcoal (DBC) and sedimentation of DBC by centrifugation. Receptor-bound radiolabeled ligand in the supernatant was determined by liquid scintillation counting. Binding was corrected for non-specific binding (NSB) and expressed relative to the corresponding solvent control. Data correspond to run D. For the solvent control and for NSB in the presence of 1 μM 17β-estradiol six replicates were performed, any other condition was studied in triplicate. Data correspond to run F. For the sake of clarity, only the curve fit, but no individual data points are given.

compounds were also tested using DMSO as solvent. Corticosterone and genistein could be dissolved in DMSO at 100 mM, but the resulting final concentration of 1 mM resulted in slight turbidity for genistein. Similarly, final concentrations of 1 mM dibutylphtalate and 100 μM *o*,*p*′-DDT resulted in strong and slight (*o*,*p*′-DDT) turbidity. Accordingly, only for corticosterone the use of DMSO finally resulted in an improved solubility.

ERα binding data for all compounds dissolved in ethanol expressed as $IC_{50}$-values are summarized in Table 4. Irrespective of limited solubility in ethanol or in the final assay, $IC_{50}$-values could be observed for all reference and test compounds except for dibutylphthalate and corticosterone that showed no affinity to the ERα. A compilation of representative displacement curves using ethanol as solvent is shown in Fig. 3. Corresponding relative binding affinities ($IC_{50}$ of 17β-estradiol divided by the $IC_{50}$ of the test chemical and multiplied by 100) are given in Table 5. Genistein, *o*,*p*′-DDT, and corticosterone together with the set of reference compounds were also tested using DMSO as solvent. A compilation of representative displacement curves using DMSO as solvent is given in Fig. 4. Corresponding $IC_{50}$-values and relative binding affinities are presented in Tables 6 and 7.

Overall, the following rank order of affinity to the ERα could be established:

17β-Estradiol > ethynylestradiol > DES > m-hexestrol > equol > norethynyodrel > genistein ≈ nonylphenol > *o*,*p*′-DDT > butylparaben

Dibutylphthalate and corticosterone were characterized as non-binders. The observed rank order of affinity to the ERα was in line with our expectations.

## 4. Discussion

The value of receptor binding assays both as a mechanistic tool to characterize receptor mediated endocrine activity, but also as an important screening assay for endocrine active compounds, is well recognized. Interestingly, after two decades of research in the field of endocrine active compounds, still no validated human recombinant (full length) ERα binding assay is available. Aim of this work was to evaluate a model protocol as a first step towards a validation of a human recombinant full length ERα binding assay.

The model protocol worked well and the general requirements were fulfilled: non-specific binding in competition experiments was clearly below the maximally tolerated 35% of total binding and specific binding roughly corresponded to 20 ± 5% relative to the added [$^3$H]-17β-estradiol for solvent controls. The upper limit was occasionally slightly exceeded, but not on a regular basis and thus was acceptable. Saturation experiments demonstrated that at a ligand concentration of 1 nM binding the receptor was still not saturated. This indicates that on the one hand competitors could readily displace [$^3$H]-17β-estradiol from the ERα, and on the other hand the ligand concentration was high enough to provide a readily measurable signal. Scatchard analysis of the saturation experiments provided a mean Kd of 0.3734 nM which is well within the range of reported Kd values for full length human recombinant ERα or ER isolated from human cells, whereas truncated recombinant ERα and possibly rat uterine ER may have higher Kd values (see Table 8). The observed Bmax of 0.3439 nM corresponds well to the added 0.5 nM receptor protein (determined by means of the hydroxyapatite methodology according to the manufacturer's information) and indicates equivalence of the charcoal and hydroxyapatite methodologies.

