Toxicogenomics-based in vitro alternatives for estrogenicity testing

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

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Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Thursday 20 June, 2013
at 11 a.m. in the Aula.

Si Wang
Toxicogenomics-based in vitro alternatives for estrogenicity testing
PhD thesis, Wageningen University, Wageningen, NL (2013)
With references, with summaries in Dutch and English
ISBN 978-94-6173-581-2

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1 Introduction

INTRODUCTION

Endocrine disruption - causes for concern

In 1996, with the publication of Our Stolen Future by Theo Colborn and co-authors, many people heard for the first time about the existence of endocrine disruptors and that these chemicals could affect health of humans and wildlife. Since then public concerns regarding the possible harmful effects posed by endocrine-disrupting chemicals (EDCs) has grown. As a consequence, the U.S. Congress passed two laws affecting the regulation of pesticides and other chemicals in 1996. Both of these laws, the Food Quality Protection Act [1] and the Safe Drinking Water Act, [2] mandated that the Environmental Protection Agency (EPA) implements the Endocrine Disruptor Screening Program (EDSP) to test the possible endocrine disruptive effects of approximately 87,000 individual chemical substances. The mandate mainly dealt with compounds exhibiting estrogenic activity, although other hormone disrupting effects (e.g., disruption of androgen or thyroid hormone activity) were considered as well.

An endocrine-disrupting compound was defined by the U.S. EPA as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes". The group of molecules identified as endocrine disruptors is highly heterogeneous and includes pharmaceuticals like diethylstilbestrol (DES), industrial by-products and pollutants like dioxins and polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT) and other pesticides, fungicides like vinclozolin and plasticizers such as bisphenol A. Although most of the endocrine-disrupting chemicals identified to date have a weak activity compared with their endogenous counterparts, they can be present in significant concentrations in human and animal food or in the environment. In addition, most of these chemicals have a strong tendency to bioaccumulate in different organisms. Exposure to chemicals that can act as hormones is not confined to those of industrial origin: some natural compounds can mimic the effects of estrogens and thus might act as EDCs too. Phytoestrogens (e.g., genistein and coumestrol), generally thought to have relatively low binding affinity to estrogen receptors (ERs), are widely consumed and are components of infant formula. [3, 4] A recent study reported that urinary concentrations of the phytoestrogens genistein and daidzein were about 500-fold higher in infants fed soy formula compared with those fed cow's milk formula. [5] Therefore, the potential adverse health effects of phytoestrogens need to be considered. Fungi can also produce estrogenic substances, e.g., zearalenone and its derivatives, which are found in a number of cereal crops and food products derived from them. [6, 7] The natural estrogens, 17ß-estradiol (E2), estrone (E1) and estriol (E3) and the synthetic estrogen used as main active ingredient in oral contraceptive pills 17α-ethinyl estradiol (EE2), have also been shown to contaminate the aquatic environment. [8, 9] As these substances are not completely removed with conventional wastewater treatment processes, there is increasing pressure to further develop and apply advanced methods for municipal wastewater treatment, such as ozonation and activated carbon treatment. [10, 11] Many of the substances listed above have estrogenic, androgenic, antiestrogenic, antiandrogenic or thyroid hormone-like activities. Moreover,

the same substance often has more than one of these properties. For example, bisphenol A may act both as an estrogen receptor agonist and an androgen receptor antagonist, [12-14] while dioxin-like chemicals appear to have even more complicated effects, i.e., not only by the aryl hydrocarbon receptor (AhR), but also on the function of steroid hormones and thyroid hormones. [15-17] Furthermore, some substances are broken down or converted into metabolites that are even more potent than the parent compound, or into substances with other disturbing properties. For instance, the estrogen agonist DDT is metabolized into the androgen antagonist DDE, both in rodents and humans. [18, 19]

In recent years, our understanding of the mechanisms by which endocrine disruptors exert their effect has increased. EDCs were originally thought to exert their actions primarily through activation of nuclear hormone receptors, including estrogen receptors (ERs), androgen receptors (ARs), thyroid receptors (TRs), progesterone receptors (PRs), glucocorticoid receptors (GRs), and retinoic acid receptors (RARs), among others. Today, basic endocrinology studies show that the mechanisms are much broader than originally recognized, as EDCs also act via e.g. membrane receptors, neurotransmitter receptors (such as the serotonin receptor, dopamine receptor and norepinephrine receptor), the enzymatic pathways involved in steroid biosynthesis and/or metabolism, and numerous other mechanisms that may affect endocrine and reproductive systems. [16, 20] EDCs thus encompass agents that cause alterations in reproduction or development through direct or indirect effects on the vertebrate hypothalamic–pituitary–thyroidal (HPT) or hypothalamic–pituitary–gonadal (HPG) axes.

A great number of laboratory and field studies have shown that the exposure to EDCs can cause developmental, reproductive, neural, immune, and other problems in a range of animals, and these compounds have been hypothesized to elicit similar responses in humans and wildlife. [21] These effects include feminization, altered sex ratios and intersex gonads in frog and fish, [22, 23] egg-shell thinning in birds induced by DDT, [18] increased tumor formation in rats, [24] and impairment of immune function in seals caused by PCBs. [25] There is also evidence that exposure to EDCs can decrease sperm quality and semen volume, increase the number of testicular germ cells and the incidence of hormone-dependent cancers. [16] However, considerable scientific uncertainty remains regarding the causes of these reported effects. EDCs may not only affect the exposed individual, but also the children and even subsequent generations. Diethylstilbestrol (DES) is a good example, showing clear effects both during embryonic development and in adult life of exposed offspring. DES is an orally active synthetic estrogen that was frequently prescribed to pregnant women in the 1940s to 1960s to prevent spontaneous abortion. However, later, better-controlled studies showed no benefit, [26] and in 1971 in utero exposure was found to be associated with a greatly increased risk of clear cell carcinoma of the vagina and cervix in first generation female offspring, known worldwide as the problem with the DES daughters. [27] Subsequently, DES treatment was found to be associated with an increased risk of breast cancer in women who took the drug, namely the mothers, [28] and the latest results suggest that DES daughters also have an increased risk of breast cancer after the age of 40 years. [29] Even the fertility of men exposed to DES before birth has been investigated and several studies have shown a misplaced opening of the penis (hypospadias), epididymal cysts (non-cancerous growth on the testicle), and undescended testicles (cryptorchidism) in first generation male

offspring. [30, 31] In adult men taking DES, the drug has been shown to cause gynaecomastia. [32, 33] In view of these overt adverse effects of EDCs, it is necessary to consider EDCs as substances of concern equivalent to carcinogens, mutagens and reproductive toxicants, as well as persistent and bioaccumulative toxic chemicals. [34]

Current EDC screening programs and strategies

Due to the possible adverse effects of EDCs, national governments and international regulatory authorities are in the process of establishing testing programs and strategies to assess the safety of currently used chemicals. One of the leading and the first national, legally-binding programs is the EDSP of the U.S. EPA, which employs a two-tier screening program consisting of a battery of in vitro and in vivo screening assays to gather information needed to identify endocrine-active substances. The Tier 1 battery assesses the potential of a chemical to interact with the endocrine system via estrogen- and androgen-mediated effects, i.e., receptor binding, transcriptional activation, steroidogenesis, and HPG axis feedback (Table 1). In addition, in vivo rodent pubertal and amphibian metamorphosis assays were included to detect direct and indirect effects on the HPT axis. These in vitro and in vivo assays are designed to complement one another, therefore the robustness and specificity of the current Tier 1 battery is based on the strengths of each individual assay and their complementary nature. However, conducting all of the 11 EDSP Tier 1 tests would require a minimum of 520 animals and the current costs are reported to range between $335,100 and $964,250 per chemical. [35, 36] Once a chemical is identified as a potential EDC in Tier 1 screening, EPA will require further testing in the Tier 2 screening, e.g., a reproductive toxicity test in rodents encompassing one or more generations. However, to date, no definite decisions have been made regarding the specific tests to be included into the Tier 2 testing stage. With the vast number of chemicals potentially targeted for testing under the current five-year time horizon of the EDSP Comprehensive Management Plan, [37] prioritization of chemicals to enter Tier 1 screening and replacing validated in vivo Tier 1 assays with effect-based validated in vitro high-throughput assays are essential. Furthermore, EPA also works together with the Organisation for Economic Co-operation and Development (OECD) to set international standards on the safety of chemicals, and issues OECD guidelines for the testing of chemicals. They have defined test guidelines (TG) e.g., the Uterotrophic Bioassay for testing estrogenic properties (TG 440), the Hershberger Bioassay for testing (anti-)androgenic properties (TG 441) and the in vitro H295R assay for testing effects on steroidogenesis (TG 456). The OECD has also amended and validated its test guideline for repeated-dose 28-day oral toxicity studies in rodents (TG 407) to include endpoints relevant to the thyroid system, including histopathology of the pituitary and thyroid, and thyroid weight. However, the TG 407 in most cases failed to identify endocrine active substances that weakly affect estrogen or androgen receptors. Thus, characterization of potential endocrine activity of a substance should not be based on this TG alone, but its results should be used in a weight of evidence approach incorporating all existing data.

To date, no mandatory testing programs for endocrine disruption such as U.S. EPA's EDSP exist in Europe. However three pieces of European Community legislation deal explicitly with

Table 1. EDSP Tier 1 Tests (Adapted from Willett et al., 2011).

Screening assays	Number of animals used/test	Estimate amount spent by EPA in the year 2010	Modes of action								OECD test guideline
			Receptor binding				Steroidogenesis				
			E[b]	Anti-E[b]	A[b]	Anti-A[b]	E-production[b]	A-production[b]	HPG-axis[c]	HPT-axis[d]	
In vitro											
ER binding - rat cytosol	?	$17,250	√	√							
ERα transcriptional activation		$15,000	√								TG 455
AR binding - rat cytosol	?	$17,250			√	√					
Steroidogenesis H295R		$13,750					√	√			TG 456
Aromatase recombinant		$25,000					√				
In vivo											
Uterotrophic	18	$43,050	√								TG 440
Hershberger	18–36	$47,400			√	√					TG 441
Pubertal male	45	$93,500			√	√		√	√	√	
Pubertal female	45	$87,100	√	√			√		√	√	
Fish short-term reproduction (male and female)	72	$92,500	√	√	√	√	√	√	√		TG 229
Amphibian metamorphosis	320	$80,597								√	TG 231
Analytical chemistry		$12,000									
Total	518–536	$544,397									

[a] Based on [36]; [b] E = estrogen and A = androgen; [c] Hypothalamic–pituitary–gonadal;
[d] Hypothalamic–pituitary–thyroidal

endocrine disrupters: The Plant Protection Product Regulation 1107/2009; [38] The Biocidal Products Directive 98/8/EC, [39] and most importantly, the European Community regulation: Registration, Evaluation, Authorization and Restriction of Chemical substances (REACH; EC 1907/2006) and their safe use. [40] Specifically, REACH requires all substances that are marketed in the European Union (EU) at more than one tonne per year to be registered by presenting an extensive dossier to the European Chemical Agency (ECHA). A recent report by Rovida and Hartung claims that the total number of animals and costs to accomplish REACH legislation requirements for reproductive toxicity testing can increase up to 48.6 million experimental animals and 6.9 billion euro, respectively, if growth of the EU and its chemical industry is taken into account, and the current test guidelines are strictly followed. [41] One way to reduce the use of laboratory animals could obviously be replacing in vivo tests with in vitro alternative methods. So far, the EU has invested more than 200 million dollars into the development of alternative methods by funding respective research, [42] apart from the funding at the national level by the Member States themselves. In addition, The U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the European Commission for the Validation of Alternative Methods (ECVAM) are also concerned about possible effects of known and yet unknown EDCs, and have closely cooperated and conducted studies to develop and validate new test methods for EDC testing. Moreover, to undertake the investigation of high production volume (HPV) chemicals in a co-operative way, the OECD Cooperative Chemicals Assessment Programme was established, addressing challenges such as: assessing more chemicals in a shorter time period, addressing all chemicals on the market and avoiding duplication with work going on in other countries. In order to meet these goals and fulfil the 3R principle of Refinement, Reduction and Replacement of animal testing, OECD expert panels on non-animal testing have set up and approved the validation of the in vitro ERα-HeLa-9903 (TG 455) and BG1Luc ER (TG 457) transcriptional activation (TA) assays for estrogenicity testing. [43, 44] The present thesis focusses on developing an in vitro integrated test system (ITS) for estrogenicity. In the next sections, the in vivo uterotrophic bioassay and in vitro assays based on different steps in the estrogen receptor-dependent signal transduction pathway, will be discussed in some more detail.

