A 155-plex High-Throughput *In Vitro* Coregulator Binding Assay for (Anti-)Estrogenicity Testing Evaluated with 23 Reference Compounds

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Summary

To further develop an integrated in vitro testing strategy for replacement of in vivo tests for (anti-) estrogenicity testing, the ligand-modulated interaction of coregulators with estrogen receptor α was assessed using a PamChip® plate. The relative estrogenic potencies determined, based on ERα binding to coregulator peptides in the presence of ligands on the PamChip® plate, were compared to the relative estrogenic potencies as determined in the in vivo uterotrophic assay. The results show that the estrogenic potencies predicted by the 57 coactivators on the peptide microarray for 18 compounds that display a clear E2 dose-dependent response (goodness of fit of a logistic dose-response model of 0.90 or higher) correlated very well with their in vivo potencies in the uterotrophic assay, i.e., coefficient of determination values for 30 coactivators higher than or equal to 0.85. Moreover, this coregulator binding assay is able to distinguish ER agonists from ER antagonists: profiles of selective estrogen receptor modulators, such as tamoxifen, were distinct from those of pure ER agonists, such as dienestrol. Combination of this coregulator binding assay with other types of in vitro assays, e.g., reporter gene assays and the H295R steroidogenesis assay, will frame an in vitro test panel for screening and prioritization of chemicals, thereby contributing to the reduction and ultimately the replacement of animal testing for (anti-)estrogenic effects.

Keywords: estrogenicity, coregulator, peptide microarray, in vivo uterotrophic assay, alternative in vitro method

Abbreviations

2,4,5-T	2,4,5-trichlorophenoxyacetic acid	LBD	ligand-binding domain
CV	coefficient of variation	MIE	molecular initiating event
DMSO	dimethyl sulfoxide	NCOA1	nuclear receptor coactivator 1
E2	17β-estradiol	NCOR1	nuclear receptor corepressor 1
EE2	17α-ethinyl estradiol	NR	nuclear receptor
EPA	Environmental Protection Agency, United States	OMIY-bisphenol	4,4'-(octahydro-4,7-methano-5h-inden-5-ylidene)bisphenol
ER	estrogen receptor	R^2	coefficient of determination
GST	glutathione S-transferase	REACH	Registration, Evaluation, Authorization and restriction of Chemicals
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods	SERMs	selective estrogen receptor modulators

Received August 14, 2012; accepted in revised form January 2, 2013

1 Introduction

Estrogens exert their physiological effects mainly through activation of the estrogen receptor (ER) in target cells (Couse and Korach, 1999; Heldring et al., 2007). Although two main forms of ER exist, ERα and ERβ, in (reproduction) toxicology the primary attention goes to the ERα, as it is the dominating type in breast and uterus tissue (Gustafsson, 1999; Harris et al., 2002). Moreover, with respect to regulatory purposes, the focus is on ERα because binding and induction of ERα is implicated as a key molecular initiating event (MIE) in estrogenicity-related adverse endpoints. ERα and ERβ, like all the members of the nuclear receptor (NR) super-family, are ligand-dependent transcription factors that work in concert with transcriptional coregulators to control target gene transcription. Upon ligand binding, the ligand-binding domain (LBD) undergoes a conformational change that leads to receptor dimerization, translocation of the ER from cytosol to nucleus, and binding to estrogen-responsive elements. Moreover, as a result of the intramolecular conformational changes induced by ligand binding, the affinity of the ER for coregulator proteins is changed, resulting in recruitment or release of transcriptional coactivator or corepressor proteins, respectively, that enhance or repress the interaction of RNA polymerase II with estrogen-responsive gene promoters and all of the subsequent reactions needed to actually induce or repress transcription of target genes (Klinge, 2000; Ascenzi et al., 2006).

In general, the transcriptional coregulator family consists of coactivators, which augment the activity of the receptors, and corepressors that mediate the repressive effects of receptors (Johnson and O'Malley, 2012; McKenna et al., 1999). The most studied group of ERα coactivators includes the p160 protein family, consisting of NCOA1 (SRC-1), NCOA2 (SRC-2), and NCOA3 (SRC-3), which interact with the activation function-2 (AF-2) domain of agonist-bound ERs through multiple LXXLL motifs present in these coactivator proteins (where L is leucine and X is any amino acid) (Klinge, 2000; Metzler et al., 2001). Structural analysis of nuclear receptor (NR) LBDs has established that agonist binding stabilizes the AF-2 helix in an active conformation to form a charge clamp pocket, which is permissive for interactions with LXXLL motifs. In contrast, ER antagonists affect the positioning of the AF-2's mobile C-terminal helix (helix 12) to form a large binding pocket that interacts with the LXXXIXXXL motifs of corepressor proteins (where I is isoleucine) such as nuclear receptor corepressor 1 (NCOR1) and nuclear receptor corepressor 2 (NCOR2), thereby disrupting the LXXLL-binding site and preventing coactivator recruitment (Shiau et al., 1998; Brzozowski et al., 1997; Kong et al., 2005).

The standard test for disruption of normal estrogen function is the *in vivo* uterotrophic assay, i.e., a test with immature or ovariectomized rodents using uterus weight as the crucial read-out parameter (Clode, 2006; Owens and Ashby, 2002). With a view to the REACH Regulation (EC, 2006) and the need to reduce, refine, and replace the use of experimental animals for safety testing (3Rs), modulation of ER activity is usually quantitatively analyzed by assaying ER binding, ER-controlled reporter genes, or other downstream events such as estrogen receptor-mediated cell proliferation (Bovee and Pikkemaat, 2009). ER binding assays are rapid and easy to perform; one of the main drawbacks, however, is that these assays are unable to distinguish receptor agonists from receptor antagonists. Moreover, the rat uterine cytosol ER binding assay, currently listed as part of the Environmental Protection Agency's (EPA) Endocrine Disruptor Screening Program Tier 1 screening battery, still requires the use of animals as a source of ERs. Unlike receptor binding assays, reporter gene assays can distinguish between agonist and antagonist activity. Several reporter gene assays have been developed and applied as screening tools to determine the estrogenic/anti-estrogenic activities of compounds, as they are cheap, fast, robust, and have been shown to produce relevant and reliable outcomes (Bovee et al., 2009; van der Burg et al., 2010; Plotan et al., 2012). Proliferation assays and low-density DNA microchip-based analysis of marker gene expression also have been shown to provide valuable tools for estrogenicity testing, and outcomes correlate well with the *in vivo* uterotrophic assay (Wang et al., 2012, 2013), but these two assays are laborious and require 3-6 days. Therefore, they are not ideal for the large-scale testing of chemicals with respect to initiatives such as REACH.