When competitive binding was studied, irrespective of the chemical nature of the compound and the solvent used, for a given compound individual $IC_{50}$-values varied by not more than a factor of 2.5 from one another. Variability of binding affinities relative to 17β-estradiol (RBA) was in the same range, only for equol (2.9) and genistein (3.3) slightly higher values were observed. In general $IC_{50}$-values for runs A and B were more comparable to each other than data from run D. However, there was no uniform trend, and $IC_{50}$-values obtained in run D were either higher, lower, or comparable (n-butylparaben) to those in the other runs. No difference in the quality of the data sets for run D was observed and $IC_{50}$-values for run D maximally differed from those of runs A and B by a factor of 2.2. This difference – in the absence of a trend – was considered as random. Mean coefficients of variation (CVs) across all compounds were 25% (ethanol as solvent) and 13% (DMSO) for $IC_{50}$-values and 24% (ethanol) and 13% (DMSO) for relative binding affinities. These low values, especially in light of the low number of independent experiments, indicate that the assay is capable of accurately assessing receptor affinities with only small variability.

Following an ICCVAM/NICEATM recommendation [9] ethanol was used as solvent whenever possible. Accordingly, only few compounds, namely the reference compounds 17β-estradiol, norethynodrel, and dibutylphthalate as well as genistein, *o*,*p*′-DDT, and corticosterone were tested using both solvents. The use of DMSO seemed to provide lower variability of the $IC_{50}$/RBA values and possibly slightly lower $IC_{50}$-values compared to the use of ethanol. However, in the light of the low number of experiments with DMSO and the small number of compounds studied, it is currently not possible to consider one solvent more appropriate than the other. Basically, both solvents can be used with the assay, and these options can be helpful to achieve optimal dissolution of test compounds.

Optimal interactions of a ligand with its receptor requires that the free ligand concentration is similar to the added concentration, i.e., total binding as the sum of specific and non-specific

**Table 4**
$IC_{50}$-values achieved for reference and test compounds using ethanol as solvent.

| Compound | Experiment | $IC_{50}$-value [nM] | Confidence interval [nM] | | Coefficient of variation [%] |
|---|---|---|---|---|---|
| | | | Lower | Upper | |
| 17β-Estradiol | A | 1.297 | 1.1800 | 1.425 | |
| | B | 1.301 | 1.1880 | 1.425 | |
| | D | 0.965 | 0.8814 | 1.057 | |
| | Mean ± S.D. | 1.188 ± 0.193 | | | 16.2 |
| Norethynodrel | A | 451.2 | 408.2 | 498.6 | |
| | B | 410.8 | 366.1 | 460.8 | |
| | D | 702.3 | 623.9 | 790.6 | |
| | Mean ± S.D. | 521.4 ± 157.9 | | | 30.3 |
| Dibutylphthalate | A | No affinity[a] | | | |
| | B | No affinity | | | |
| | D | No affinity | | | |
| | Mean ± S.D. | – | | | – |
| 17α-Ethynylestradiol | A | 1.939 | 1.673 | 2.246 | |
| | B | 2.508 | 1.930 | 3.259 | |
| | D | 1.294 | 1.199 | 1.397 | |
| | Mean ± S.D. | 1.914 ± 0.607 | | | 31.7 |
| DES | A | 7.961 | 6.980 | 9.081 | |
| | B | 7.723 | 6.611 | 9.021 | |
| | D | 5.550 | 4.931 | 6.247 | |
| | Mean ± S.D. | 7.078 ± 1.329 | | | 18.8 |
| m-Hexestrol | A | 34.62 | 30.58 | 39.18 | |
| | B | 36.07 | 20.39 | 63.81 | |
| | D | 16.02 | 13.16 | 19.50 | |
| | Mean ± S.D. | 28.90 ± 11.18 | | | 38.7 |
| Equol, racemic | A | 93.78 | 75.03 | 117.2 | |
| | B | 91.09 | 67.00 | 123.8 | |
| | D | 197.2 | 180.0 | 216.0 | |
| | Mean ± S.D. | 127.4 ± 60.50 | | | 47.5 |
| Genistein | A | 422.6 | 350.2 | 509.9 | |
| | B | –[b] | – | – | |
| | D | 1049 | 788.8 | 1394 | |
| | Mean | 735.8 | | | Not calculated |
| Nonylphenol | A | 868.8 | 628.7 | 1201 | |
| | B | 1024 | 692.6 | 1514 | |
| | D | 859.8 | 589.2 | 1255 | |
| | Mean ± S.D. | 917.5 ± 92.3 | | | 10.1 |
| *o,p′*-DDT | A | –[b] | – | – | |
| | B | 1333 | 950.0 | 1872 | |
| | D | 2399 | 1937 | 2970 | |
| | Mean | 1866 | | | Not calculated |
| n-Butylparaben | A | $228.4 \times 10^2$ | $166.6 \times 10^2$ | $313.1 \times 10^2$ | |
| | B | $254.0 \times 10^2$ | $183.0 \times 10^2$ | $352.7 \times 10^2$ | |
| | D | $232.0 \times 10^2$ | $162.0 \times 10^2$ | $332.4 \times 10^2$ | |
| | Mean ± S.D. | $238.1 \times 10^2 \pm 13.85 \times 10^2$ | | | 5.8 |
| Corticosterone | A | No affinity[a] | | | |
| | B | No affinity | | | |
| | D | No affinity | | | |
| | Mean ± S.D. | – | | | – |