Uterotrophic Bioassay

Presently, the standard test for disruption of normal estrogen function is the Allen and Doisy test, an uterotrophic assay in immature or ovariectomised rodents with uterus weight as a crucial read-out parameter. [45, 46] The uterotrophic assay is a short-term screening test that originated in the 1930's [47, 48] and was validated through a 4-year international validation program starting from July 2009 under the auspices of the OECD. [49] The biological and scientific basis for the uterotrophic assay is the central role of endogenous estrogens in the natural estrous cycle. The endogenous steroidal estrogens control cell division for the tissues of the female reproductive tract, i.e., the uterus, cervix, and vagina, resulting in easily measurable levels of tissue growth. In rodents like mice and rats, this tissue growth response takes place within a period of approximately two days and the entire cycle of growth and

regression is complete in 4-5 days. The time-frame for conducting the uterotrophic assay roughly corresponds to this time, and the assay measures increases in uterine tissue weight after 3 consecutive days of test substance administration. The uterotrophic assay relies for its sensitivity on an animal test system in which the hypothalamic-pituitary-ovarian axis is not functional, leading to low homeostatic regulation against exogenous estrogenic stimuli. [50] When the animal is immature or ovariectomised, it lacks its main natural source of estrogens and also the feed-back response upon the administration of exogenous estrogenic compounds through the HPG axis is absent (when ovariectomised) or has little effect (when immature). As a result, the uterus becomes sensitive to external sources of estrogens. Subsequently, when exposed to an estrogenic substance the animals' uterus will increase in weight as a result of the absorption of fluid and cell proliferation induced by the administered estrogen. For animal welfare reasons preference should be given to the method using immature rodents, avoiding surgical pre-treatment of the animals. The uterotrophic assay also presents the opportunity to assay estrogen antagonists, however, its use for antagonist detection is much less common than for agonists. To detect estrogen antagonists, a potent reference estrogen (e.g., EE2) in a dose causing a submaximal response is co-administered with the test substance and the response is compared to a control group given reference estrogen-only. The mean uterine weight of the test substance groups relative to the reference estrogen-only group is assessed for a statistically significant alteration. A statistically significant decrease in uterine weight indicates that the test substance has an ability to reduce or block the action of portent estrogens, and, therefore, is considered to be a potential antiestrogen. [51]

The main advantage of the in vivo uterotrophic assay is, that it determines an overall biological effect by allowing for interactions between cells and between different components of the endocrine system, thereby being able to detect complex modes of action that may occur only in the intact animal. In addition, it takes account of the absorption, distribution, metabolism and elimination (ADME) parameters of the test chemicals. The uterotrophic assay has been extensively employed in pharmaceutical research within the drug discovery process for over 60 years. However, the current intended application of the uterotrophic assay is shifting from potent pharmacological substances to the identification of far less potent estrogen agonists and antagonists for large-scale regulatory chemical screening programs, e.g., REACH. Due to the fact that the in vivo methods are in general expensive, labour-intensive, time-consuming, and for obvious ethical reasons, this animal test is not suitable for routine high-throughput screening purposes. Several in vitro assays have been developed to determine the potential estrogenic activities of compounds corresponding to the different steps in the estrogen receptor-dependent signal transduction pathway. The cellular mechanism of action of estrogens is discussed in the next section.

Cellular mechanism of action of estrogens

Estrogens are steroid hormones that are primarily biosynthesized in the female ovaries. Estrogens influence the growth, differentiation and function of many target organs, such as mammary gland, uterus, ovary, vagina, as well as testis and prostate. [52, 53] They also play a role in bone maintenance, the central nervous system and the cardiovascular system. [54] Estrogens

exert their physiological effects mainly through activation of the nuclear ER in target cells, exerting its effect through upregulation of the transcription of estrogen-responsive element (ERE)-controlled genes. Part of the effects of estrogens is also thought to be mediated by plasma membrane-associated ERs. Although two main forms of the ER exist, ERα and ERβ, in (reproduction) toxicology the main attention goes out to the nuclear ERα, as activation of nuclear ERα is the driving force behind the uterus response in the uterotrophic assay and ERα knockout animals have an irresponsive uterus. [52] Moreover, the focus on ERα, with respect to regulatory purposes, is because in contrast to ERβ, binding and activation of ERα is implicated as a key molecular initiating event (MIE) in estrogenicity-related adverse endpoints. [44]

ERs consist of several distinct functional regions (A-F; Figure 1). The A/B region located in the N-terminal part of the protein encompasses the activation function 1 (AF-1) domain responsible for ligand-independent transactivation. The most conserved domain among ERs is the DNA-binding domain (DBD) corresponding to the C region, which is responsible for binding to a specific DNA sequence, called ERE, in gene promoter regions. The C-terminal protein part (E/F region) includes the ligand-binding domain (LBD) together with the ligand-dependent transactivation domain AF-2. [53] The nuclear ERα and ERβ, like all 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. The unoccupied ER is found in the cytoplasm (Figure 2). Upon ligand binding, the LBD undergoes a conformational change that leads to receptor dimerization, translocation of the ER from the cytoplasm into the nucleus, and binding to estrogen-responsive elements. Moreover, as a result of the intramolecular conformational changes induced by ligand binding, the affinity of the

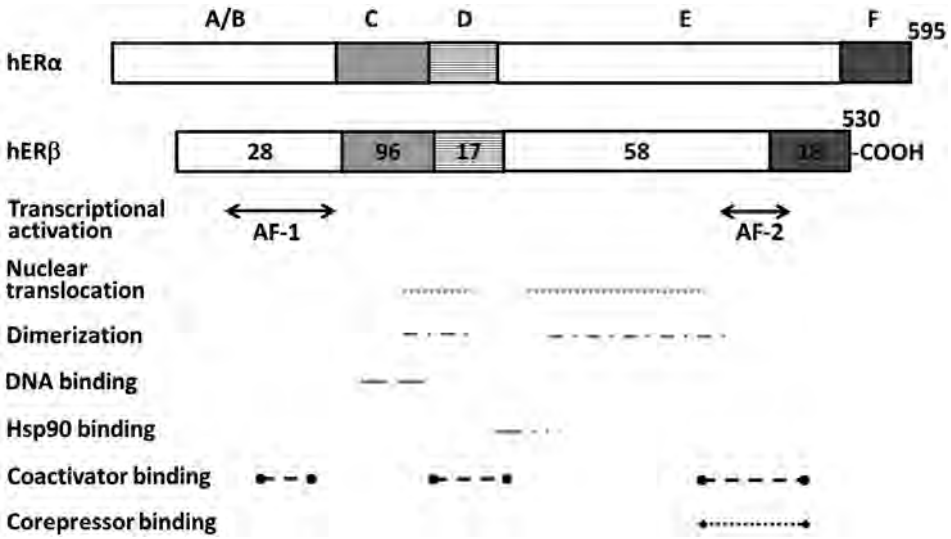

Figure 1. Schematic representation of the domain structure of the estrogen receptors, ERα and ERβ. The percentage of amino acid homology between regions A-F in ERα and ERβ is indicated by the numbers. Adapted from: Klinge, 2000. [55]

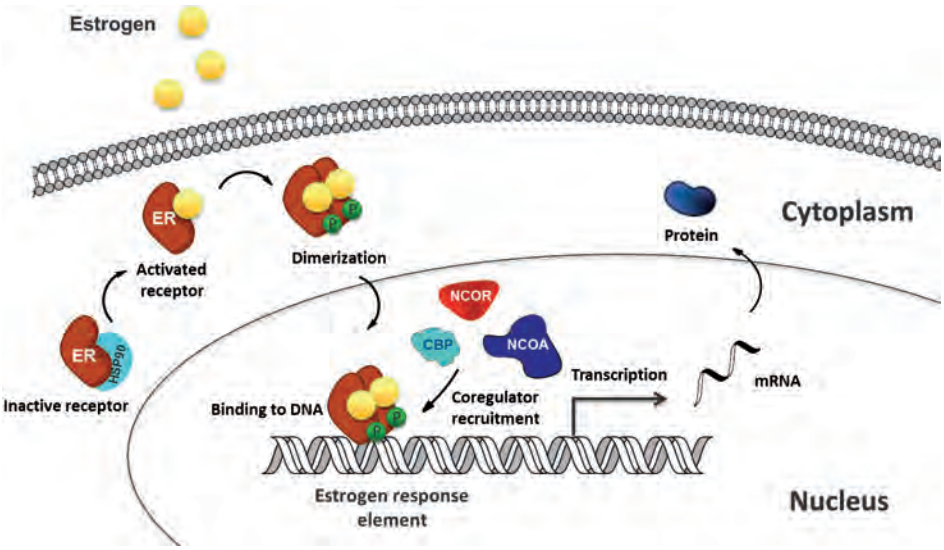

Figure 2. Schematic representation of the mechanism of action of estrogens.

ER for coregulator proteins is changed resulting in recruitment or release of transcriptional coactivator or corepressor proteins, respectively, that enhance or repress interaction of RNA polymerase II with estrogen-responsive gene promoters needed to actually induce or repress transcription of target genes. [55, 56] However, it is also possible that chemicals elicit their estrogenic effects through mechanisms that are not directly mediated by the ER, e.g., alteration of the hormonal steroidogenic pathway or cross-talk between ER and growth factor signaling, such as insulin-like growth factor-1 and epidermal growth factor. [53]

In vitro bioassays for the detection of estrogen (ant)agonist

Due to the great variety of chemicals with estrogenic properties, both immunochemical and analytical chemical methods have the drawback that they are only able to detect structurally related compounds or target compounds already identified to be estrogenic and are not able to detect biological activity of unknown compounds and their metabolites. This is in contrast to biological assays which are based on the molecular or cellular mechanism of action of estrogens, and thus can detect unknown compounds with estrogenic activity. Many in vitro assays have been developed to determine the potential estrogenic activities of compounds corresponding to the different steps in the estrogen receptor-dependent signal transduction pathway, e.g., ER binding, ER-controlled reporter gene expression or other downstream events such as estrogen receptor-mediated cell proliferation. These in vitro assays were recently reviewed by Bovee and Pikkemaat, showing that every assay type has its own specific advantages and disadvantages. [57]

In vitro ER competitive binding assays have been well established and extensively used to investigate ER-ligand interactions. ER competitive binding assays measure the displacement

of a receptor-bound probe molecule by a test compound, allowing to determine the relative binding affinity of the test compound as a ligand of the ER. Conventional ER binding assays are rapid and easy to perform. However, one of the main drawbacks 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 (EPA) Endocrine Disruptor Screening Program Tier 1 screening battery, still requires the use of animals as a source of ERs. Although studies using a surface plasmon resonance biosensor technology-based receptor binding assay have shown that agonists and antagonists exhibited very different association kinetics for the ER, [58] the use of such a binding assay to detect estrogen antagonists, still has to be proven and validated using a larger set of compounds.

Reporter gene assays (or transcriptional activation assays), based on stably transfected cells (either mammalian or yeast cells), provide a more specific endpoint measurement, i.e., are based on the estrogen receptor-mediated mechanism of gene transcription activation. [59] These reporter-gene assays measure the induction of a reporter gene, the expression of which is easy to quantify and is put under transcriptional control of one or more EREs and therefore depends on the level of activation of the estrogen receptor. To date, two reporter gene assays have been validated by OECD and are being proposed for screening and prioritization purposes, i.e., the ERa-HeLa-9903 cell line, derived from a human cervical tumor, and the BG1Luc ER TA assay using the human ovarian BG1Luc-4E2 cell line. [43, 44] These reporter gene assays are used to identify chemicals that activate the ER following ligand binding, after which the receptor-ligand complex binds to EREs and activates the expression of the reporter gene, resulting in increased cellular expression of a marker enzyme (e.g. luciferase). In the presence of the substrate luciferin, the enzyme luciferase will use the energy from ATP to oxidize luciferin and release photons. The emitted light can be quantitatively measured with a luminometer. In addition to the OECD-validated reporter gene assays, several other reporter gene assays have been developed and applied as screening tools to determine the estrogenic/anti-estrogenic activities of compounds and have been shown to produce relevant and reliable outcomes. [60-62]

Proliferation assays have also been shown to provide valuable tools for estrogenicity testing. The E-screen is a proliferative assay based on the human MCF-7/BOS breast cancer cell line and is widely used as a screening tool to detect environmental and xenobiotic estrogens. [63, 64] However, crosstalk can occur due to the fact that MCF-7/BOS cells also express androgen, progesterone, glucocorticoid and retinoid receptors, which are also known regulators of cell proliferation. [65]

As discussed above, the estrogenic effect of a chemical can be due to direct activation of ER but can be also due to alteration of normal hormone levels by affecting hormone biosynthesis, transport, or metabolism. The H295R steroidogenesis assay recently validated by the OECD provides an in vitro methodology to evaluate the potential interference of exogenous compounds with endogenous steroid hormone synthesis. [66] Compared to in vivo testing, the H295R steroidogenesis assay is one of the most complete and simple in vitro assays, since it is based on the human H295R adrenocarcinoma cell line which expresses all key

enzymes and can synthesize all steroid hormone precursors involved in adrenal and gonadal steroidogenesis. Moreover, regulation of steroidogenesis and hormone secretion patterns in H295R cells highly reflect those of freshly isolated adrenal cells.[67, 68] However, the current OECD-approved protocol of the H295R steroidogenesis assay only measures testosterone (T) and estradiol (E2) levels after exposure to a test chemical and results of the assay are expressed as relative changes in hormone production compared with the solvent controls. Thus, this assay can only provide a YES/NO answer with regard to the potential of a chemical to induce or inhibit the production of T and E2 and does not provide specific mechanistic information concerning the interaction of the test substance with the endocrine system. [66]

OUTLINE OF THESIS

To date, more than one hundred different in vitro assays have been described for the screening of estrogenic compounds. [69] However, there is as yet no single standalone or a panel of in vitro assay defined for replacement of the in vivo uterotrophic assay for estrogenicity testing. The aim of the research described in this thesis was to develop an integrated testing strategy (ITS) based on existing and newly developed in vitro assays for estrogenicity, allowing easy high-throughput screening and prioritization of chemicals, thereby contributing to reduction and ultimately replacement of current animal testing for estrogenic effects.