Thus far, studying nuclear receptor interactions with coregulators has been performed mainly for theoretical reasons and for drug development (Lonard and O'Malley, 2012; Hsia et al., 2010; McDonnell and Wardell, 2010). However, a high-throughput *in vitro* assay enabling quantification of coactivator or corepressor recruitment by receptors upon ligand binding would have the potential to add relevant information to an integrated *in vitro* strategy for (anti-)estrogenicity testing, aiming at prioritization of chemicals and reduction of *in vivo* animal experiments needed for initiatives such as REACH. In the present study, the ligand-modulated interaction of coregulators with ERα was assessed using a PamChip® plate consisting of 96 identical arrays, each array containing 155 immobilized nuclear receptor (NR) coregulator peptides harboring either LXXLL (coactivator) or LXXXIXXXL (corepressor) motifs. A set of 23 reference compounds was tested in the coregulator binding assay based on the PamChip® plate. Twenty-one of these compounds were selected from the 78 compounds listed by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for validation of *in vitro* ER binding and transcriptional activation assays assays (ICCVAM, 2003). The objective was to determine to what extent this coregulator binding assay correctly predicts the estrogenic/anti-estrogenic activities and potencies of the test compounds when compared to the outcomes obtained in the *in vivo* uterotrophic assay.

2 Materials and methods

Chemicals

17β-Estradiol (E2), diethylstilbestrol, meso-hexestrol, coumestrol, dienestrol, zearalenone, corticosterone, tamoxifen, 4-hydroxytamoxifen, bisphenol A, ethyl paraben, *o,p'*-DDT, *p*-n-nonylphenol, and apigenin were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). 17α-Ethinyl estradiol (EE2), progesterone, and testosterone

(T) were purchased from Steraloids (Newport, RI, USA), while genistein was obtained from Apin Chemicals (Abingdon, Oxon, UK). 4,4'-(Octahydro-4,7-methano-5h-inden-5-ylidene) bisphenol (OMIY-bisphenol) was from Acros Organics (Fisher Emergo B.V., Landsmeer, The Netherlands). Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany). Kepone and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Butylbenzyl phthalate and di-*n*-butyl phthalate were purchased from TCI Europe N.V. (Zwijndrecht, Belgium).

Nuclear receptor-coregulator interaction profiling

Ligand-modulated interaction of coregulators with ERα-LBD was assessed using a PamChip® plate described previously (Houtman et al., 2012). The PamChip® plate consists of 96 identical arrays, each array containing 155 NR coregulator peptides harboring either LXXLL (coactivator) or LXXXIXXXL (corepressor) motifs (PamGene International B.V., 's-Hertogenbosch, The Netherlands). The coregulator peptides are immobilized on a porous metal oxide carrier by piezo technology as previously used in kinase assays (Hilhorst et al., 2009; Lemeer et al., 2007). The PamChip® plate was used in combination with the glutathione S-transferase (GST)-labeled ERα-LBD to screen dilution series of a set of 23 compounds. The peptide microarray was incubated with the test solution containing ERα-LBD-GST in the absence or presence of ligand by pumping the sample up and down the three-dimensional metal oxide carrier (Fig. 1A). In short, assay mixtures were prepared on ice in a master 96-well plate with 5 nM GST-tagged human ERα-LBD (PamGene International B.V.), 25 nM Alexa 488-conjugated GST antibody (Invitrogen, Breda, The Netherlands), and ligand at the indicated concentration in reaction buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 0.2% BSA, 0.05% Tween-20). All assays were performed in a fully automated PamStation®-96 (PamGene International B.V.) at 20°C applying two cycles per minute. The initial blocking was carried out by incubating each array for 20 cycles with 25 µl blocking buffer (TBS with 1% BSA, 0.01% Tween-20, and 0.3% skimmed milk powder). Subsequently, the blocking buffer was removed and 25 µl assay mix was transferred to each array and incubated for 80 cycles (~40 min). Eight concentrations with tenfold serial dilution in dimethyl sulfoxide (DMSO; final concentration 2%) of each compound were tested in singular. After removal of the unbound receptor by washing the plate with 25 µl TBS, tiff images were obtained by a CCD camera-based optical system integrated in the PamStation®-96 instrument. The total set of compounds was tested over two PamChip® plates and by using E2 as a reference compound on each plate.

Data analysis

Image analysis was performed using BioNavigator software (PamGene International B.V.), which performs automated array grid finding and subsequent quantification of signal and local background for each individual peptide. In short, the boundaries of a spot are determined and the median fluorescent signal is quantified within the spot (signal) as well as that in a defined area surrounding it (background). The signal-minus-background value is used subsequently as the quantitative parameter of binding. Ligand dose-response relations were analyzed using the DRC package in R (version 2.12.0, http://www.r-project.org). A sigmoidal 4-parameter logistic model was fitted to the dose-response data and the goodness-of-fit parameter and EC_{50} values as calculated by the DRC package were recorded. Relative binding potency (RBP) values were obtained from the ratio of the concentration of E2 needed to achieve 50% of maximal ERα-LBD binding to the coregulator and the concentration of the test compounds required to achieve a similar effect. This ratio subsequently is multiplied by 100. The RBP value of E2 is thus 100, resulting in a logRBP of 2.0. A cut-off value of -5.0 is listed for compounds showing no effect. The estrogenicity data used for comparisons with the current ERα-coregulator binding data were ER binding data in the review published by ICCVAM (2003), and the BG1Luc ER TA data reported by the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and ICCVAM (2011). For comparison of the presented *in vitro* ERα-coregulator binding data with estrogenicity *in vivo*, uterotrophic assay data were used that were derived from the Endocrine Disruptor Knowledge Base (EDKB), designed and produced by the National Center for Toxicological Research, USA (Ding et al., 2010).

3 Results

Twenty-one of the 23 compounds tested were selected from the 78 compounds listed by ICCVAM for validation of *in vitro* ER binding and transcriptional activation assays, representing the main groups of compounds with estrogenic activity, i.e., natural steroids, synthetic steroids, flavonoids, phenols, organochlorines, and phthalates (ICCVAM, 2003). Figure 1B shows the dose-response curves of ERα-LBD binding to the 155 coregulator peptides as induced by 17β-estradiol (E2). Most of the coregulator spots showed an increased binding signal with increasing E2 concentrations, e.g., NCOA1_677_700, NCOA2_628_651 and NCOA3_673_695, which all have the LXXLL motif signature sequence and are known to function as coactivators. As an example, the E2-induced dose-response curve of ERα-LBD binding to coactivator peptide NCOA1_677_700 is shown in Figure 1C. The lowest concentration of the potent E2 that resulted in a detectable binding of ERα-LBD to NCOA1_677_700 was 0.19 nM, reaching a half maximal binding level (EC_{50}) at approximately 0.7 nM and binding was saturated above 20 nM. However, not all the coactivator peptides immobilized on the peptide microarray showed an E2 concentration-dependent binding response of ERα-LBD, e.g., chromodomain-helicase-DNA-binding protein 9 (CHD9_855_877) and centromere protein R (CENPR_1_18) did not show any E2-induced binding. As expected, the corepressor peptides with the LXXXIXXXL motif, e.g., NCOR1_1925_1946 and NCOR2_2330_2352, did not show an ERα-LBD binding response upon co-incubation with E2. E2 was used as a reference compound on each of the two PamChip® plates. To assess the reproducibility of the assay, all duplicate E2 data together were plotted against each other, which resulted in a correlation with coefficient of determination (R^2) of 0.944. Moreover, the coefficient of variation (CV) was calculated at a binding-saturating E2