ERα (nominally 0.5 nM) was incubated with 1 nM [$^3$H]-17β-estradiol and increasing concentrations of the indicated compounds. Following incubation overnight, unbound radiolabeled ligand was removed by absorption to dextran-bound charcoal (DBC) and sedimentation of DBC by centrifugation. ERα-bound radiolabel in the supernatant was determined by liquid scintillation counting. Binding was corrected for non-specific binding (NSB) and expressed relative to the corresponding solvent control. For the solvent control and NSB in the presence of 1 μM 17β-estradiol six replicates were performed, any other condition was studied in triplicate. $IC_{50}$-values were calculated by means of the software R-package `drc` using a three-parametric log-logistic function. Results from three independent experiments A, B, and D are given.

[a] Dibutylphthalate and corticosterone were tested up to 100 μM.

[b] No $IC_{50}$-value was calculated due to unclear dose response (displacement curves were comparable to corresponding experiments, but contained one or two aberrant data points a low concentrations).

binding should not exceed 10%. However, assay miniaturization in order to allow intermediate or high throughput, increases the risk of ligand depletion (where the actual free ligand concentration is significantly lower than the added ligand concentration), since receptor concentrations are rather high in these assays in order to achieve adequate signal sizes [10]. In competition experiments, ligand depletion results in a shift of competition curves/$IC_{50}$-values to the right, i.e., to higher concentrations [11]. With regard to the experiments reported herein, total binding in vehicle controls of competition experiments corresponded to 26–35% of the added ligand (see Table 3). Thus, in all experiments slight ligand depletion had occurred. Modeling of ligand depletion [11] indicated that the impact on competition experiments of a depletion up to 38% was small. Accordingly, competition experiments reported herein was sufficiently accurate in terms of ligand depletion and no corrective action is needed. The use of relative binding affinities with all com-