To reach this aim, several presently available and newly developed in vitro bioassays were selected and evaluated for optimal representation of the (anti)estrogenic effects occurring in the uterus/endometrium in vivo. These assays cover a broad range of endpoints and measure different steps in the estrogen-signaling pathways (i.e., receptor binding, receptor-coregulator binding, reporter-gene transcriptional activation, targeted gene expression, and cell proliferation). **Chapter 2** presents studies investigating the predictive value of ER-controlled cell proliferation assays for the in vivo uterotrophic effect. Four cell lines derived from three different estrogen-sensitive female tissues, i.e., breast (MCF-7/BOS and T47D), endometrial (ECC-1) and ovarian (BG-1) cells, were investigated using a series of reference compounds in order to select a possible candidate for the in vitro test battery that shows the best correlation with in vivo outcomes. In **chapter 3,** the suitability of reporter gene assays for being part of an integrated in vitro testing strategy for the detection of estrogenic activity was studied. To this end, a set of reference compounds was chosen and tested in a battery of reporter gene assays in order to determine which assay most accurately predicts the uterotrophic effect observed in vivo. In **chapter 4,** a newly developed low-density DNA microchip, which contains probes for 11 different estrogen-responsive marker genes, was evaluated for the detection of estrogenic compounds and their relative potencies. **Chapter 5** presents the results of a 155-plex high-throughput in vitro coregulator binding assay for estrogenicity testing. Using this coregulator binding assay the ligand-modulated interaction of coregulators with ERα was assessed for a series of selected reference compounds and results obtained were correlated with results obtained in the OECD validated BG1Luc ER TA assay and in vivo uterotrophic assay. In **chapter 6**, the reproducibility and robustness of the coregulator binding assay was investigated by assessing the binding profiles of a set

of model compounds, listed in the U.S. EPA endocrine disruptor screening program test guideline (OPPTS 890.1300). **Chapter 7** describes an integrated in vitro testing panel for estrogenicity. This panel contains assays selected from previous chapters, but also includes in vitro assay for testing antiandrogenic properties and effects on steroidogenesis that might potentially also result in estrogenic or estrogen-like effects in vivo. Finally, **chapter 8** summarizes and discusses the results of the present thesis and provides future perspectives on endocrine disruption testing.

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2 Proliferation assays for estrogenicity testing with high predictive value for the in vivo uterotrophic effect

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Based on: The Journal of Steroid Biochemistry and Molecular Biology, 128, 98-106 (2012)

ABSTRACT

Proliferation assays based on human cell lines are the most used in vitro tests to determine estrogenic properties of compounds. Our objective was to characterise to what extent these in vitro tests provide alternatives for the in vivo Allen and Doisy test, a uterotrophic assay in immature or ovariectomised rodents with uterus weight as a crucial read-out parameter. In the present study four different human cell lines derived from three different female estrogen-sensitive tissues, i.e. breast (MCF-7/BOS and T47D), endometrial (ECC-1) and ovarian (BG-1) cells, were characterised by investigating their relative ERα and ERβ amounts, as the ERα/ERβ ratio is a dominant factor determining their estrogen-dependent proliferative responses. All four cell lines clearly expressed the ERα type and a very low but detectable amount of ERβ on both the mRNA and protein level, with the T47D cell line expressing the highest level of the ERβ type. Subsequently, a set of reference compounds representing different modes of estrogen action and estrogenic potency were used to investigate the proliferative response in the four cell lines, to determine which cell line most accurately predicts the effect observed in vivo. All four cell lines revealed a reasonable to good correlation with the in vivo uterotrophic effect, with the correlation being highest for the MCF-7/BOS cell line ($R^2 = 0.85$). The main differences between the in vivo uterotrophic assay and the in vitro proliferation assays were observed for tamoxifen and testosterone. The proliferative response of the MCF-7/BOS cells to testosterone was partially caused by its conversion to estradiol by aromatase or via androstenedione to estrone. It is concluded that of the four cell lines tested, the best assay to include in an integrated testing strategy for replacement of the in vivo uterotrophic assay is the human MCF-7/BOS breast cancer cell line.

INTRODUCTION

Reproduction toxicology is an important field of chemical hazard characterisation and management and in the light of REACH many compounds need to be investigated. Presently, the standard test for disruption of normal estrogen function is the Allen and Doisy test, a uterotrophic assay in immature or ovariectomised rodents with uterus weight as a crucial read-out parameter. [1, 2] The main advantage of the Allen and Doisy test is that it determines an overall biological effect by allowing for interactions between cells and between different components of the endocrine system thereby being able to detect complex modes of action that may occur only in the intact animal. However, this assay is labour-intensive, expensive and use of laboratory animals may raise ethical concerns. There are many alternative in vitro assays to determine the potential hormonal activities of compounds. These alternative in vitro assays were recently reviewed by Bovee and Pikkemaat, [3] showing that every assay type has its own specific advantages and disadvantages. From the cell-based assays, reporter gene assays based on either mammalian or yeast cells and proliferation assays based on human cell lines are among the most used. The E-screen is a proliferative assay based on the human MCF-7/BOS breast cancer cell line and has been used to determine the estrogenic characteristics of pesticides and alkyl phenols. [4, 5] However, the E-screen has certain drawbacks due to the fact that the MCF-7 cells also express androgen, progesterone, glucocorticoid and retinoid receptors. This may compromise drawing straightforward conclusions from the assay results when testing compounds for anti-estrogenicity or when testing complex mixtures or sample extracts for estrogenicity, which are able to activate these receptors, as it has been shown that androgens, progestins, and glucocorticoids can antagonise estradiol induced cell proliferation. [3, 6] In addition, breast cancer cell lines often respond differently to estrogens and anti-estrogens compared to endometrial cell lines, and even between different breast cancer cell lines the response to estrogens and anti-estrogens may be different. [3] A well-known example of a compound that displays different estrogen signaling activities in different cell models is the selective estrogen receptor modulator (SERM) tamoxifen. This compound is mainly known from its anti-proliferative effect on human breast cancer cells and its estrogenic effect in the in vivo uterotrophic assay, which is considered the gold standard for estrogenicity testing. Tamoxifen not only suppresses the growth of estrogen sensitive breast cancer cells, e.g. MCF-7 and MDA-MB-134, but is also able to induce proliferation in the E-screen, however, not in the MDA-MB-134 cell line. [7, 8]

In the present study a set of reference compounds was chosen and tested in a battery of in vitro proliferative assays in order to determine which cell line most accurately predicts the uterotrophic effect observed in vivo. Outcomes were therefore compared with the outcomes of the in vivo uterotrophic assay. Twelve reference compounds were chosen, including 17β-estradiol (E2), 17α-ethinylestradiol (EE2) and dienestrol as positive controls and corticosterone, progesterone and 17β-testosterone as negative controls. Dienestrol was added because this compound is only known as an estrogen receptor (ER) agonist, while EE2 is not only an ER agonist but also an androgen receptor (AR) antagonist and E2 is not only an ER agonist but also a partial AR agonist and AR antagonist. [9] Tamoxifen was chosen as a model compound for the selective estrogen receptor modulators (SERMs),

i.e. compounds displaying both ER agonistic and antagonistic properties, depending on the physiological context. Carbon tetrachloride (CCl_4) was chosen as a compound that not directly acts via the ER itself, but displays an in vivo estrogenic effect by elevating the endogenous E2 level, as it inhibits the metabolism of E2. [6] Epidermal growth factor (EGF) also displays an indirect mode of action. It activates a membrane receptor that eventually increases the phosphorylation of the ER, by which the latter becomes more active. [10] Besides the MCF-7/BOS cell line, used for the E-screen, another breast cancer cell line, T47D, was included in the study. The T47D cell line is not often used for proliferative assays, but it is the host cell in many developed transcriptional activation assays. [11-13] The ECC-1 cell line is an endometrium cell line that also proliferates under the influence of estrogens and was included because it is derived from a target tissue, which is actually examined in the in vivo uterotrophic assay. [14] In addition, the BG-1 cell line was included because it represents the ovary, another main female estrogen-sensitive tissue. [15]

MATERIALS AND METHODS

Chemicals

Estradiol (E2), dienestrol, zearalenone, corticosterone, tamoxifen, 4-hydroxytamoxifen, EGF and β-mercaptoethanol were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Ethynyl estradiol (EE2), progesterone and testosterone 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 from Acros Organics (Fisher Emergo B.V., Landsmeer, The Netherlands), carbon tetrachloride from Supelco Inc. (Bellefonte, PA, USA). Dimethylsulfoxide (DMSO), sodium chloride, Tween 20 and glycine were purchased from Merck (Darmstadt, Germany). Fetal bovine serum (FBS), charcoal-stripped fetal bovine serum (csFBS), ultrapure Tris, Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM (DMEM/F12) with L-glutamine and 15 mM HEPES and DMEM/F-12 with L-glutamine and 15 mM HEPES (without phenol red) were obtained from Gibco/Invitrogen (Breda, The Netherlands). Dulbecco's modified Eagle's medium (DMEM) was purchased from Lonza (Breda, The Netherlands). Methanol was from Biosolve (Valkenswaard, The Netherlands).

Cell lines and cell culture conditions

MCF-7/BOS human breast cancer cells were kindly provided by Dr. Ana M. Soto (Tufts University, Boston, MA, USA) and maintained in DMEM supplemented with 10% FBS. T47D human breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM/F-12 with glutamine and 15 mM HEPES supplemented with 10% FBS. ECC-1 human endometrial cancer cells were kindly provided by Dr. Leen J. Blok (Erasmus Medical Center, Rotterdam, The Netherlands) and cultured in DMEM/F12 medium with glutamine and 15 mM HEPES, without phenol red supplemented with 5% FBS. BG-1 human ovarian cancer cells were kindly provided by Dr. Kenneth S. Korach (NIEHS, National Institute of Health, Research Triangle Park, NC, USA) and

cultured in DMEM/F-12 with glutamine and 15 mM HEPES supplemented with 10% FBS. All cell lines are routinely maintained in 75 cm^2 canted-neck tissue culture flasks (Greiner, Gloucestershire, UK) in a humidified incubator at 37 °C and 5% CO_2. Cells were sub-cultured at approximately 80% confluence and regularly tested negative for mycoplasma.

Cell proliferation assays

Proliferation of the human MCF-7/BOS breast cancer cells for testing the estrogenic activity of a given compound was basically quantified as described by Soto et al. for the E-screen. [4] Briefly, cells were plated into 24-well plates at an initial density of 25,000 cells/well in 500 µl assay medium (DMEM/F12 medium, without phenol red supplemented with 5% csFBS) and allowed to attach for 24h. After 24h, the assay medium was renewed with fresh medium containing test compounds dissolved in DMSO (maximum 0.2%). DMSO and E2 (300 pM) were included in each plate as, respectively, a negative and positive control. Each compound concentration was tested in threefold and cells were incubated for 6 days. After 6 days the cells were trypsinised and harvested. The amount of cells was counted using a Coulter Counter (Beckman Coulter Mijdrecht, The Netherlands) and proliferation of the cells in percentages was determined by dividing the amount of cells exposed to the compound by the amount of cells exposed to DMSO control and subsequently multiplied by 100%.

Proliferation of the ECC-1 cells was quantified as described by Gielen et al. [14] with some minor modifications. Cells were cultured in assay medium (DMEM/F12 medium, without phenol red supplemented with 5% csFBS) for 5 days before starting the experiment. Next, cells were plated into 24-well plates at an initial density of 5,000 cells/well and allowed to attach for 24h. After 24h, the assay medium was renewed with fresh medium containing test compounds dissolved in DMSO (maximum 0.2%). DMSO and E2 (300 pM) controls were included in each plate. Each compound concentration was tested in threefold and cells were incubated for 7 days. After 7 days the cells were trypsinised and counted as described above for the MCF-7/BOS cells. Proliferation of the T47D and BG-1 cells were measured using the method from MCF-7/BOS assay with two modifications: T47D and BG-1 cells were plated into 24-well plates at an initial concentration of 50,000 and 5,000 cells/well, respectively and both cell lines were exposed for 5 days.

LDH cytotoxicity test

At the end of the proliferation assay, lactate dehydrogenase (LDH) released by damaged cells into the cell culture medium was determined using a LDH cytotoxicity detection kit (Roche Diagnostics, Germany), according to the manufacturer's instructions. Cells exposed to the highest concentration used in the proliferation assays were tested for LDH release. The cell culture medium was removed and centrifuged for 5 min at 800 × g. Aliquots of 100 µl of the centrifuged medium was transferred to a 96-well plate and LDH substrate (100 µl) was added to each well and incubated for 30 min at room temperature, protected from light. The absorbance of the samples was measured at 490 nm and corrected for background absorption at 630 nm.

RNA isolation and reverse transcription

Total RNA was isolated from cultured cells using the QIAshredder and RNeasy Mini kits (Qiagen, Venlo, The Netherlands) with RNase-free DNase treatment according to the manufacturer's protocols. In short: cells were seeded in 6-well plate in culture medium. When cells reached 80% confluence, the medium was removed and the cells were lysed in 600 µl RLT buffer with 1% β-mercaptoethanol. After extraction with QIAshredder and RNeasy Mini kits, the RNA concentration and purity were determined by absorbance at 260/280 nm using a NanoDrop spectrophotometer (Nanodrop Technologies, Montchanin, DE, USA). For cDNA synthesis, 0.5 µg of oligo(dT) primer (Invitrogen) and pd(N)6 random hexamer (GE Healthcare, Roosendaal, The Netherlands) were added to the sample prior to heating. The mix was placed directly on ice and 200 units of M-MVL reverse transcriptase, RNase H Minus, Point Mutant (Promega, Leiden, The Netherlands), 10 nmol of each dNTP and RT buffer were added to a final volume of 20 µl. The mix was incubated for 10 min at 25 °C, 50 min at 40 °C and finally 10 min at 65 °C to inactivate the enzyme. All cDNA samples were stored at -20 °C until assayed.