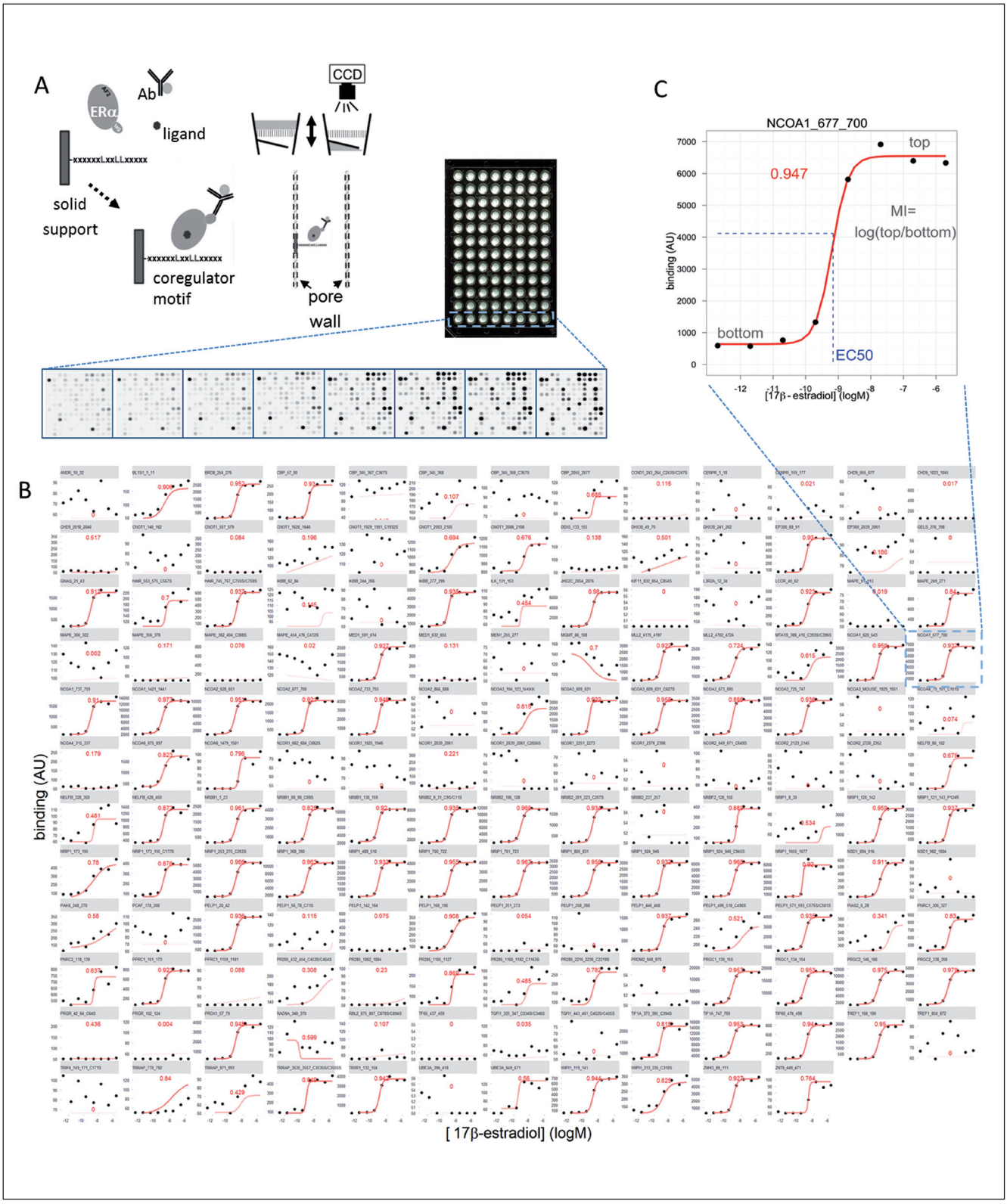

Fig. 1: Analysis of ERα-LBD binding to coregulators induced by 17β-estradiol

A. Schematic overview of the PamChip peptide microarray technology. B. Dose-response curves for ERα-LBD binding to the 155 coregulator-derived receptor binding motifs induced by 17β-estradiol. C. Enlargement of the dose-response curve for 17β-estradiol-induced binding of ERα-LBD to coactivator NCOA1_677_700.

concentration (20 nM) over each pair of duplicate E2 data. This resulted in a median intraplate CV of 8.0%.

All known estrogenic compounds tested on the peptide microarray (e.g., EE2, dienestrol, diethylstilbestrol, and *meso*-hexestrol) resulted in coregulator binding profiles similar to that of E2. Except for testosterone, the negative controls (i.e., corticosterone and progesterone) showed no statistically significant binding changes compared with the solvent control DMSO (data not shown), while the selective estrogen receptor modulators (SERMs), i.e., tamoxifen, 4-hydroxytamoxifen, and (OMIY-bisphenol), showed a completely different coregulator binding profile. As shown in Figure 2, compared with the solvent control DMSO and the known estrogen agonists, tamoxifen strongly inhibited binding of ERα-LBD to almost all coactivator peptides, and similar repression of binding was observed with 4-hydroxytamoxifen and OMIY-bisphenol (see supplementary files 1 and 2 at www.altex-edition.org). Surprisingly, no binding was observed for these SERMs to the corepressors present on the peptide microarray. Figure 3 shows the dose-response curves of 23 compounds based on three coregulators NCOA1_677_700, NRIP1_173_195, and PNRC2_118_139, resulting in curve fittings with a goodness of fit of 0.94, 0.78 and 0.63, respectively for E2. The relative coregulator binding potency (RBP) values of these 23 compounds were calculated for these three coactivator peptides and listed in Table 1. To allow comparison with the observed *in vivo* effects, the median log relative potency (logRP) of these compounds as determined previously in the *in vivo* uterotrophic assay with mice or rats are included and shown in Table 1. Figure 4 shows the comparison between the logRP values as determined in the *in vivo* uterotrophic assay and the logRBP values as determined in the coregulator binding assay based on coactivators of NCOA1_677_700, NRIP1_173_195, and PNRC2_118_139. Although tamoxifen had a clear effect on the binding of these three coactivators, its effect is mostly opposite to that of E2. As tamoxifen is a SERM that mainly shows its antagonistic properties when tested on the current coregulator binding assay (Fig. 2), it is not possible to compare its observed antagonistic binding effects with the agonistic binding effects obtained with E2. The same is valid for the SERMs 4-hydroxytamoxifen and OMIY-bisphenol, which also show antagonistic coregulator binding effects. Therefore, for the comparison of the *in*