**Table 5**
Relative binding affinities for reference and test compounds using ethanol as solvent.

| Compound | Experiment No. | Relative binding affinity [%] | Coefficient of variation [%] |
|---|---|---|---|
| 17β-Estradiol | | 100 | – |
| Norethynodrel | A | 0.2875 | |
| | B | 0.3167 | |
| | D | 0.1374 | |
| | Mean ± S.D. | 0.2472 ± 0.0962 | 38.9 |
| Dibutylphthalate | A | No affinity | |
| | B | No affinity | |
| | D | No affinity | |
| | Mean ± S.D. | – | – |
| 17α-Ethynylestradiol | A | 66.89 | |
| | B | 51.87 | |
| | D | 74.57 | |
| | Mean ± S.D. | 64.45 ± 11.55 | 17.9 |
| DES | A | 16.29 | |
| | B | 16.85 | |
| | D | 17.39 | |
| | Mean ± S.D. | 16.84 ± 0.55 | 3.3 |
| m-Hexestrol | A | 3.746 | |
| | B | 3.607 | |
| | D | 6.024 | |
| | Mean ± S.D. | 4.459 ± 1.357 | 30.4 |
| Equol, racemic | A | 1.383 | |
| | B | 1.428 | |
| | D | 0.489 | |
| | Mean ± S.D. | 1.100 ± 0.530 | 48.2 |
| Genistein | A | 0.3069 | |
| | B | – | |
| | D | 0.0920 | |
| | Mean | 0.1995 | Not calculated |
| Nonylphenol | A | 0.1493 | |
| | B | 0.1271 | |
| | D | 0.1122 | |
| | Mean ± S.D. | 0.1295 ± 0.0186 | 14.4 |
| *o,p'*-DDT | A | – | |
| | B | 0.0976 | |
| | D | 0.0402 | |
| | Mean | 0.0689 | Not calculated |
| n-Butylparaben | A | 0.00568 | |
| | B | 0.00512 | |
| | D | 0.00416 | |
| | Mean ± S.D. | 0.00499 ± 0.00077 | 15.4 |
| Corticosterone | A | No affinity | |
| | B | No affinity | |
| | D | No affinity | |
| | Mean ± S.D. | – | – |

Relative binding affinities were calculated from data given in Table 4.

pounds being tested under the same conditions should even further minimize a small impact of ligand depletion.

An important step in the evaluation of an assay is the comparison of assay data with data from similar assays observed for the same test compounds. In Table 9, results from the investigation reported herein are compared to ERα binding data from several sources. With regard to data generated for full length human recombinant ERα [12,13] and for a human recombinant ERα ligand binding domain as receptor source (by the Chemicals Evaluation and Research Center (CERI) in Japan with funding by the Japanese Ministry of Economy, Trade and Industry [14]), a good to very good fit for the binding relative to estradiol (RBA) was observed. For compounds with weak affinity to the ERα the concurrence was excellent, and also for compounds with stronger affinity, the ranking was comparable, although in absolute terms in our hands the relative binding values for DES, ethynylestradiol and m-hexestrol were lower mainly compared to the CERI data. A similar constellation was observed when comparing our RBAs with those observed for rat uterine cytosol as receptor source [15,16]. It remains to be elucidated further whether these lower RBAs are characteristic of the human recombinant full length ERα as opposed to a human truncated or rat receptor or are related to other reasons such as the solvent used or assay buffer composition (e.g., in aqueous media DES can be converted to its much weaker cis-isomer). If this was the case for a large portion of DES, the lower RBA of DES could readily be explained [17]. The CERI assay utilized DMSO as the solvent in all experiments, while this assay used EtOH as the solvent for DES. Finally, as reported here and by others [14,15], dibutylphthalate and corticosterone did not show affinity for the ERα.

On the whole, our data demonstrate that the assay was robust and reliably ranked compounds with strong, weak, and without affinity for the ERα with high accuracy. Using a human recombinant full length estrogen receptor-α, it avoids the manipulation and use of animals, i.e., the preparation of uterine cytosol from ovariectomized rats, and thus contributes to the 3R concept. Furthermore, in contrast to other assays, our assay could be adjusted

**Table 6**
$IC_{50}$-values achieved for reference and test compounds using dimethylsulfoxide as solvent.

| Compound | Experiment No. | $IC_{50}$-value [nM] | Confidence interval [nM] | | Coefficient of variation [%] |
|---|---|---|---|---|---|
| | | | Lower | Upper | |
| 17β-Estradiol | E | 0.9857 | 0.8443 | 1.151 | |
| | F | 0.8193 | 0.7280 | 0.9220 | |
| | G | 0.7654 | 0.6624 | 0.8845 | |
| | Mean ± S.D. | 0.8568 ± 0.1148 | | | 13.4 |
| Norethynodrel | E | 424.5 | 384.5 | 468.8 | |
| | F | 353.4 | 308.9 | 404.4 | |
| | G | 365.5 | 298.6 | 447.4 | |
| | Mean ± S.D. | 381.1 ± 38.0 | | | 10.0 |
| Dibutylphthalate | E | No affinity[a] | | | |
| | F | No affinity | | | |
| | G | No affinity | | | |
| | Mean ± S.D. | – | | | – |
| Genistein | E | 744.5 | 611.3 | 906.6 | |
| | F | 776.4 | 678.6 | 888.4 | |
| | G | 633.1 | 520.1 | 770.7 | |
| | Mean ± S.D. | 718.0 ± 75.2 | | | 10.5. |
| *o,p′*-DDT | E | 1405 | 1081 | 1825 | |
| | F | 1770 | 1339 | 2340 | |
| | G | 1225 | 923.6 | 1625 | |
| | Mean ± S.D. | 1467 ± 278 | | | 19.0 |
| Corticosterone | E | No affinity[a] | | | |
| | F | No affinity | | | |
| | G | No affinity | | | |
| | Mean ± S.D. | – | | | – |