Quantitative PCR

Quantitative real-time PCR was performed with specific primers (listed in table 1) and was carried out in 25 µl reaction mixture containing 5 µl 20 times diluted cDNA, 12.5 µl of power SYBR green mix (Applied Biosystems, Maarssen, The Netherlands), 1 µl of each 10 µM primer and 5.5 µl of milliQ. QPCR measurements were performed with the ABI7900HT Sequence Detection System (Applied Biosystems) with the following conditions: 10 min denaturation at 95 °C followed by 40 cycles consisting of 65 °C for 1 min for annealing, 95 °C for 15 s for denaturation and extension at 65 °C for 1 min. PCR products were checked by melting curve analysis applying an increment of 0.5 °C per 5 s from 60 °C to 95 °C. Gene expression was assayed in triplicate for each sample and normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or heterochromatin protein 1 (HP-1) mRNA levels.

Western blotting

Cells were seeded in 6-well plate in culture medium. When cells reached 80% confluence, the medium was removed and the cells were lysed with M-PER mammalian extraction buffer supplemented with phosphatase and protease inhibitors (Thermo Fisher Scientific,

Table 1. Sequences of the primers used in Q-PCR.

Gene	Forward primer	Reverse primer
ERα	5'-CCTAACTTGCTCTTGGACAGGA-3'	5'-GCCAGCAGCATGTCGAAGAT-3'
ERβ	5'-CGACAAGGAGTTGGTACACATGA-3'	5'-CCAAGAGCCGCACTTGGT-3'
CYP19A1	5'-AGGTGCTATTTGTCATCTGCTC-3'	5'-TGGTGGAATCGGGTCTTTATGG-3'
HP-1	5'-CCCACGTCCCAAGATGGAT-3'	5'-CTGATGCACCACTCTTCTGGAA-3'
GAPDH	5'-AGGTCGGAGTCAACGGATTTGG-3'	5'-GCTCCTGGAAGATGGTGATGGG-3'

Etten-Leur, The Netherlands), according to the manufacturer's instructions. The protein concentration was determined according to Lowry, [16] using the BioRad DC protein assay (BioRad, Veenendaal, The Netherlands) and BSA as a standard. Next, 10 µg of the protein sample was mixed with an equal volume of Laemmli sample buffer (BioRad) supplemented with 5% β-mercaptoethanol. After denaturation at 95 °C for 3 min, 20 µl sample was loaded on 10% Mini-PROTEAN® TGX™ precast gel (BioRad). Electrophoresis was carried out at 50V for 30 min followed by 100V for 30 min using anode buffer (0.2M Tris-HCl, pH 8.9) and cathode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.2). Protein was transferred to PVDF membrane (Immobilon-FL, 0.45 µm pore size, Millipore B.V., Amsterdam Zuidoost, The Netherlands) at 100V for 1 h in Tris-glycine buffer (BioRad) containing 20% (v/v) methanol. Subsequently, membranes were blocked with Odyssey blocking buffer (LI-COR, Bad Homburg, Germany) for 1 h after which the membranes were probed overnight for proteins of interest with ERα, ERβ or β-actin primary antibodies (Cell Signaling, Bioké, Leiden, The Netherlands) diluted 1:1000 in Tris-buffered saline containing 1% Tween 20 (TBS-T) and 5% BSA. The following day the membranes were washed with TBS-T and incubated with infrared dye-labeled IRDye 800CW donkey anti-mouse IgG and IRDye 680 donkey anti-rabbit IgG (LI-COR). The final protein expression was detected using the Odyssey infrared imaging system (Westburg, Leusden, The Netherlands).

Aromatase enzyme assay

Aromatase activity in four different cell lines was measured by the tritiated water release assay, as previously described. [17] Briefly, cells were plated into 6-well plate at 1×10^6 cells/well in assay medium. Three days later, culture plate was washed with PBS. One ml of serum-free DMEM/F12 medium containing 50 nM [1β-^{3}H(N)]-androst-4-ene-3,17-dione (15–30 Ci/mmol, Perkin-Elmer Life Sciences) was then added to each well in triplicate. After 24h incubation at 37 °C, the reaction mixture was collected and extracted with two volumes of chloroform to extract unused substrate. After 2 min centrifugation at 11,000 × g, the aqueous phase was treated with an equal volume of dextran activated charcoal to eliminate residual steroids. After 15 min at 11,000 × g centrifugation, radioactivity was assessed by liquid scintillation counting. Aromatase activity was calculated as fmol estrogen formed/mg protein/h.

Data analysis

The relative proliferative potency (RPP), defined as the ratio of the concentration of E2 needed to achieve 50% of maximal cell yield and the dose of the test compounds required to achieve a similar effect and then multiplied by 100, was calculated from proliferation dose-response curves fitted using nonlinear regression analysis (sigmoidal dose-response curve, Graphpad Prism software version 5.04). The RPP value for E2 is thus set at 100. The relative proliferative effect (RPE) is defined as the maximal induction of cell proliferation by a compound relative to the maximal induction by E2 and is used to discriminate between full and partial estrogen receptor agonists. The log relative potency (logRP) values based on mouse or rat uterotrophic assay results were derived from the endocrine disruptor knowledge base (EDKB) designed and produced by the National Center for Toxicological Research (NCTR, USA). [18]

RESULTS

Characterisation of the cell lines

The estrogen receptor (ER) is present in the human body in two subtypes. The ERα subtype is mainly present in the sex organs, e.g. uterus, prostate (stroma), ovary (theca cells) and mammary gland, while the ERβ is mainly present in the colon, prostate (epithelium), testis, ovary (granulosa cells), bladder, lung and bone. [19-21] It is generally assumed that in cells expressing both receptor subtypes, the ERβ is a regulator/inhibitor of the ERα and thus affects the response of such cell types. [22-24] The relative levels of both receptor subtypes were therefore investigated at mRNA and protein level in the four human cell lines used in the present study, i.e. the MCF-7/BOS and T47D breast cells, the endometrial ECC-1 cells and the ovarian BG-1 cells. The aromatase enzyme catalyses the conversion of androgens to estrogens. It has been reported by Sonne-Hansen and Lykkesfeldt that the aromatase activity in the MCF-7 cells was sufficient to aromatise testosterone to estrogen, resulting in significant cell growth stimulation. [25] Therefore, in addition to ER levels, aromatase activity was characterised as well in each of the four cell line at the mRNA level and by measuring activity.

ERα, ERβ and aromatase mRNA levels

Total RNA was isolated from each of the four cell line and the levels of ERα, ERβ and aromatase mRNA were determined by quantitative real-time PCR analysis using specific primers for each ER subtype and the aromatase gene. Figure 1 shows the ERα, ERβ and aromatase mRNA levels as determined in the four human cell lines. Figure 1A shows that, compared to the MCF-7/BOS cell line, the T47D, ECC-1 and BG-1 cell lines have about 1.5 to 2.0 times higher ERα mRNA amounts and that the T47D cell line expresses a relative high amount of the ERβ

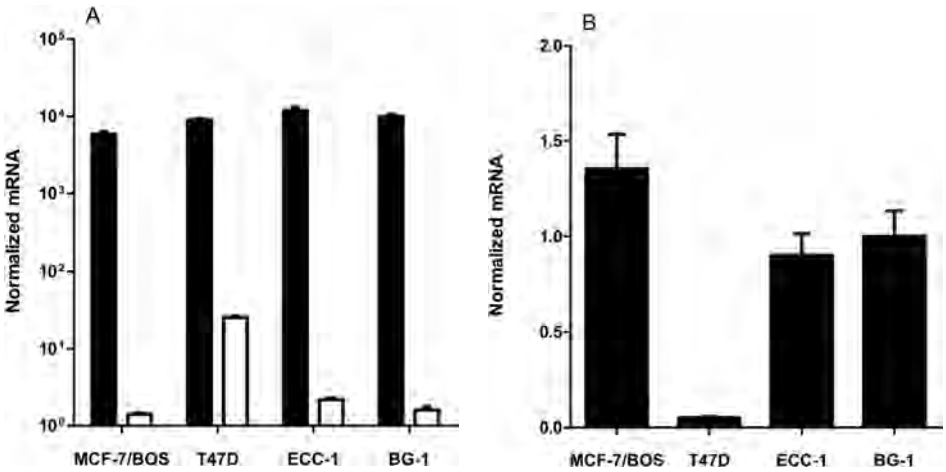

Figure 1. Relative estrogen receptor and aromatase mRNA expression measured in MCF-7/BOS, T47D, ECC-1 and BG-1 cells. (A) ERα (black column) and ERβ (white column) expression are normalised to HP-1 mRNA. (B) aromatase expression is normalised to GAPDH mRNA. Average fold change and standard deviation were calculated from three biological replicate samples, each measured in triplicate.

mRNA. Figure 1B shows that the MCF-7/BOS, ECC-1 and BG-1 cell lines have relatively high levels of the aromatase mRNA, while the T47D cell line hardly expresses this mRNA.

Western blotting and aromatase activity

Levels in mRNA not necessarily reflect the actual protein levels. Additional western blots were therefore performed for ERα and ERβ and an enzyme assay was carried for detecting aromatase activity. Figure 2 shows the western blots and demonstrates that the four human cell lines clearly express the ERα protein and small but detectable amounts of ERβ protein. Figure 3 shows the aromatase activity as determined in the four cell lines. A similar pattern was observed as obtained for the aromatase mRNA levels, with only the aromatase activity in the ECC-1 cell line being about 2-fold higher than what would be expected based on the mRNA level.

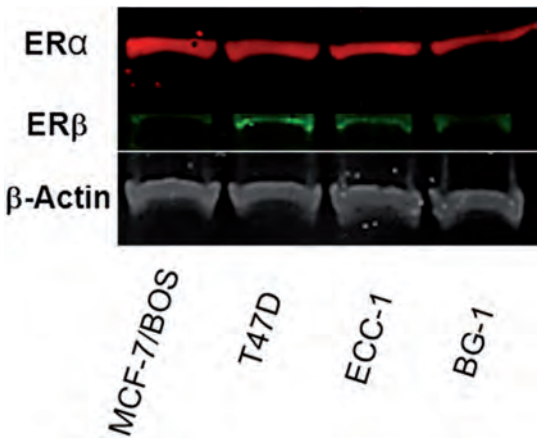

Figure 2. ERα and ERβ protein expression measured in MCF-7/BOS, T47D, ECC-1 and BG-1 cells. Upper part shows a Western blot of a total protein extract after treatment with ER antibodies, and the lower part the same blot after treatment with β-actin antibodies to show that equal amounts of protein had been loaded for each cell sample.

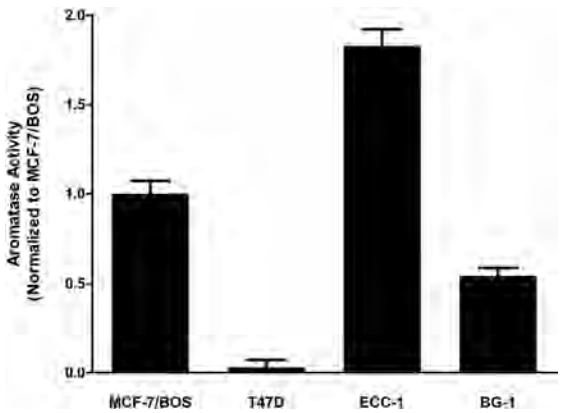

Figure 3. Aromatase enzyme activity measured in MCF-7/BOS, T47D, ECC-1 and BG-1 cells. Aromatase activity was measured as described in materials and methods. Results are presented as the mean aromatase enzyme activity ± SD (n=3) expressed as fmol estrogen formed/mg protein/h.

Cell proliferation assays

Figure 4 shows the dose–response curves of the natural estrogen 17β-estradiol (E2) as obtained in proliferation assays with the four different human cell lines derived from three different female estrogen-sensitive tissues. The maximal response (expressed as % of the DMSO control) was reached at 300 pM to 1 nM E2 for all four cell lines. The fold increase in cell proliferation ranged from 2.5 times for the endometrial ECC-1 cell line to 8 times for the T47D breast cell line. However, this fold increase is relative to a DMSO control and does not necessarily reflect the absolute increase in cell numbers. Although the relative fold increase of the T47D cell line seems high compared to the ECC-1 cell line, it should be emphasised that the T47D cell line is the only one of the tested cell types that essentially shows no proliferation in the absence of estradiol. At higher E2 concentrations a slight inhibition of the cell growth was observed with all four cell lines. This was not due to cytotoxicity, as no LDH leakage was observed at the highest concentrations of E2 tested (data not shown). The EC_{50}, i.e. the E2 concentration giving a half-maximum proliferation response, was calculated as a mean ± SEM from at least five independent experiments and resulted in EC_{50} values of 25 ± 4.3 pM, 41 ± 5.2 pM, 15 ± 1.5 pM and 9 ± 2.8 pM for the MCF-7/BOS, T47D, ECC-1 and BG-1 cell line, respectively.