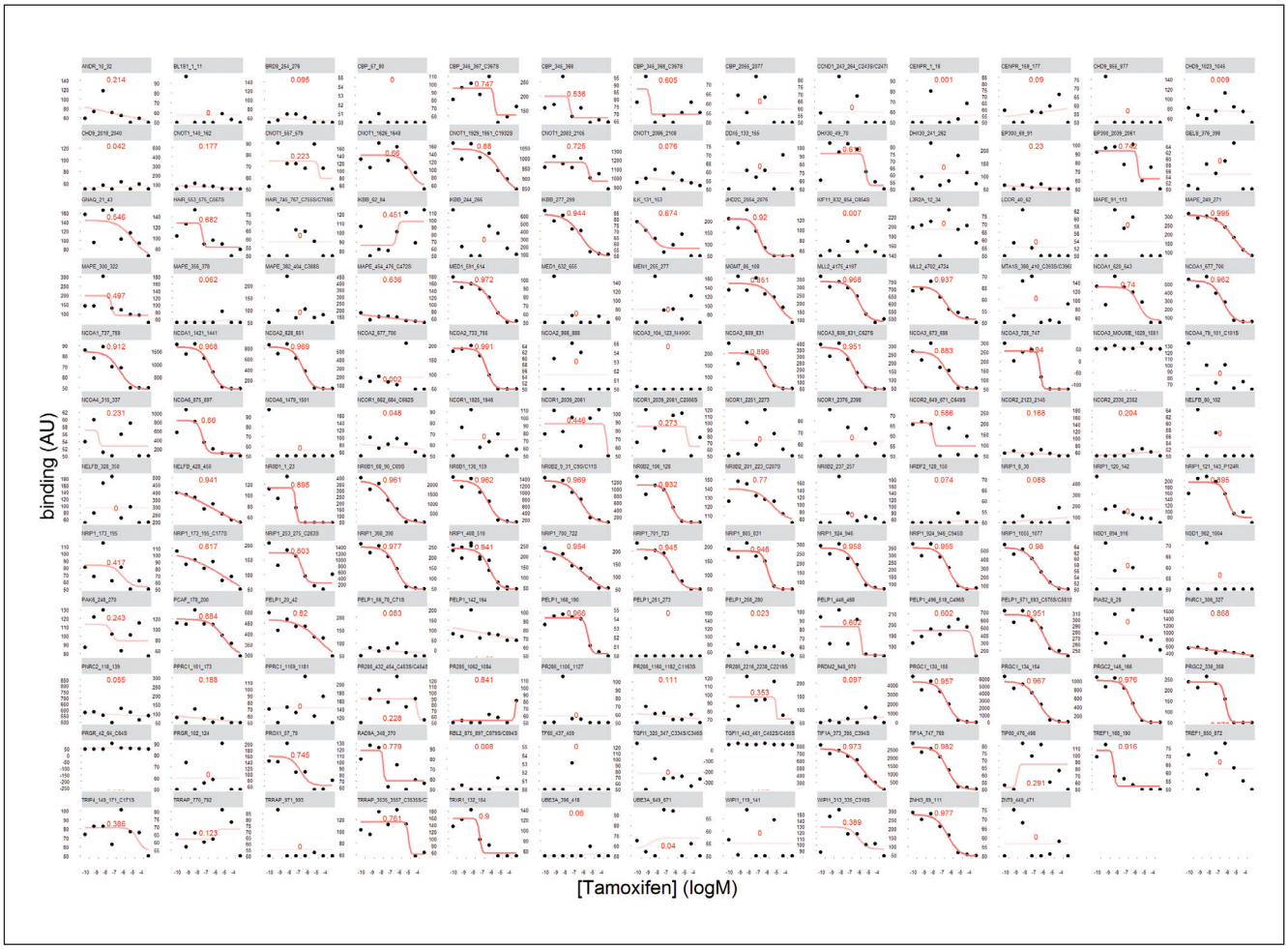

Fig. 2: Analysis of ERα-LBD binding to coregulators induced by tamoxifen

vitro coregulator binding assay with the *in vivo* uterotrophic assay, tamoxifen, 4-hydroxytamoxifen, and OMIY-bisphenol were left out (Fig. 4 and Tab. 2). Instead, to provide an overall measure of the antagonistic binding potency for the SERMs, the median of the IC_{50} values were calculated over all those coregulators showing a good fit of the standard dose-response model used (goodness-of-fit of a sigmoidal 4-parameter logistic model of 0.85 or higher). The number of coregulators meeting this requirement are 29 for OMIY-bisphenol, 41 for tamoxifen and 50 for 4-hydroxytamoxifen, resulting in median IC_{50} of 1.39×10^{-7} M, 2.55×10^{-7} M, and 1.82×10^{-9} M, respectively. In addition to the SERMs, apigenin and 2,4,5-T also were left out for the comparison of the coregulator binding assay with the *in vivo* uterotrophic assay. For 2,4,5-T there are no uterotrophic data available in the literature, and although apigenin has been shown to display estrogenic activities in *in vitro* reporter gene assays (Willemsen et al., 2004; Long et al., 2008), it was negative for estrogenic effects on the uterus in the uterotrophic assay. However, this is probably due to the poor bioavailability of apigenin in rodents (Breinholt et al., 2000). In the coregulator binding assay apigenin clearly induced binding of ERα-LBD to similar coactivators as E2. Figure 4 illustrates that using all dose-response curves and corresponding coregulator binding potencies (logRBP), the NCOA1_677_700 coactivator peptide-based logRBP values correlated very well with the *in vivo* relative potencies (logRP), values determined in the uterotrophic assay (R^2=0.89, $p < 0.0001$, n=18). Binding of coactivator NRIP1_173_195, with an intermediate E2 dose-response