ERα (nominally 0.5 nM) was incubated with 1 nM [$^3$H]-17β-estradiol and increasing concentrations of the indicated compounds. Following incubation overnight, unbound radiolabeled ligand was removed by absorption to dextran-bound charcoal (DBC) and sedimentation of DBC by centrifugation. ERα-bound radiolabel in the supernatant was determined by liquid scintillation counting. Binding was corrected for non-specific binding (NSB) and expressed relative to the corresponding solvent control. For the solvent control and NSB in the presence of 1 μM 17β-estradiol six replicates were performed, any other condition was studied in triplicate. $IC_{50}$-values were calculated by means of the software `R`-package `drc` using a three-parametric log-logistic function. Results from three independent experiments E, F, and G are given.

[a] Dibutylphthalate was tested up to 100 μM, corticosterone up to 1 mM.

to an intermediate/high throughput format. In terms of the ECVAM modular approach of validation, the data set presented herein corresponds to the first two modules, namely test definition and within-laboratory variability [18]. Further testing of the protocol in collaboration with OECD in additional laboratories is ongoing and will provide an in-depth evaluation of transferability and inter-laboratory variability. The assay may be used to screen for endocrine-modulating compounds with affinity for the ERα, to further investigate equivocal results from other *in vitro* assays such as transactivation assays, and it may also be used as a mechanistic tool in order to characterize effects on the endocrine system observed in animal studies. Whether ERα binding studies can reliably predict the outcome of corresponding *in vivo* screening assays such as the uterotrophic assay remains to be explored.

**Table 7**
Relative binding affinities for reference and test compounds using dimethylsulfoxide as solvent.

| Compound | Experiment No. | Relative binding affinity [%] | Coefficient of variation [%] |
|---|---|---|---|
| 17β-Estradiol | | 100 | – |
| Norethynodrel | E | 0.2322 | |
| | F | 0.2318 | |
| | G | 0.2094 | |
| | Mean ± S.D. | 0.2245 ± 0.0131 | 5.8 |
| Dibutylphthalate | E | No affinity | |
| | F | No affinity | |
| | G | No affinity | |
| | Mean ± S.D. | – | – |
| Genistein | E | 0.1324 | |
| | F | 0.1055 | |
| | G | 0.1209 | |
| | Mean ± S.D. | 0.1196 ± 0.0135 | 11.3 |
| *o,p′*-DDT | E | 0.0702 | |
| | F | 0.0463 | |
| | G | 0.0625 | |
| | Mean ± S.D. | 0.0596 ± 0.0122 | 20.5 |
| Corticosterone | E | No affinity | |
| | F | No affinity | |
| | G | No affinity | |
| | Mean ± S.D. | – | – |

Relative binding affinities were calculated from data given in Table 5.