A set of 12 reference compounds, including E2, was chosen and their selection was based on their different modes of action leading to positive and negative outcomes in the in vivo uterotrophic assay (Allen and Doisy test). The RPP and RPE values of these 12 compounds as calculated from the fitted proliferative responses on the four human cell lines are listed in Table 2. No LDH leakage was observed at the highest concentrations of the compounds tested (data not shown). To allow comparison with the observed in vivo effects, the relative potency (RP) of these compounds as determined previously in the in vivo mouse or rat uterotrophic assay are included and shown in the second column of Table 2. Figure 5 shows the comparison between the logRP values as determined in the in vivo mouse or rat uterotrophic assay and the logRPP values as determined in the in vitro proliferation assays using the four different human cell lines. From these data it becomes

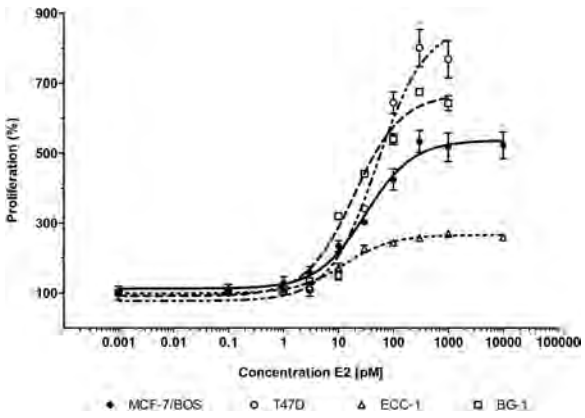

Figure 4. Proliferative response of 17β-estradiol obtained in the four estrogen-sensitive human cell lines. Results are representative of at least five independent experiments and are presented as the mean proliferative response ±SD (n=3) expressed as a percentage of the solvent (DMSO) control.

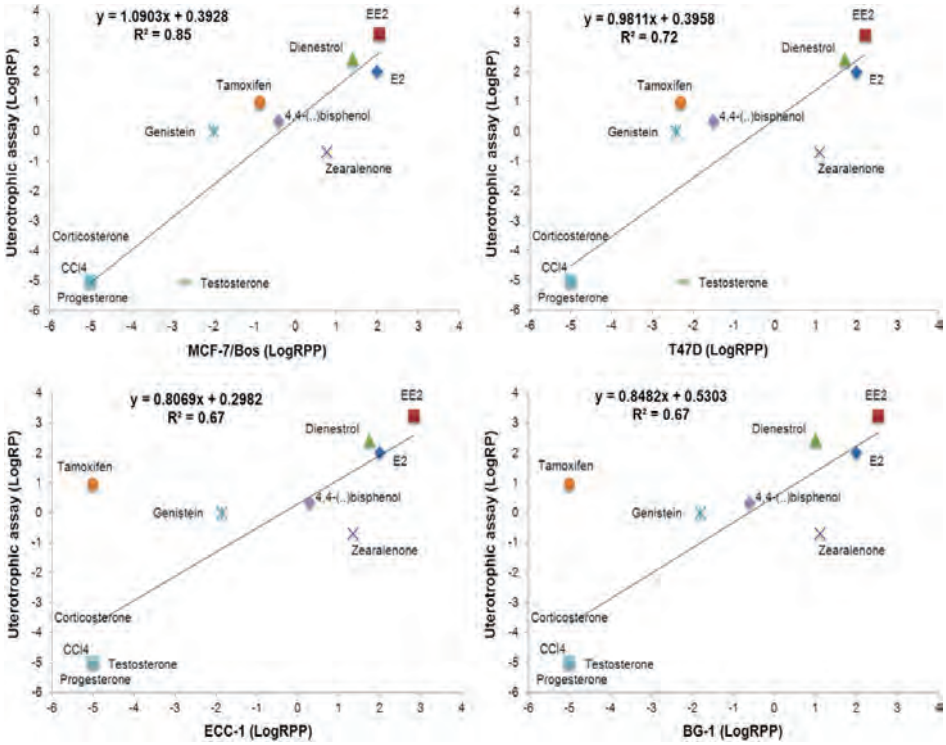

Figure 5. Comparison of the in vivo uterotrophic assay with the proliferation assays using four different estrogen-responsive human cell lines. With tamoxifen excluded, the ECC-1 and BG-1 cell lines show a $R^2 = 0.92$, y = 0.97x+0.06 and $R^2 = 0.91$, y = 1.02x+0.34, respectively.

clear that the estrogens E2, EE2, dienestrol, zearalenone, genistein and 4,4'-(octahydro-4,7-methano-5h-inden-5-ylidene) bisphenol show similar relative potencies in the different proliferation assays. The negative controls, i.e. the androgen testosterone, the (pro)gestagen progesterone and the (glycol)corticoid corticosterone, are almost inactive in all five assay types. The only exception is testosterone, as a full dose response curve was obtained for testosterone in the MCF-7/BOS proliferation assay and also a clear response with T47D cell line, while EGF could not be compared as the in vivo data for this compound are yet unknown. However, EGF was clearly active in the proliferation assays, showing the highest potency in the BG-1 cell line (logRPP 1.4) and the most extensive proliferative effect in the ECC-1 cell line (RPE of 65). In addition, a MCF-7/BOS proliferation study with testosterone and the aromatase inhibitor letrozole was performed. Figure 6 shows that letrozole partially inhibited the proliferative response induced by testosterone.

The main differences between the in vivo and in vitro assays were observed for tamoxifen. This compound is clearly estrogenic in vivo (logRP of 0.9), but is almost inactive in the proliferation assays using the ECC-1 and BG-1 cell lines (logRPP of -5.0). Tamoxifen was clearly estrogenic in the proliferation assays using the MCF-7/BOS and, to a lesser

Table 2. Comparison of the relative potencies obtained in the in vivo uterotrophic assay with those obtained in proliferation assays using four human cell lines derived from three female estrogen sensitive tissues.

	Uterotrophic assay	MCF-7/BOS		T47D		ECC-1		BG-1	
Compounds	**logRP[a]**	**logRPP[b]**	**RPE[c]**	**logRPP**	**RPE**	**logRPP**	**RPE**	**logRPP**	**RPE**
Estradiol	2.0	2.0	100	2.0	100	2.0	100	2.0	100
Ethinyl estradiol	3.2	2.0[e]	92[e]	2.2	93	2.8	103	2.5	103
Dienestrol	2.4	1.4	99	1.7	92	1.7	94	1.0	98
Zearalenone	-0.7	0.7[e]	100[e]	1.1	110	1.3	100	1.1	96
Genistein	-2.7	-2.0	98	-2.4	84	-1.8	96	-1.8	108
4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol	0.3[d]	-0.4	47	-1.5	18	0.3	50	-0.6	38
Tamoxifen	0.9	-0.9	36	-2.3	19	-5.0	-	-5.0	-
EGF	uterotrophic effect[f]	-1.2	24	0.9	16	-0.4	65	1.4	36
Carbon tetrachloride	-5.0	-5.0	-	-5.0	-	-5.0	-	-5.0	-
Corticosterone	-5.0	-5.0	-	-5.0	-	-5.0	-	-5.0	-
Progesterone	-5.0	-5.0	-	-5.0	-	-5.0	-	-5.0	-
Testosterone	--5.0	-2.9	92	-2.2	35	-5.0	<5	-5.0	<15

[a] Relative potency values based on mouse or rat uterotrophic assay results, derived from EDKB (NCTR,USA). E2 is used as a reference chemical and is defined to have a relative potency of 100 ($\log_{10}100=2$) [18]. A logRP cut-off value of -5.0 is listed for compounds showing no effect.
[b] Relative proliferative potency values are obtained from the ratio of the concentration of E2 needed to achieve 50% of maximal cell yield (proliferation) and the dose of the test compounds required to achieve a similar effect, and this ratio is subsequently multiplied by 100. The RPP value of E2 is thus 100, resulting in a logRPP of 2 ($\log_{10}100=2$). A cut-off value of -5.0 is listed for compounds showing no effect.
[c] Relative proliferative effect values are defined as the maximal cell proliferation induced by a compound relative to the maximal cell proliferation obtained with E2.
[d] logRP of 4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol was calculated from a minimal active dose described by Yamasaki et al. 2003. [26]
[e] Obtained from Fang et al. 2000. [27]
[f] EGF is able to mimic the uterotrophic effects of estrogen in the rodent. [28]

extent, the T47D breast cancer cell lines (logRPP of -0.9 and -2.3, respectively). The best correlation with the in vivo uterotrophic assay was therefore obtained with the human MCF-7/BOS breast cancer cell line ($R^2 = 0.85$).

Tamoxifen is not a pure estrogen receptor agonist or antagonist, but is a selective estrogen receptor modulator (SERM) and mainly reported to act as an ER agonist in uterus and as an ER antagonist in breast. [29] However, tamoxifen is also able to inhibit the effect caused by EE2 in the uterotrophic assay and to induce proliferation in the E-screen. [26, 27] In order to select the cell line showing proliferative responses with the best correlation with the outcomes of the in vivo the uterotrophic assay, the antagonistic properties of several

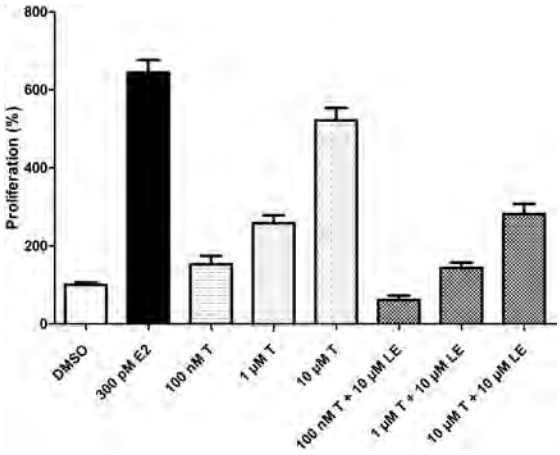

Figure 6. Effect of the aromatase inhibitor letrozole (LE) on testosterone induced cell proliferation in MCF-7/BOS cells. Results are representative of at least three independent experiments and are presented as the mean proliferative response ±SD (n=3) expressed as a percentage of the solvent (DMSO) control.

compounds were studied as well. Figure 7 shows the proliferative responses of the four cell lines upon exposure to 300 pM E2 giving 80-100% proliferation rate, in combination with different concentrations of tamoxifen. These results clearly show that tamoxifen acts as an ER antagonist in all four cell lines too. The antagonistic or additive effects of tamoxifen, 4-hydroxytamoxifen, 4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol, CCl_4 and EGF with respect to E2 are summarised in Table 3. The results presented reveal that in combination with E2, tamoxifen, 4-hydroxytamoxifen and 4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene) bisphenol all acted as antagonists both in vivo and in vitro, while CCl_4 and EGF only in vivo resulted in effects that were additive to those of E2.

DISCUSSION

A set of 12 reference compounds representing diverse modes of estrogenic action and chemical classes were tested in four cell proliferation assays in order to establish which cell line most accurately predicts the in vivo observed effects. These four cell lines were derived from three different female estrogen-sensitive tissues: i.e. breast (the MCF-7/BOS and T47D cell lines), endometrium (the ECC-1 cell line) and ovary (the BG-1 cell line). These cell lines were first characterised with respect to their relative amounts of ERα and ERβ both at the mRNA and protein level, as it is known that the relative ERα/ERβ level affects the proliferation response of cells that express both receptor types. [22-24] All four cell lines clearly expressed the ERα type and a very low but detectable amount of ERβ at both the mRNA and protein level. The results conform to the expectations, as the ERα is known to be the predominant ER type expressed in these estrogen-sensitive tissues, [19-21] and in agreement with the general findings that most ER-positive tumours appear to exhibit increased ratios of ERα/ERβ, due to lower expression of ERβ. [30, 31] Given that the rat and mouse uterus are known to predominantly express the ERα and almost no ERβ, [32, 33] these data on the relative expression levels of ERα and ERβ in the four cell lines investigated indicate that the T47D

Table 3. Reference compounds tested for antagonistic or additive effect on proliferation of the four human cancer cell lines upon exposure in combination with the prototype ER agonist E2.

Compounds	Behaviour of test compounds in uterotrophic assay	MCF-7/BOS IC_{50}	T47D IC_{50}	ECC-1 IC_{50}	BG-1 IC_{50}
Tamoxifen+E2	Antagonist	670 nM	650 nM	220 nM	240 nM
4OH-TAM+E2	Antagonist	18 nM	5.2 nM	6.5 nM	2 nM
4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol+E2	Antagonist	400 nM	230 nM	390 nM	130 nM
CCl_4+E2	Enhanced [a]	NE [b]	NE	NE	NE
EGF+E2	Enhanced	NE	NE	NE	NE

[a] Enhanced uterotrophic effect as compared to E2 alone.
[b] No antagonistic or additive effect is observed on cell proliferation as compared to E2 alone.