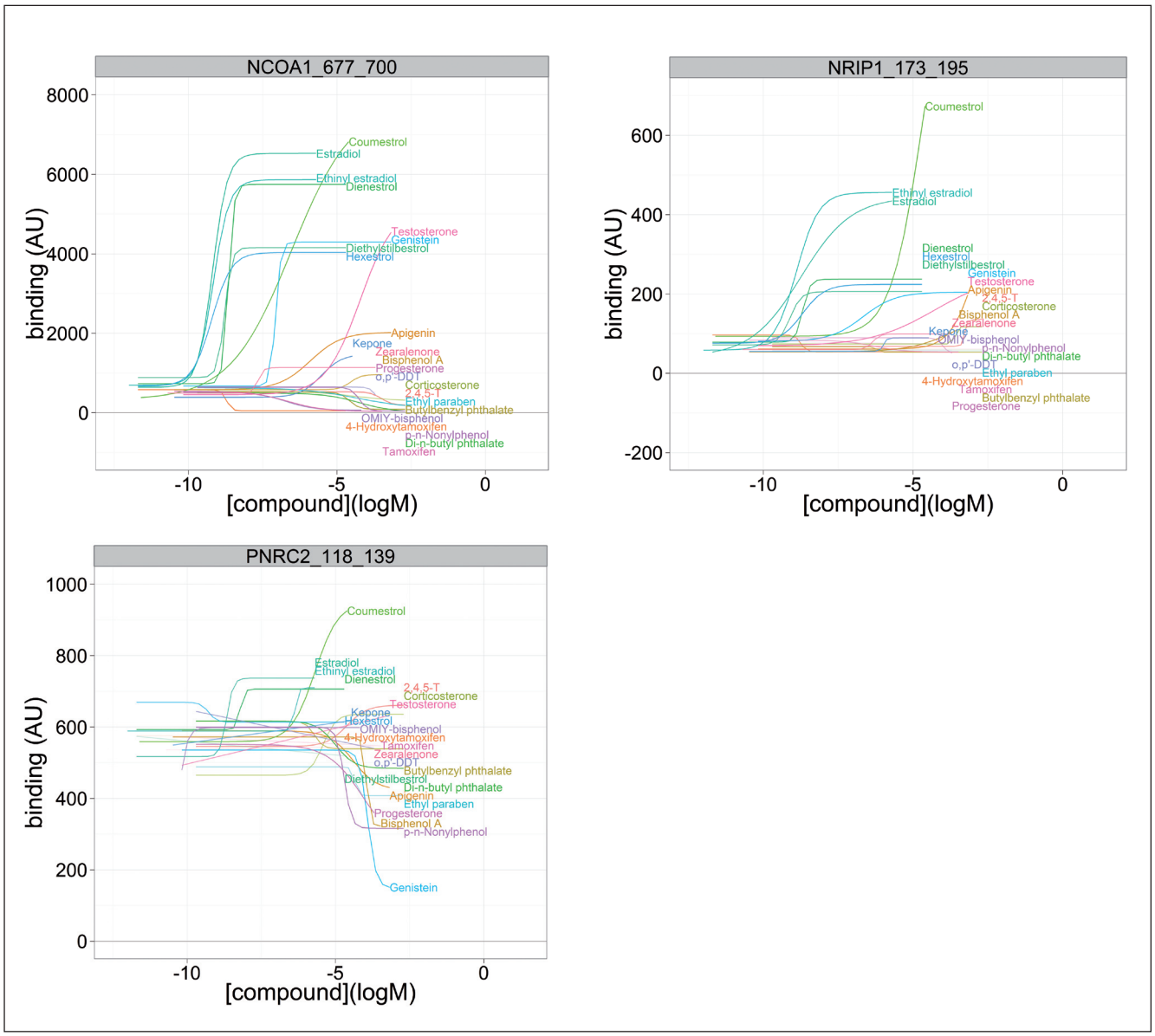

Fig. 3: Dose-response curves of ERα-LBD binding to the NCOA1_677_700, NRIP1_173_195, and PNRC2_118_139 coactivator peptides for the 23 compounds

curve goodness of fit of 0.78, still resulted in a relatively good correlation (R^2=0.79, $p < 0.0001$, n=18), whereas the binding of PNRC2_118_139, which has a relatively low E2 dose-response curve goodness of fit of 0.63, showed no correlation with the *in vivo* determined logRP values of the uterotrophic assay (R^2=0.01, p =0.698, n=18).

Next, the dose-response curve goodness-of-fit value for each coregulator of each compound was calculated. Out of the 155 coactivator peptides, 57 gave E2 curve fittings higher than or equal to 0.9. The determined EC_{50} values for E2 derived from these 57 curves were all in the low nanomolar range and showed a median EC_{50} of 0.9 nM. Subsequently, similar to what is shown in Table 1 and Figure 4, the logRBP values based on these 57 coactivators were calculated for each compound and correlated with different relative potency values from literature, i.e., logRBA values obtained in the ER binding assay (n=19), logREP values obtained in the BG1Luc ER transcriptional activation assay (n=16), and logRP values obtained in the uterotrophic assay (n=18). The resulting R^2 values are shown in Table 2. In general, the estrogenic potencies predicted by the coregulator binding assay correlated well with the ER binding assay, as well as with the BG1Luc ER transcriptional activation assay, which was recently approved by OECD as a test method for identifying estrogen receptor agonists and antagonists. As shown in Table 2, 33 coactivators showed an R^2 value higher than or equal to 0.80 with the ER binding assay, and 32 coactivators showed an R^2 value higher than or equal to 0.80 with the BG1Luc ER transcriptional activation assay. For the correlation with the *in vivo* uterotrophic assay, in total 48 coactivators showed an R^2 value higher than or equal to 0.8, and among these, 30 coactivators showed an R^2 value higher than or equal to 0.85 (e.g., NCOA1_677_700, NCOA3_673_695, and NR0B2_106_128). A low correlation coefficient was observed for BL1S1_1_11 (R^2=0.49).

4 Discussion

The aim of the present study was to investigate the potential of the PamChip® plate based coregulator binding assay as part of an integrated *in vitro* testing strategy for detection of (anti-) estrogenic activity. To this end a set of 23 reference compounds was investigated using the peptide microarray in combination with the GST-labeled ligand-binding domain of ERα. As a concentration series consisting of eight concentrations with tenfold serial dilution was tested for each compound, the dose-response relation could be determined with great statistical accuracy as demonstrated by the goodness of fit data. With a median EC_{50} of 0.9 nM, an intraplate coefficient of variation of 8.0% at a saturating binding concentration of 20 nM E2, and an excellent correlation (R^2=0.944) between duplicate E2 measurements, the sensitivity and reproducibility of the coregulator binding assay was well within the range observed for other commonly used *in vitro* ER functional assays. Moreover, the coregulator binding assay uses fluorescence as an endpoint measurement, which offers several advantages in comparison to radioligand receptor binding assays, such as low costs and avoiding problems related to health hazards of radiation exposure and radioactive waste disposal. Dose-response analysis of the binding of ERα-LBD to coregulators in the presence of the ligand showed that the known estrogens (e.g., EE2, dienestrol, diethylstilbestrol, and *meso*-hexestrol) resulted in coregulator binding profiles similar to the reference compound E2, but each compound showed its own specific potency resulting in different EC_{50} values. Except for testosterone, the negative controls (i.e., corticosterone and progesterone) showed no significant binding changes compared with the solvent control DMSO. The SERMs tested on the peptide microarray (i.e., tamoxifen, 4-hydroxytamoxifen, and OMIY-bisphenol) showed no binding on the corepressors present on the peptide microarray and showed decreased coac-