**Table 8**
Kd values for estrogen receptors isolated from various sources.

| Estrogen receptor | Expression system | Receptor preparation | Methodology[a] | Kd [nM][b] | Reference |
|---|---|---|---|---|---|
| Full length human recombinant ERα | Insect cells | Purified protein | Charcoal | 0.37 | This investigation |
| Full length human recombinant ERα | Insect cells | Extract | Charcoal | 0.22 | [12] |
| Human ER (mainly α-subtype) from MCF-7 cells | – | Cytosol | Charcoal | 0.13 | [19] |
| Human ER (mainly α-subtype) from MCF-7 cells | – | Cell extract | No information | 0.36 | [20] |
| Full length human recombinant ERα | Yeast | Yeast extract | No information | 0.35 | [20] |
| Human recombinant ERα–ligand binding domain | Yeast | Yeast extract | No information | 1.00 | [20] |
| Human recombinant ERα–ligand binding domain | Bacteria | Bacteria extract | No information | 1.49 | [20] |
| Full length quail recombinant ERα | Insect cells | Extract | Charcoal | 0.58 | [12] |
| Full length alligator recombinant ERα | Insect cells | Extract | Charcoal | 0.44 | [12] |
| Full length salamander recombinant ERα | Insect cells | Extract | Charcoal | 0.28 | [12] |
| Full length fathead minnow recombinant ERα | Insect cells | Extract | Charcoal | 0.58 | [12] |
| Rat ER (mainly α-subtype) from rat uterus, laboratory X | – | Cytosol | Hydroxyapatite | 0.15–1.18 | [16] |
| Rat ER (mainly α-subtype) from rat uterus, laboratory Y | – | Cytosol | Hydroxyapatite | 0.04–0.24 | [16] |
| Rat ER (mainly α-subtype) from rat uterus, laboratory Z | – | Cytosol | Hydroxyapatite | 0.8–1.5 | [16] |

[a] Methodology used to separate receptor-bound from unbound ligand.
[b] [$^3$H]-17β-estradiol was used as ligand for Kd determinations.

**Table 9**
Comparison of relative ERα binding affinities to published data.

| Compound | Investigation | | | | | |
|---|---|---|---|---|---|---|
| | This investigation (full length human recombinant) RBA [%] | Rider et al. [12] (full length human recombinant) RBA [%] | Kuiper et al. [13] (full length human recombinant) RBA [%] | METI/CERI [14] (human recombinant ligand binding domain) RBA [%] | Blair et al. [15] (rat uterus cytosol) RBA [%] | EPA validation [16] (rat uterus cytosol) RBA [%] |
| 17β-Estradiol | 100 | 100 | 100 | 100 | 100 | 100, 100, 100[a] |
| Ethynylestradiol | 64.5 | 91.2 | Not done | 142 | 190 | 165, 268, 113 |
| DES | 16.8 | Not done | 236 | 88.9 | 400 | 590, 284, equivocal[b] |
| m-Hexestrol | 4.46 | Not done | Not done | 37.6 | 300 | 345, 2090, 270 |
| Norethynodrel | 0.247/0.225$^{DMSO}$ | Not done | Not done | 0.282 | 0.204 | 0.135, 0.147, 0.130 |
| Butylparaben | 0.00499 | Not done | Not done | 0.018 | Not done | 0.00416, 0.0105, 0.00086 |
| Equol | 1.10 | Not done | Not done | Not done | Not done | 0.0812, 0.0542, 0.039 |
| Genistein | 0.199/0.120$^{DMSO}$ | Not done | 4/0.7 | 0.12 | Not done | 0.971, 4.81, 0.402 |
| Nonylphenol | 0.130 | Not done | 0.05 | 0.108 | 0.019–0.037 | 0.125, 0.101, equivocal |
| *o,p′*-DDT | 0.0689/0.0596$^{DMSO}$ | Not done | 0.01 | Not done | 0.001 | Negative, 14,400, negative |
| Dibutylphthalate | No affinity | Not done | Not done | No $IC_{50}$, RBA<0.001 | No affinity | Not done |
| Corticosterone | No affinity | No affinity | Not done | Not done | No affinity | 0.00103, 2.04, equivocal |

For our data, solvent is ethanol, if not otherwise indicated.
[a] Data from three laboratories (X, Y, and Z).
[b] Equivocal: displacement of labeled ligand was observed, however, no characteristic binding curve was observed.

## Conflict of interest

There are no conflicts of interest.

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