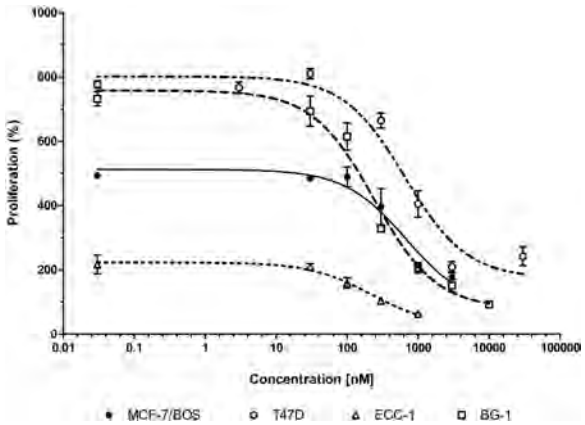

Figure 7. Antagonistic effect of tamoxifen measured in MCF-7/BOS, T47D, ECC-1 and BG-1 cells. Cells were treated with 300 pM 17β-estradiol in combination with different concentrations of tamoxifen. Results are expressed as the mean proliferative response ±SD (n=3) presented as a percentage of the solvent (DMSO) control.

breast cancer cell line, with its relatively high ERβ levels, may be less suitable as a member of a panel of bioassay to replace the in vivo uterotrophic assay. It is important to note that it has been reported that long term estrogen deprivation can affect estrogen receptor levels in breast cancer cells, i.e. after culturing cells in estrogen free medium for several months. [34] In the present study the proliferation assays are performed by culturing cells in estrogen free medium for 24 h or 5 days before exposure to the test compounds, and it cannot be fully excluded that during the prolonged pre-treatment of the ECC-1 cells, receptor levels may have changed somewhat more than in the other cell lines. However, given that all compounds in all cell lines were compared on a relative scale with estradiol used as the standard, the influence on the ultimate outcome is expected to be limited if any.

The logRPP values of the tested compounds were calculated from the dose–response relation determined by curve-fitting and they showed a good correlation with logRP values as obtained in the in vivo uterotrophic assay. Surprisingly, the best correlation between the in vivo uterotrophic assay and a proliferation assay was obtained with the MCF-7/BOS breast cell line

and not with the ECC-1 endometrial cell line. The highest R^2 value (0.85) was thus obtained with the MCF-7/BOS proliferation assay. Compounds on the line, figure 5, have about the same relative potency in the in vitro proliferation assay as obtained in the in vivo uterotrophic assay, compounds clearly above the line are relatively more potent in vivo than in vitro, while compounds below the line are relatively more potent in the in vitro proliferation assays than in the in vivo uterotrophic assay. Deviations from the line have different causes, as will be discussed for testosterone and tamoxifen. Although the T47D cell line expresses a similar ERα and a much lower level of ERβ but still relatively high compared to the other three cell lines, the relative proliferative potencies observed for T47D do not much differ from the other cell types that express almost no ERβ. However, the fact that the T47D cell line showed the highest EC_{50} for E2 and was not able to grow in the absence of estrogens, while the other cell lines were able to grow in assay medium (the amount of ECC-1 cells in the DMSO control even increased more than 10 times) might be due to the inhibitory effect of the relatively high levels of ERβ on the ERα activity in the T47D cell line as compared to the other cell lines. Testosterone was inactive in the in vivo uterotrophic assay and slightly active in ECC-1 and BG-1 cell proliferation assays, but induced a clear proliferative response in the MCF-7/BOS and T47D cells. In theory this proliferative response to testosterone might be due to aromatase activity in the latter two cell types. To test this hypothesis the four cell lines were characterised by determination of their aromatase mRNA levels and by aromatase activity measurements. The mRNA levels correlated well with the determined aromatase activities and the observed aromatase activity in the MCF-7/BOS cells might explain its proliferative response to testosterone. However, the aromatase inhibitor letrozole only partially inhibited the proliferative response as obtained with testosterone in MCF-7/BOS cells. In addition, aromatase activity was not observed in T47D cells, but still testosterone induced a clear response. These results indicating that other or combined modes of action underlay the proliferative effect of testosterone in the MCF-7/BOS and T47D cell proliferation assays. For instance, the proliferative effect of testosterone could be due to the formation of other estrogenic metabolites than estradiol and estrone, i.e. androstenediol, hydroxytestosterone or dehydroepiandrosterone, which might also activate ER and induce cell proliferation. On the other hand, the proliferative effect of testosterone in the T47D assay might be caused by crosstalk from the androgen receptor (AR) as it is known that the T47D expresses a high amount of AR. [13, 35, 36] The ECC-1 and BG-1 cells show clear aromatase activities, but did not give clear proliferative response when exposed to testosterone. The reason for this unexpected observation remains to be elucidated.

The main reason for the best correlation with the MCF-7/BOS cell line is due to the outcomes with tamoxifen. Tamoxifen is a selective estrogen receptor modulator (SERM), depending on the cell tissue type and the intrinsic E2 levels, it can act both as an agonist or an antagonist. [29] In order to select the cell line showing proliferative responses with the best correlation with the outcomes of the in vivo uterotrophic assay, the antagonistic properties of tamoxifen in these assay types were studied and compared as well. The results obtained showed that tamoxifen acted as an ER antagonist in all four cell lines and also in vivo, whereas the ER agonistic properties of tamoxifen, also observed in the uterotrophic assay, are only revealed by the MCF-7/BOS and T47D cell lines. This means that the ECC-1 and BG-1

cell lines are only capable to detect the antagonistic properties of tamoxifen. It is at least surprising that in the uterotrophic assay tamoxifen is a strong ER agonist and is not active as an agonist in the ECC-1 cell line, which is actually derived from uterus tissue (endometrium). The differences observed with tamoxifen might be due to differences in cofactor (repressor) concentrations in the different cell types, however, even today and despite the fact that tamoxifen is already used as a drug for more than three decades, its mechanism of action is still not fully understood. [29, 37] Differences in metabolism might be another explanation for the differences observed with tamoxifen, as it is known that tamoxifen is converted in vivo into the more active 4-hydroxytamoxifen (4OH-TAM) and 4-hydroxy-N-desmethyl-tamoxifen (endoxifen). It has been reported that 4OH-TAM possesses a high affinity for ERs and 30- to 100-fold more antagonist potency than tamoxifen in suppressing estrogen-dependent cell proliferation. [38] Several studies have shown that endoxifen is equipotent to 4OH-TAM with respect to inhibition of estradiol induced cell proliferation in vitro. [39, 40] If tamoxifen was metabolised to a substantial amount to 4OH-TAM or endoxifen in the four cell lines, exposure of the cells to tamoxifen and 4OH-TAM would have resulted in similar IC_{50} values for the two compounds when testing the antagonistic activity. However, when tested alone 4OH-TAM was only active as an agonist in the MCF-7/BOS and T47D cell line but not active in the ECC-1 and BG-1 cell line (data not shown). In addition, our results show that 4OH-TAM was 30-150 times more potent as an antagonist than tamoxifen in the four cells lines. Thus, taken together these data indicate that conversion of tamoxifen to 4OH-TAM or endoxifen does not occur in the cell lines tested and cannot explain the deviating behaviour of tamoxifen in the various cell lines. The in vitro/in vivo discrepancy observed with tamoxifen and 4OH-TAM is most likely due to the differences in the expression and/or activity of co-regulators in the different cell lines. It is worth noting that 4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol shows both agonistic and antagonistic effects in the uterotrophic assay and when tested in the proliferation assays it also behaves as an agonist and antagonist, demonstrating a nearly identical biological effect profile as tamoxifen. This might indicate that it is to be expected that broad window screening of unknown compounds might involve many more cases of partial agonists/antagonists and/or SERMs, which an ideal assay battery should be capable to correctly classify with respect to estrogenic properties. This supports the notion that in the end it might require more than one cell type to correctly classify estrogens in general, including the SERM class, because of the mechanistic factors in addition to just ER-interaction involved in the estrogenic action of SERMs.

In conclusion, the MCF-7/BOS proliferation assay showed the best correlation with the in vivo uterotrophic assay and based on the 12 compounds tested in this study, was shown to be suited to be part of a panel of in vitro bioassays to replace this in vivo test. However, there are still compounds that cannot correctly be predicted such as the additive effect of carbon tetrachloride and EGF. Other in vitro assay formats, such as transcription activation assays based on cells that express no endogenous hormone receptors in order to avoid crosstalk or the H295R steroidogenesis assay in order to detect estrogenic effects of compounds that are not caused by a direct interaction with the ERα, are needed in such a panel of assays to increase its predictivity for the outcomes as observed in the uterotrophic assay.

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3 Towards an integrated in vitro strategy for estrogenicity testing

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Submitted for publication to Journal of Applied Toxicology

ABSTRACT

In order to define an in vitro integrated testing strategy (ITS) for estrogenicity, a set of 23 reference compounds representing diverse chemical classes, were tested in a series of in vitro assays including proliferation and reporter gene assays. Outcomes of these assays were combined with published results for estrogen receptor (ER) binding assays and the OECD validated BG1Luc ER transcriptional activation (TA) assay and compared with the outcomes of the in vivo uterotrophic assay to investigate which assays most accurately predict the in vivo uterotrophic effect and to identify discrepancies between the in vitro assays and the in vivo uterotrophic assay. All in vitro assays used revealed a reasonable to good correlation (R^2 = 0.62 - 0.87) with the in vivo uterotrophic assay but the combination of the yeast estrogen bioassay with the U2OS ERα-CALUX assay seems most promising for an ITS for in vitro estrogenicity testing. The main outliers identified when correlating data from the different in vitro assays and the in vivo uterotrophic assay were 4-hydroxytamoxifen, testosterone and to a lesser extent apigenin, tamoxifen and kepone. Based on the modes of action possibly underlying these discrepancies it becomes evident that to further improve the ITS and ultimately replace animal testing for (anti-)estrogenic effects, the selected bioassays have to be combined with other types of in vitro assays, including for instance in vitro models for digestion, bioavailability and metabolism of the compounds under investigation.

INTRODUCTION

Testing chemicals for their endocrine-disrupting potential, including interference with estrogen receptor (ER) signaling, is an important aspect to assess the safety of currently used chemicals. With the vast number of chemicals potentially targeted for endocrine-disruption testing, millions of animals are needed under the U.S. Environmental Protection Agency (EPA) Endocrine Disruptor Screening Program (EDSP) [1] and the European Union (EU) legislation on Registration, Evaluation, Authorization and Restriction of chemical substances (REACH). [2] In order to fulfill the 3R principle of Refinement, Reduction and Replacement of animal testing and reduce the costs, there is a clear need to develop high-throughput in vitro methods to efficiently screen chemicals and prioritize limited testing resources.

Presently, the standard test for disruption of normal estrogen function is the Allen and Doisy test, a uterotrophic assay in immature or ovariectomised rodents with uterus weight as a crucial read-out parameter. [3, 4] As alternatives, a variety of in vitro bioassays has been developed to determine the potential estrogenic activities of compounds based on different steps in the estrogen receptor-dependent signal transduction pathway, e.g., ER binding, ER-controlled reporter gene expression or more downstream events such as ER-mediated cell proliferation. These in vitro assays have been shown to produce relevant and reliable outcomes. Sonneveld et al. (2006), for example, reported that the results obtained with the human U2OS osteosarcoma cell based reporter gene assay correlate well with the results from the in vivo uterotrophic assay for a set of 31 steroids. [5] In addition, Kolle et al. (2010) compared experimental data from a yeast estrogen screen (YES) assay with the literature data from the uterotrophic assay and showed that the YES assay has a very high concordance (87%) for predicting estrogenic effects in vivo. [6] Moreover, in a previous study, it was demonstrated that of four different proliferation assays, data obtained in the MCF-7/BOS proliferation assay correlate better with the data from the in vivo uterotrophic assay than the proliferative responses of three other human cell lines, i.e., another cell line derived from breast (T47D), one derived from the endometrium (ECC-1) and one from the ovary (BG-1). [7] These results indicate that in vitro assays may have high predictive value for in vivo estrogenic effects. However, outliers do occur, and this is due, at least to some extent, to the fact that these assays are generally based on a single estrogen-responsive human cell type cultured in vitro, and do not reflect the biological complexity of a whole animal, missing e.g. influences of bioavailability, metabolism or effects on steroidogenesis. In addition, a variety of cell types are used as hosts for different in vitro assays, including mammalian and yeast cells. This complicates the interpretation of the test results as the same compound may act differently and even show contradictory results in different in vitro assays. The aim of the present study was to further investigate which in vitro assays most accurately predict the in vivo observed uterotrophic effect and to identify the discrepancies between in vitro data and results from the in vivo uterotrophic assay, in order to better define gaps that have to be bridged by additional in vitro tests when developing an in vitro integrated testing strategy (ITS) for estrogenicity.

To this end, a set of 23 reference compounds was chosen and tested in the MCF-7/BOS proliferation assay and a battery of reporter gene assays. The MCF-7/BOS proliferation assay was chosen as it has been shown to correlate the best of a series of 4 proliferation assays with

the in vivo uterotrophic outcomes based on data for a set of 12 compounds. In the current study, this assay was further evaluated using a larger set of 23 compounds. The ER-CALUX assay based on the human T47D breast carcinoma cell was chosen as a model, because this cell line endogenously expresses both estrogen receptor α and β, [8] and therefore is likely to be more representative for the uterus and other estrogen-responsive human tissues expressing both ER subtypes. For comparison purposes, the ERα-CALUX assay based on the human U2OS osteosarcoma cell line stably transfected with an ERα expression vector and a luciferase reporter construct [9, 10] was chosen and added to test panel. Compared to mammalian cell lines, yeast cells are easy to culture. In addition, the simplicity of yeast-based assays can be an advantage when studying the estrogenic effect of chemicals as yeast-based assays do not suffer from crosstalk from other nuclear receptors and are devoid of steroid metabolism. Therefore, the third assay included in the present study was an estrogen bioassay based on yeast cells expressing yeast Enhanced Green Fluorescent Protein (yEGFP) as a marker protein. [11] In these cells both the human estrogen receptor α (hERα) expression and yEGFP reporter construct were stably transfected into the genome. Finally, the published results of ER binding assays and the OECD-validated BG1Luc ER TA reporter gene assay were also included and compared with the outcomes as obtained in the selected in vitro assays and in the in vivo uterotrophic assay. Special attention was directed at possible outliers and their possible mode of action since this was expected to provide further insight into possible remaining gaps and thus future assays required for further improvement of an in vitro ITS for estrogenicity.