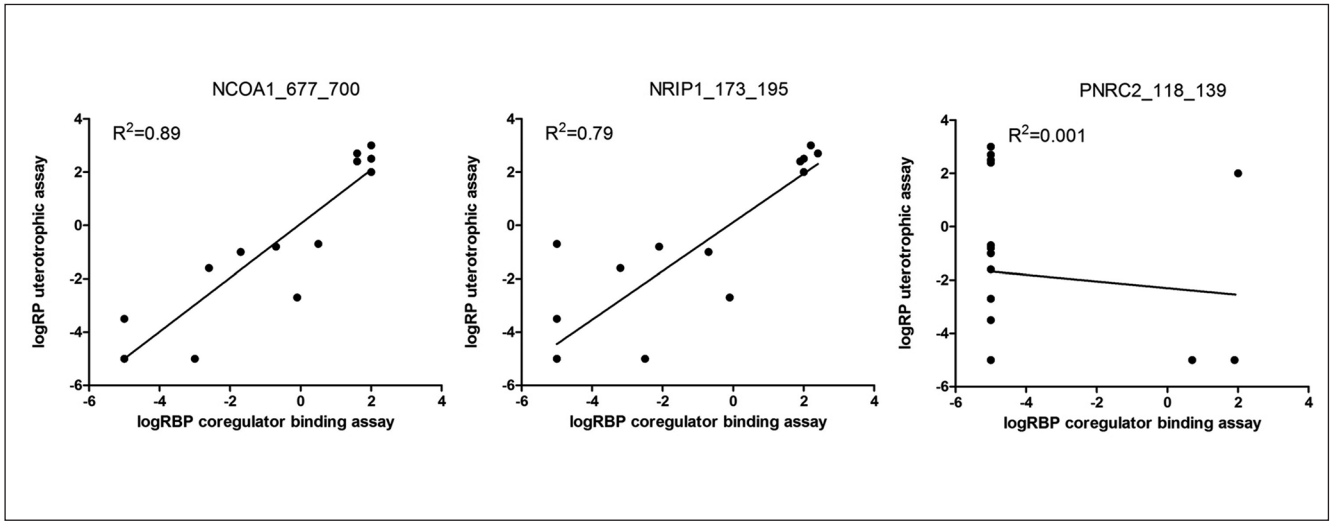

Fig. 4: Comparison of the log relative potencies (logRP) measured in the *in vivo* uterotrophic assay with the log relative coregulator binding potency (logRBP) as determined in the coregulator binding assay for the NCOA1_677_700, NRIP1_173_195, and PNRC2_118_139 coactivator peptides for the 18 compounds

Tamoxifen, 4-hydroxytamoxifen, OMIY-bisphenol, apigenin and 2,4,5-T were excluded from the comparison.

Tab. 1: Comparison of the relative potencies obtained in the ER binding assay, BG1Luc ER transcriptional activation assay, and *in vivo* uterotrophic assay with those obtained in the coregulator binding assay for the 23 compounds

Compounds		CAS nr.	ER binding assay log RBA[a]	BG1Luc ER TA logREP[e]	Uterotrophic assay logRP[f]	Coregulator binding assay logRBP[h]		
						NCOA1_ 677_700	NRIP1_ 173_195	PNRC2_ 118_139
Steroids and synthetic estrogens	17β-Estradiol	50-28-2	2.0	2.0	2.0	2.0	2.0	2.0
	17α-Ethinyl estradiol	57-63-6	2.2	1.7	3.0	2.0	2.2	-5.0
	Diethylstilbestrol	56-53-1	2.1	1.2	2.7	1.6	2.4	-5.0
	Dienestrol	84-17-3	2.0[b]	NA	2.4	1.6	1.9	-5.0
	meso-Hexestrol	84-16-2	2.4	1.3	2.5	2.0	2.0	-5.0
	Corticosterone	50-22-6	-5.0[c]	-5.0	-5.0	-5.0	-5.0	-5.0
	Progesterone	57-83-0	-3.5	-5.0	-5.0	-5.0	-5.0	-5.0
	Testosterone	58-22-0	-1.6	-3.2	-5.0	-3.0	-2.5	0.7
Phytoestrogens (natural products)	Coumestrol	479-13-0	1.1	-2.6	-0.8	-0.7	-2.1	-5.0
	Genistein	446-72-0	0.2	-2.9	-2.7	-0.1	-0.1	-5.0
	Apigenin	520-36-5	0.1	-3.6	-5.0	-1.3	-3.8	-5.0
	Zearalenone	17924-92-4	1.2	NA	-0.7	0.5	-5.0	-5.0
Phenols	OMIY-bisphenol	1943-97-1	NA[d]	NA	-0.3[g]	-	-	-
	p-n-Nonylphenol	104-40-5	-1.5	NA	-5.0	-5.0	-5.0	-5.0
	Bisphenol A	80-05-7	-1.5	-3.1	-1.6	-2.6	-3.2	-5.0
Organochlorines	Kepone	143-50-0	-1.5	-3.2	-1.0	-1.7	-0.7	-5.0
	o,p'-DDT	789-02-6	-1.7	-3.1	-3.5	-5.0	-5.0	-5.0
	2,4,5-T	93-76-5	-5.0	NA	NA	-5.0	-5.0	-5.0
Phthalates	Butylbenzyl phthalate	85-68-7	-2.7	-3.8	-5.0	-5.0	-5.0	-5.0
	Di-*n*-butyl phthalate	84-74-2	-2.6	-2.6	-5.0	-5.0	-5.0	-5.0
Paraben	Ethyl paraben	120-47-8	-3.2	-4.9	-5.0	-5.0	-5.0	1.9
SERMs	Tamoxifen	10540-29-1	0.6	NA	1.0	-	-	-
	4-Hydroxy-tamoxifen	68047-06-3	2.2	NA	1.0	-	-	-

[a] Logarithm of the median ER relative binding affinity values listed in the review of ICCVAM (ICCVAM, 2003).

[b] Median logRBA value derived from the EDKB (National Center for Toxicological Research, USA) (Ding et al., 2010).

[c] A cut-off value of -5.0 is listed for compounds showing no effect.

[d] Data not available

[e] Relative estrogenic potency is defined as the ratio between the EC_{50} of 17β-estradiol and the EC_{50} of the compound, and this ratio is subsequently multiplied by 100, calculated based on the BG1Luc ER TA data reported by ICCVAM (ICCVAM, 2011). The REP value of 17β-estradiol is thus 100, resulting in a logREP of 2.0.

[f] Median relative potency values based on uterotrophic assay in mouse or rat, derived from the EDKB (NCTR, USA). 17β-Estradiol is used as a reference chemical and is defined to have a relative potency of 100 (logRP=2.0) (Ding et al., 2010).

[g] LogRP of 4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol was calculated based on the minimal active dose described by Yamasaki et al. (2003).

[h] Relative binding potency values are obtained from the ratio of the concentration of 17β-estradiol needed to achieve 50% of maximal binding of ERα-LBD to coregulator and the concentration of the test compounds required to achieve a similar effect, and this ratio is subsequently multiplied by 100. The RBP value of 17β-estradiol is thus 100, resulting in a logRBP of 2.0.

[i] Compound showing only antagonist binding effect; therefore, the logRBP value cannot be determined.

tivator binding signals compared to the known ER agonists and the solvent control DMSO. The binding profiles of these SERMs are thus unique, as they are almost opposite to the profiles obtained with the known ER agonists and different from the profiles obtained with the negative controls and DMSO solvent control, indicating that the antagonistic properties of these SERMs mainly result from blocking the interaction of ERα with coactivator peptides rather than recruitment of corepressors. These findings are in agreement with other studies, showing that tamoxifen and 4-hydroxytamoxifen bind to the ligand-binding domain of ERα and cause a conformational shift of helix 12 into an adjacent coactivator site, which in turn prevents ERα from binding a coactivator (Shiau et al., 1998; Klinge et al., 2001; Pike et al., 1999; Konge et al., 2005; Kojetin et al., 2008). Moreover, the calculated IC_{50} values, based on all the coregulators showing a very good fit of the standard dose-response model, were 2.55×10^{-7} M and 1.82×10^{-9} M for tamoxifen and 4-hydroxytamoxifen, respectively. These IC_{50} values are also in line with data reported by ICCVAM, i.e., 7.12×10^{-7} M and 4.94×10^{-9} M for tamoxifen and 4-hydroxytamoxifen, respectively, in the BG1Luc ER transcriptional activation assay, demonstrating that the coregulator binding assay also is useful to test anti-estrogenic properties of compounds. Although tamoxifen and 4-hydroxytamoxifen are mainly reported to act as ER antagonists in breast and as ER agonists in uterus tissue (Shang and Brown, 2002), they are also able to inhibit the effect caused by EE2 in the uterotrophic assay and to induce breast cell proliferation in the E-screen (Fang et al., 2000; Yamasaki et al., 2003; Wang et al., 2012). The OMIY-bisphenol shows both agonistic and antagonistic effects in the uterotrophic assay, and when tested in proliferation assays it also behaves as an agonist and antagonist, demonstrating a biological effect profile nearly identical to tamoxifen (Wang et al., 2012). Thus, transcriptional activation assays, cell proliferation assays, and the *in vivo* uterotrophic assay are capable of displaying both the ER agonistic and ER antagonistic properties of tamoxifen, 4-hydroxytamoxifen, and OMIY-bisphenol. However, when tested on the peptide microarray, these three compounds showed only antagonistic effects. It is highly unlikely that the profiles will reveal the agonistic properties of these SERMs when co-exposed with E2 or EE2.

The androgen testosterone (T) was used as a negative control, as it is inactive in the *in vivo* uterotrophic assay. In the coregulator binding assay, T clearly induced the binding of ERα-LBD to several coactivators (e.g., binding of NCOA1_677_700 resulted a logRBP value of -3.0). However, in several studies it was shown that T can induce cell proliferation in MCF-7/BOS cells (E-screen), and it has been demonstrated that this atypical response was mediated by activation of the ER. More specifically, the proliferative response induced by testosterone in the E-screen is partially due to its conversion into 17β-estradiol by aromatase (Wang et al., 2012), partially due to formation of other estrogenic metabolites (Wang et al., 2013), and also partially due to T, i.e., activation of ERα. These findings are in line with the observations in the present study, i.e., T is capable of activating ERα-LBD and induces subsequent binding of several coactivators. Moreover, when tested in a yeast estrogen bioassay lacking steroid metabolism and steroidogenesis enzymes T was also shown to elicit weak responses at very high concentrations (Bovee et al., 2004), once again confirming that T is able to activate the ERα *in vitro*.

The environmental pollutant p-*n*-nonylphenol (CAS nr.104-40-5) was included by ICCVAM as a positive control in a set of reference compounds for validation of *in vitro* ER binding and transcriptional activation assays (ICCVAM, 2003). In our coregulator binding assay, this compound did not show any response. However, although others reported *p*-n-nonylphenol to be active in transcriptional activation assays based on either yeast cells or mammalian cells (Gaido et al., 1997; Legler et al., 1999), a thorough review of these studies showed that a technical mixture like the one available from Fluka (approximately 85-92.7% of branched isomers) or p-nonylphenol (CAS No. 84852-15-3) was used instead of the unbranched nonyl chain (CAS nr.104-40-5). Thus, the ICCVAM report may need to be updated in this regard. Meanwhile, it has been shown that the *p*-n-nonylphenol (CAS nr.104-40-5) was inactive in a yeast estrogen bioassay, while a technical mixture of nonylphenol from Fluka was active in the estrogen yeast bioassay (Bovee et al., 2004). From this, it could be concluded that *p*-n-nonylphenol is not estrogenic and that the estrogenicity of the technical mixture is due to one or more isomers with a branched side-chain. A similar conclusion was presented by Pedersen et al. (1999) studying the induction of plasma vitellogenin in rainbow trout by linear and technical nonyl- and octylphenol. In a more recent study, *p*-n-nonylphenol was even used as a negative control for the validation of a recombinant yeast estrogen receptor agonist assay (Kolle et al., 2010). Moreover, butylbenzyl phthalate, di-*n*-butyl phthalate, and ethyl paraben were reported to bind weakly to the ER and were also slightly active in the BG1Luc ER transcriptional activation assay (ICCVAM, 2003, 2011). In the coregulator binding assay, although they may also bind weakly to the ERα-LBD, detectable coregulator recruitment was not induced by these compounds under the concentrations tested. In the *in vivo* uterotrophic assay, butylbenzyl phthalate, di-*n*-butyl phthalate and ethyl paraben also are not able to induce uterotrophic effects (Zacharewski et al., 1998; Sik Kim et al., 2005; Hossaini et al., 2000). This demonstrated that the human cancer cell line based reporter gene assays are more sensitive than both the *in vivo* uterotrophic assay and the current coregulator binding assay, which is an advantage for screening estrogenicity in food or environmental samples. However, for prioritization and testing pure chemicals, all these *in vitro* assays are sensitive enough to measure potent to weak estrogens. Compounds that are negative in the coregulator binding assay, although slightly active in the BG1Luc ER assay, may not have the highest priority to be further tested for estrogenicity in the *in vivo* uterotrophic assay. In addition, *o,p'*-DDT is able to induce a uterotrophic effect in the rat (Shelby et al., 1996; Newbold et al., 2001), but did not show an effect in the current coregulator binding assay. This is probably due to the narrow concentration range tested (20 pM-200 μM), resulting in poor fitting of the applied dose-response model to the data, and as a consequence, classification of the response of *o,p'*-DDT as negative. With excellent correlation with the estrogen receptor binding as-

Tab. 2: Correlation of the relative potencies obtained in the ER binding assay, BG1Luc ER transcriptional activation assay, and *in vivo* uterotrophic assay with those obtained in the coregulator binding assay based on 57 coactivators showing a clear sigmoidal dose-response relation for 17β-estradiol-induced binding of ERα-LBD as reflected by a goodness-of-fit value of 0.9 or higher