MATERIALS AND METHODS

Chemicals

Apigenin, bisphenol A (BPA), corticosterone, coumestrol, dienestrol, diethylstilbestrol (DES), diphenylamine, 17ß-estradiol (E2), ethyl paraben, *meso*-hexestrol, zearalenone, tamoxifen, 4-hydroxytamoxifen, *o,p*'-DDT, *p*-n-nonylphenol, type I calf thymus DNA (sodium salt) and 1,2-diaminocyclohexanetetraacetic acid monohydrate were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). 17α-Ethinyl estradiol (EE2), progesterone (P4) and testosterone (T) were purchased from Steraloids (Newport, RI, USA), while genistein was obtained from Apin Chemicals (Abingdon, Oxon, UK). Acetaldehyde and 4,4'-(octahydro-4,7-methano-5h-inden-5-ylidene)bisphenol (OMIY-bisphenol) were from Acros Organics (Fisher Emergo B.V., Landsmeer, The Netherlands). 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). Dimethylsulfoxide (DMSO) and perchloric acid were purchased from Merck (Darmstadt, Germany), d-Luciferin from Duchefa (Haarlem, The Netherlands) and ATP from Roche (Mannheim, Germany). Dextran-coated charcoal-filtered fetal bovine serum (DCC-FBS), fetal bovine serum (FBS), ultrapure Tris, Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM (DMEM/F12) with l-glutamine and 15 mM HEPES and DMEM/F-12 with L-glutamine and 15 mM HEPES (without phenol red) were obtained from Gibco/Invitrogen (Breda, The Netherlands), while Dulbecco's

modified Eagle's medium (DMEM) was purchased from Lonza (Breda, The Netherlands). Chemicals to prepare the growth media for yeast were described previously [11].

Cell culture

The MCF-7/BOS human breast cancer cell line was kindly provided by Dr. Ana M. Soto (Tufts University, Boston, Massachusetts, USA) and was cultured in DMEM supplemented with 10% FBS. The T47D.Luc cell line, as described by Legler et al., [8] is a reporter gene variant of the estrogen-responsive human T47D breast carcinoma cell line, stably transfected with a pEREtata-Luc construct. The T47D cells were maintained in DMEM/F-12 supplemented with 7.5% FBS and non-essential amino acids solution (Sigma-Aldrich). The ERα U2OS. Luc cell line, provided by BioDetection Systems B.V. (Amsterdam, The Netherlands), is a human U2OS line stably co-transfected with an expression construct for the human ERα (pSG5-neo-hERα) and a pGL3 (Promega)-based reporter construct containing 3 EREs and a TATA box in front of a *luc*+ luciferase reporter gene(pGL3-3xERE*tata*Luc) as described by Sonneveld et al. [9] The U2OS cells were cultured in DMEM/F-12 supplemented with 7.5% FBS, 200 µg/mL geneticin and 50 µg/mL hygromycin. All cell lines were routinely maintained in 75 cm^2 canted-neck tissue culture flasks (Greiner, Gloucestershire, UK) in a humidified incubator at 37 °C and 5% CO_2. Cells were sub-cultured at approximately 80% confluence and tested negative for mycoplasma.

MCF-7/BOS cell proliferation assay

Proliferation of the human MCF-7/BOS breast cancer cells for estrogenicity testing was performed and quantified as described previously, but with some modifications. [12] Briefly, cells were cultured in assay medium (DMEM/F12 medium, without phenol red supplemented with 5% csFBS) for 3 days before starting the experiment. Next, cells were plated into 96-well plates at an initial density of 2,000 cells/well in 200 µL assay medium. After 24 h, the assay medium was renewed with fresh medium containing test compounds dissolved in DMSO (maximum 0.2% DMSO, final concentration). Each test compound at each concentration was tested in quadruplicate. After 6 days of exposure, the amount of DNA per well, a measure of the amount of cells, was assayed using a microplate modification of the Burton diphenylamine assay. [13, 14]

ER-CALUX reporter gene assays

Human cell-based CALUX reporter gene assays were carried out as described previously. [8] In short, T47D.Luc and ERα U2OS.Luc cells were plated in 96-well plates with DMEM/F12 medium without phenol red and supplemented with 5% DCC-FBS for 48 h with a refreshment of the medium after 24 h. At 48 h post plating, the medium was refreshed and cells were incubated with the test compounds added from a stock solution in DMSO (maximum 0.2% DMSO, final concentration). Each test compound at each concentration was tested in quadruplicate. After 24 h of exposure, media was removed, cells were washed with 0.5 x PBS and were lysed with 30 µL of hypotonic low-salt buffer (10 mM Tris, 2 mM DDT, and 2 mM CDTA; pH 7.8). Plates were put on ice for 15 min to allow swelling of the cells and subsequently frozen at -80 °C for at least 30 min to lyse the cells. Plates were thawed on ice and shaken

for 2 min at room temperature. Luciferase activity in cellular lysates was measured with a Labsystems Luminoskan RS ® luminometer (Thermo Electron, Breda, The Netherlands) by adding 100µl flash mix (20 mM Tricine, 1.07 mM $(MgCO_3)4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 2.0 mM dithiothreitol, 470 µM luciferine, 5.0 mM ATP) per well.

Yeast estrogen bioassay

The yeast cytosensor expressing the human estrogen receptor α (hERα) and yeast enhanced green fluorescent protein (yEGFP) in response to estrogens was developed in-house and the assay was performed as described previously. [11, 15] In short, the day before running the assay, a single colony of the yeast ERα cytosensor was used to inoculate 10 mL of minimal medium supplemented with 60mg/L L-leucine (MM/L medium). This culture was grown overnight at 30 °C with vigorous orbital shaking at 225 rpm. At the late log phase, the yeast ERα cytosensor was diluted in the selective MM/L medium to an optical density (OD) value at 630 nm between 0.04 and 0.06. Subsequently, aliquots of 200 µL of the diluted yeast culture were pipetted into each well of a 96-well plate and 2 µL of test compound from a stock solution in DMSO (maximum 0.2% DMSO, final concentration) was added in triplicate. Exposure was performed for 24 h at 30 °C and orbital shaking at 125 rpm. Fluorescence and OD were measured at 0 and 24 h directly in a Synergy™ HT Multi-Detection Micro-plate Reader (BioTek Instruments Inc., USA) using excitation at 485 nm and measuring emission at 530 nm. The ODs at 630 nm were determined in order to check whether a sample was toxic for the yeast cells. Normally, the density at 630 nm increases from 0.05 at 0 h to about 0.9 at 24 h. If the OD at 24 h was below 0.7, the concentration of the compound tested was considered to cause cytotoxicity and rejected for analysis.

Data analysis

The proliferation of the MCF-7/BOS cells was determined by dividing the mean value of DNA content per well from exposed and non-exposed (DMSO control) wells, subsequently multiplied by 100%. The relative proliferative potency (RPP), defined as the ratio of the concentration of E2 needed to achieve 50% of maximal cell yield and the dose of the test compounds required to achieve a similar effect and then multiplied by 100, was calculated from fitted proliferation dose-response curves using nonlinear regression analysis (four parameter sigmoidal dose-response curve, Graphpad Prism software, version 5.04). For the luciferase reporter gene assays, luciferase activity per well was measured as relative light units (RLU). Fold induction was calculated by dividing the mean value of light units from exposed and non-exposed (DMSO control) wells. For the yeast estrogen bioassay, the fluorescence signals were corrected for the signal obtained with the MM/L medium containing DMSO solvent only. Regarding the three reporter gene assays, the relative estrogenic potency (REP), defined as the ratio of the concentration of E2 needed to achieve 50% of maximal response and the concentration of the test compounds required to achieve a similar effect, multiplied by 100, was calculated from fitted dose–response curves (four parameter sigmoidal dose-response curve, Graphpad Prism software version 5.04). The REP value for E2 is hereby set at 100 for all three reporter gene assays. The estrogenicity data used for comparisons with the current proliferation assay and reporter gene assay data

were the median ER binding affinities (from several ER binding assays with ER obtained from e.g. rat/mouse uterine cytosol or intact MCF-7 cells, or with semi-purified human ERα protein) published in the review by ICCVAM, [16] the BG1Luc ER TA reporter gene transactivation assay data that were published by ICCVAM [17] and are based on BG-1 immortalized adenocarcinoma cells that endogenously express both human ERα and ERβ, and the uterotrophic assay data that were derived from the Endocrine Disruptor Knowledge Base (EDKB), developed by the National Center for Toxicological Research (NCTR, USA). [18]

RESULTS

A set of 23 reference compounds were tested in the MCF-7/BOS proliferation assay and three reporter gene assays, i.e., the T47D ER-CALUX, the U2OS ERα-CALUX and the yeast estrogen bioassay. Twenty-one of the 23 compounds tested were selected from the ICCVAM list of compounds defined for validation of in vitro tests for estrogenicity testing and representing a diverse range of chemicals, i.e., natural steroids, synthetic steroids, flavonoids, phenols, organochlorines, and phthalates. [16] Dose-response data were generated and for each compound the EC_{50} value, i.e., the concentration giving a half maximum response, was calculated from the fitted dose-response curve. Estradiol (E2) was used as a reference compound in each assay. The relative proliferative potency (logRPP) and relative estrogenic potency (logREP) values of these 23 compounds, as calculated from the determined EC_{50} values, are listed in Table 1. To allow comparison with data from other in vitro and in vivo assays, the relative binding affinity (logRBA), the relative estrogenic potency (logREP) and the relative potency (logRP) of these compounds as determined in the ER binding assays, the OECD validated BG1Luc ER TA reporter gene assay and the in vivo mouse or rat uterotrophic assay, respectively, are also listed in Table 1. In total, 19 compounds mentioned in the ICCVAM BG1Luc ER TA assay evaluation report overlapped with the 23 compounds used in the current study, while ER binding assays data for only one compound (OMIY-bisphenol) were not available in the ICCVAM review report. In addition, at 10 μM, the organochlorine pesticide kepone was toxic to the yeast cells and this compound was therefore excluded from correlation analysis of the yeast estrogen bioassay with other assays. The selective estrogen receptor modulator (SERM) 4-hydroxytamoxifen (4-OHTAM) was slightly active in the MCF-7/BOS proliferation assay, however, the relative proliferative effect, i.e., the ratio between the highest cell yield obtained with 4-OHTAM to that obtained with E2, multiplied by 100, was less than 10%. As a result, the logRPP of 4-OHTAM could not be reliably determined and this compound was excluded from correlation analysis of the MCF-7/BOS proliferation assay with other assays. Moreover, ethyl paraben and *o,p*'-DDT induced a higher maximal response than E2 (being 100%), i.e., around 150% and 220%, respectively, in the luciferase-based reporter gene assays.

Subsequently, all the relative potency values were used to construct a correlation matrix. Table 2 shows the coefficient of determination (R^2) between the different data sets. In general, reasonable to good correlations were observed between the in vitro and in vivo assays but also among the in vitro assays. The yeast estrogen bioassay showed the best correlation with the in

Table 1. Comparison of the relative potency values obtained in various in vitro assays with the in vivo uterotrophic for the 23 compounds.

Compound	CAS nr.	Uterotrophic assay logRP [a]	MCF-7/BOS proliferation		BG1Luc ER TA logREP [f]
			EC_{50}	log REP	
17ß-Estradiol	50-28-2	2.0	2.0E-11	2.0	2.0
EE2	57-63-6	3.0	9.7E-12	2.3	1.7
DES	56-53-1	2.7	3.8E-11	1.7	1.2
Dienestrol	84-17-3	2.4	3.8E-11	1.7	-
meso-Hexestrol	84-16-2	2.5	3.8E-10	0.7	1.3
Corticosterone	50-22-6	-5.0 [b]	-	-5.0	-5.0
Progesterone	57-83-0	-5.0	-	-5.0	-3.6
Testosterone	58-22-0	-5.0	2.1E-06	-3.0	-3.2
Coumestrol	479-13-0	-0.8	1.3E-08	-0.8	-2.6
Genistein	446-72-0	-2.7	4.6E-08	-1.4	-2.9
Apigenin	520-36-5	-5.0	6.2E-07	-2.5	-3.6
Zearalenone	17924-92-4	-0.7	1.5E-10	1.1	-
OMIY-bisphenol	1943-97-1	-0.3 [c]	2.3E-09	-0.1	-
p-n-Nonylphenol	104-40-5	-5.0	-	-5.0	-
Bisphenol A	80-05-7	-1.6	3.6E-07	-2.3	-3.1
Kepone	143-50-0	-1.0	4.9E-07	-2.4	-3.1
o,p'-DDT	789-02-6	-3.5	3.8E-07	-2.3	-3.1
2,4,5-T	93-76-5	- [d]	-	-5.0	-5.0
Butylbenzyl phthalate	85-68-7	-5.0	2.0E-06	-3.0	-3.8
Di-*n*-butyl phthalate	84-74-2	-5.0	3.0E-06	-3.2	-4.1
Ethyl paraben	120-47-8	-5.0	1.4E-05	-3.8	-4.9
Tamoxifen	10540-29-1	1.0	4.1E-09	-0.3	-2.3
4-OHTAM	68047-06-3	1.0	presumed positive [e]	-	-5.0