Coregulator binding assay Peptide ID[a]	Motif	Uniprot Accession	Coefficient of determination (R^2)		
			ER binding assay (n=19)	BG1Luc ER TA (n=16)	*In vivo* uterotrophic assay (n=18)
BL1S1_1_11	LxxLL2	P78537	0.51	0.54	0.49
BRD8_254_276	LxxLL267	Q9H0E9	0.74	0.80	0.87
CBP_57_80	LxxLL70	Q92793	0.65	0.84	0.71
EP300_69_91	LxxLL81	Q09472	0.73	0.82	0.84
GNAQ_21_43	LxxLL34	P50148	0.80	0.81	0.86
HAIR_745_767_C755S/C759S	LxxLL758	O43593	0.46	0.53	0.46
IKBB_277_299	LxxLL289	Q15653	0.80	0.78	0.82
JHD2C_2054_2076	LxxLL2066	Q15652	0.85	0.79	0.88
LCOR_40_62	LxxLL53	Q96JN0	0.64	0.84	0.81
MED1_591_614	LxxLL604	Q15648	0.69	0.80	0.81
MLL2_4175_4197	LxxLL4188	O14686	0.65	0.81	0.79
NCOA1_620_643	LxxLL633	Q15788	0.82	0.80	0.87
NCOA1_677_700	LxxLL690		0.87	0.76	0.89
NCOA1_737_759	LxxLL749		0.78	0.81	0.83
NCOA1_1421_1441	LxxLL1435		0.84	0.74	0.89
NCOA2_628_651	LxxLL641	Q15596	0.86	0.75	0.88
NCOA2_677_700	LxxLL690		0.84	0.80	0.87
NCOA2_733_755	LxxLL745		0.82	0.81	0.87
NCOA3_609_631	LxxLL621	Q9Y6Q9	0.79	0.82	0.85
NCOA3_609_631_C627S	LxxLL621		0.86	0.78	0.89
NCOA3_673_695	LxxLL685		0.85	0.78	0.89
NCOA3_725_747	LxxLL738		0.85	0.78	0.89
NR0B1_1_23	LxxML13	P51843	0.78	0.81	0.82
NR0B1_136_159	LxxLL146		0.83	0.75	0.83
NR0B2_9_31_C9S/C11S	LxxLL21	Q15466	0.82	0.76	0.84
NR0B2_106_128	LxxIL118		0.83	0.71	0.89
NR0B2_201_223_C207S	LxxVL214		0.78	0.80	0.82
NRBF2_128_150	LxxLL141	Q96F24	0.64	0.77	0.66
NRIP1_120_142	LxxLL133	P48552	0.78	0.80	0.85
NRIP1_121_143_P124R	LxxLL133		0.64	0.81	0.78
NRIP1_253_275_C263S	LxxLL266		0.81	0.80	0.88
NRIP1_368_390	LxxLL380		0.84	0.73	0.84
NRIP1_488_510	LxxLL501		0.83	0.80	0.89

Coregulator binding assay Peptide ID[a]	Motif	Uniprot Accession	Coefficient of determination (R^2)		
			ER binding assay (n=19)	BG1Luc ER TA (n=16)	*In vivo* uterotrophic assay (n=18)
NRIP1_700_722	LxxLL713		0.81	0.80	0.87
NRIP1_701_723	LxxLL713		0.81	0.81	0.88
NRIP1_805_831	LxxLL819		0.81	0.80	0.88
NRIP1_924_946	LxxLL936		0.80	0.82	0.87
NRIP1_924_946_C945S	LxxLL936		0.84	0.80	0.88
NRIP1_1055_1077	LxxML1068		0.84	0.79	0.89
NSD1_894_916	FxxLL907	Q96L73	0.62	0.81	0.74
PELP1_20_42	LxxLL33	Q8IZL8	0.76	0.79	0.84
PELP1_168_190	LxxLL181		0.80	0.75	0.83
PELP1_446_468	LxxLL459		0.77	0.81	0.82
PELP1_571_593_C575S/C581S	LxxLL584		0.83	0.71	0.84
PPRC1_151_173	LxxLL164	Q5VV67	0.77	0.80	0.88
PRGC1_130_155	LxxLL144	Q9UBK2	0.73	0.61	0.82
PRGC1_134_154	LxxLL144		0.80	0.72	0.80
PRGC2_146_166	LxxLL156	Q86YN6	0.85	0.74	0.88
PRGC2_338_358	LxxLL343		0.83	0.80	0.88
PROX1_57_79	LxxLL70	Q92786	0.80	0.82	0.87
TIF1A_747_769	LxxLL760	O15164	0.83	0.74	0.82
TIP60_476_498	LxxLL489	Q92993	0.78	0.82	0.84
TREF1_168_190	LxxLL181	Q96PN7	0.76	0.80	0.87
TRRAP_3535_3557_C3535S/C3555S	LxxLL3548	Q9Y4A5	0.60	0.71	0.74
TRXR1_132_154	LxxLL145	Q16881	0.81	0.83	0.89
WIPI1_119_141	LxxLL132	Q5MNZ9	0.60	0.76	0.78
ZNHI3_89_111	LxxLL101	Q15649	0.82	0.80	0.88

[a] ID as follows: [coregulator]_[aa start]_[aa end of peptide], bold coactivators have been shown previously to bind to endogenous ERα in cell lysates and in breast tumors in the presence of E2 (Houtman et al., 2012).

say (33 coactivators with $R^2 \geq 0.80$, n=19), BG1Luc ER transcriptional activation assay (32 coactivators with $R^2 \geq 0.80$, n=16), and the *in vivo* uterotrophic assay (30 coactivators with $R^2 \geq 0.85$, n=18), the coregulator binding assay demonstrated its usefulness in screening substances for *in vitro* ER agonistic activity. Moreover, 25 coactivators have been shown to bind to endogenous ERα in cell lysates and in breast tumors when tested on a PamChip® plate peptide microarray in the presence of E2 (Houtman et al., 2012). Twenty-one of these 25 coactivators (highlighted in Tab. 2) also display a high correlation coefficient with the estrogenicity observed in the uterotrophic assay, indicating the biological relevance of the correlation found with these coactivators.

In summary, the obtained results in this study with the SERMs indicate that the coregulator binding assay based on the PamChip® plate is able to distinguish receptor agonists from antagonists. Moreover, in transcriptional activation assays, cell proliferation assays and the *in vivo* uterotrophic assay, the effects of ER antagonists generally are measured in combination with potent estrogens such as E2 or EE2, while in the coregulator binding assay, the antagonist properties can be measured directly, i.e., without the addition of a potent ER agonist. However, although the *in vivo* antagonist effects of the SERMs (e.g., tamoxifen, 4-hydroxytamoxifen and OMIY-bisphenol) could be predicted correctly, the coregulator binding assay is not able to reveal the ER agonist properties of these SERMs. Therefore,

other types of *in vitro* assays, e.g., reporter gene assays and the H295R steroidogenesis assay, are needed to build a panel of *in vitro* assays to increase the predictive power and to reach a similar performance in qualifying compounds as that achieved by the *in vivo* uterotrophic assay. The present study thus shows that the coregulator binding assay is useful within such a panel of *in vitro* test systems for estrogenicity testing, allowing easy high-throughput screening and prioritization of chemicals, thereby contributing to the reduction – and ultimately the replacement – of current animal testing for (anti-)estrogenic effects.

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Acknowledgements

This project was financially supported by the Netherlands Genomics Initiative (Netherlands Toxicogenomics Centre, grant number 6162500134).

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