[a] Median relative potency values based on uterotrophic assay in mouse or rat, derived from the EDKB (NCTR,USA). 17ß-estradiol is used as a reference chemical and is defined to have a relative potency of 100 (logRP=2.0). [18]
[b] A cut-off value of -5.0 is listed for compounds showing no effect.
[c] logRP of 4,4'-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol (OMIY-bisphenol) was calculated based on the minimal active dose described by Yamasaki et al. 2003. [19]
[d] Data not available.
[e] The EC_{50} of 4-Hydroxytamoxifen could not be reliably determined.

vivo uterotrophic assay (R^2 = 0.87), followed by MCF-7/BOS proliferation assay (R^2 = 0.85). The three human cell-based reporter gene assays showed a significant but relatively lower correlation with ER binding assays and the in vivo uterotrophic assay (R^2 ranging from 0.54 to 0.70). These low correlations were mainly caused by 4-OHTAM (see Figure. 1C-E). As shown in Table 1, a relatively high potency values was observed for 4-OHTAM in the

T47D ER-CALUX		U2OS ERα-CALUX		Yeast estrogen bioassay		ER binding
EC_{50}	log REP	EC_{50}	log REP	EC_{50}	log REP	log RBA[h]
5.0E-12	2.0	8.6E-12	2.0	6.0E-10	2.0	2.0
2.7E-12	2.3	5.6E-12	2.2	5.0E-10	2.1	2.2
1.8E-11	1.4	8.1E-11	1.0	6.0E-10	2.0	2.1
5.9E-11	0.9	8.5E-11	1.0	1.8E-09	1.5	2.0[i]
6.9E-11	0.9	1.7E-10	0.7	2.8E-09	1.3	2.4
-	-5.0	-	-5.0	-	-5.0	-5.0
-	-5.0	-	-5.0	-	-5.0	-3.5
-	-5.0	8.5E-07	-3.0	3.0E-05	-2.7	-1.6
5.2E-09	-1.0	4.4E-08	-1.7	1.4E-07	-0.4	1.1
1.3E-07	-2.4	6.8E-08	-1.9	2.0E-06	-1.5	0.2
4.1E-07	-2.9	5.8E-07	-2.8	2.4E-04	-3.6	0.1
2.3E-10	0.3	4.2E-10	0.3	1.3E-07	-0.3	1.2
3.2E-10	0.2	1.2E-08	-1.1	1.0E-07	-0.2	-
-	-5.0	-	-5.0	-	-5.0	-1.5
7.7E-07	-3.2	2.2E-07	-2.4	3.0E-06	-1.7	-1.5
6.7E-07	-3.1	8.5E-07	-3.0	-	Toxic[g]	-1.5
4.1E-07	-2.9	7.2E-07	-2.9	6.0E-06	-2.0	-1.7
-	-5.0	-	-5.0	-	-5.0	-5.0
5.7E-06	-4.1	1.0E-05	-4.1	-	-5.0	-2.7
1.7E-05	-4.5	1.9E-05	-4.3	-	-5.0	-2.6
5.5E-06	-4.0	4.2E-05	-4.7	1.0E-03	-4.2	-3.2
1.5E-08	-1.5	2.1E-08	-1.4	3.0E-06	-1.7	0.6
-	-5.0	-	-5.0	2.0E-06	-1.5	2.2

[f] Relative estrogenic potency is defined as the ration between the median EC_{50} of 17ß-estradiol and the median EC_{50} of the compound, and this ratio is subsequently multiplied by 100 calculated based on the BG1Luc ER TA data reported by ICCVAM. [17] The REP value of 17ß-estradiol is thus 100, resulting in a logREP of 2.0.
[g] Toxic to the yeast cells at concentrations higher than 10 µM.
[h] Logarithm of the median ER relative binding affinity values listed in the review report of ICCVAM. [16]
[i] Median logRBA value derived from the EDKB (NCTR,USA). [18]

uterotrophic assay (logRP = 1.0) and an even higher median value in the ER binding assays (logRBA = 2.2). However, 4-OHTAM is not active in the three human cell-based bioassays. Only the yeast estrogen bioassay and the MCF7/BOS cell proliferation assay were able to display the ER-agonistic properties of 4-OHTAM and produce qualitative outcomes that are comparable to that obtained in vivo. When 4-OHTAM was excluded from the comparison,

the correlation of the uterotrophic assay or ER binding assays with the three human cell-based reporter gene assays increased to R^2 between 0.76 and 0.87. In addition to 4-OHTAM, several other compounds also revealed discrepancies, e.g., testosterone was negative in the T47D ER-CALUX assay and the uterotrophic assay but positive in the other three reporter gene assays and the ER binding assays; butylbenzyl phthalate and di-*n*-butyl phthalate were positive in the ER binding assays, the three CALUX assays, and the proliferation assay, but negative in the yeast estrogen bioassay and the uterotrophic assay. These discrepancies also have influences on the overall correlation between different bioassays, but to a lesser extent than observed with 4-OHTAM. To visualize these discrepancies, Figure 1 shows the comparison of different in vitro assays with the in vivo uterotrophic assay. Compounds on the dotted diagonal line depicted in all graphs of Figure 1, representing exact potency equality, have about the same relative potency in the in vitro assay as obtained in the in vivo uterotrophic assay, compounds clearly above the line are relatively more potent in vivo than in vitro, while compounds below the line are relatively more potent in the in vitro assays than in the in vivo uterotrophic assay. From Figure 1 it becomes clear that the main outliers between different in vitro assays and the in vivo uterotrophic assay are 4-OHTAM, testosterone (T), and, to a lesser extent, apigenin, tamoxifen (TAM) and kepone. The yeast estrogen bioassay showed the best correlation with the in vivo uterotrophic assay ($R^2 = 0.87$), and moreover, the majority of the compounds lie close to the diagonal line of potency equality (Figure 1E). Although the relative binding affinities of TAM and 4-OHTAM to the ER correlated well with the relative estrogenic potencies of these two compounds as observed in the uterotrophic assay, most of the other compounds shown in Figure 1F lie below the dotted diagonal line of equal potency, included in all graphs in Figure 1. This indicates that most compounds show a weaker estrogenic potency relative to E2 in the uterotrophic assay than would be expected on the basis of their relative ER binding tendency. Moreover, the wider dispersion of the data points around the regression line in Figure 1F reveals an overall relatively low correlation of the results from the ER binding assays with in vivo estrogenicity displayed in the uterotrophic assay ($R^2 = 0.75$). Altogether, the results presented in Figure 1 and Table 2 reveal that the in vitro assays showing the most accurate prediction of in vivo estrogenicity as observed in the uterotrophic assay are the yeast estrogen bioassay and the MCF-7/BOS proliferation assay.

DISCUSSION

The aim of the present study was to further investigate which of a series of in vitro assays for estrogen activity most accurately predicts the uterotrophic effects observed in vivo. The outcomes of ER binding assays, four reporter gene assays and the MCF-7/BOS cell proliferation assay were compared with the outcomes of the in vivo uterotrophic assay. All together, these assays cover a broad range of endpoints representing different phases of the estrogen signaling pathway, i.e., receptor binding, target gene transcriptional activation, cell proliferation and in vivo uterotrophic effects. Results from these comparisons provide useful information to select the most suitable in vitro assays for estrogenicity testing and lead to the following considerations to be taken into account when defining an ITS for in vitro estrogenicity testing.

Table 2. Coefficient of determination (R^2) between different bioassay used in the study. Values given in brackets were obtained after 4-hydroxytamoxifen was excluded from the comparisons.

	Uterotrophic assay n=22	**MCF-7/BOS proliferation n=22**	**BG1Luc ER TA n=19**	**T47D ER-CALUX n=23**	**U2OS ERα-CALUX n=23**	**Yeast estrogen bioassay n=22**	**ER binding assays n=22**
Uterotrophic assay	-	0.85	0.62 (0.82)	0.70 (0.87)	0.68 (0.86)	0.87	0.75
MCF-7/BOS proliferation	0.85	-	0.88	0.95	0.97	0.92	0.86
BG1Luc ER TA	0.62	0.88	-	0.91	0.94	0.80	0.54
T47D ER-CALUX	0.70	0.95	0.91	-	0.95	0.85	0.63
U2OS ERα-CALUX	0.68	0.97	0.94	0.95	-	0.87	0.64
Yeast estrogen bioassay	0.87	0.92	0.80	0.85	0.87	-	0.78
ER binding	0.75	0.86	0.54 (0.76)	0.63 (0.83)	0.64 (0.85)	0.78	-

In vitro ER competitive binding assays have been well established and extensively used to investigate ER-ligand interactions. These assays measure the displacement of a receptor-bound probe molecule, e.g., [^{3}H]E2, by a test compound and subsequently determine the relative binding affinity of the test compound for ER. The ER binding assays are rapid and easy to perform, but only the strength of the binding of a compound to the receptor is determined and not the activation or inhibition of activation of the receptor. Thus, compounds with high binding affinities to the ER might show lower estrogenic activities in cell-based and in vivo assays. This was clearly demonstrated in the current study, by the fact that several compounds showed high binding affinities to ER, but were inactive in the in vivo uterotrophic assay. It was also illustrated by the fact that -as shown in Figure 1F- most of the data points for the correlation between the logRBA from the ER binding assays and the logRP from the uterotrophic assay lie below the dotted diagonal line representing equal potency. In addition, conventional ER binding 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 (EPA) Endocrine Disruptor Screening Program Tier 1 screening battery, still requires the use of animals as a source of ERs. This test is therefore considered to be less suited for an in vitro high-throughput approach for screening of compounds for estrogenic activity.

Proliferation assay based on the human MCF-7/BOS breast cancer cell line is widely used as a screening tool to detect environmental and xenobiotic estrogens. [12, 20] Based on 12 compounds, the MCF-7/BOS proliferation assay has previously been shown to correlate better with the in vivo uterotrophic assay (R^2 = 0.85) than the proliferative responses of three other human cell lines derived from breast, endometrium and ovary. [7] The correlation with the uterotrophic assay as obtained in the present study with the extended set of 23 compounds (R^2 = 0.85), is identical to the correlation obtained in the previous study in which only 12 compounds were studied.

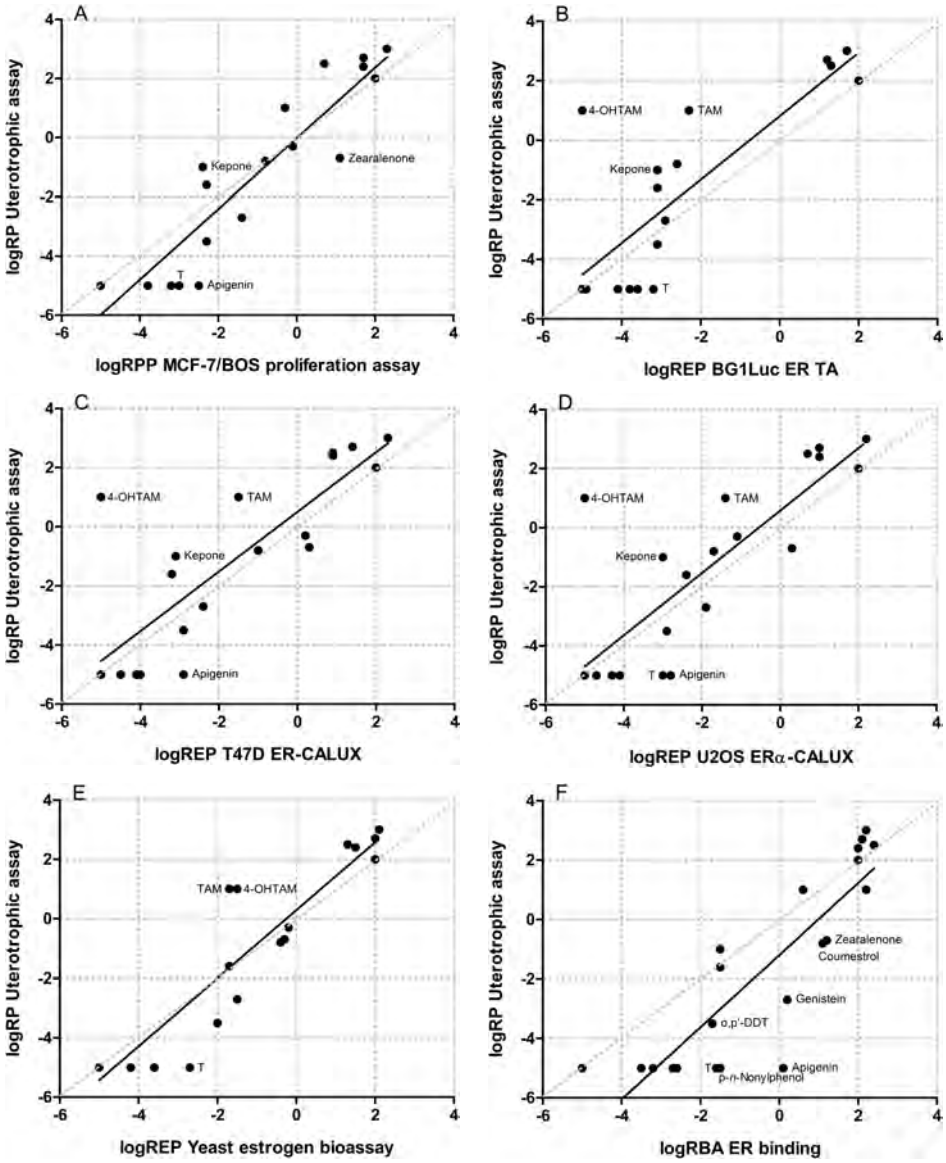

Figure 1. Comparison of the relative potency values obtained from different in vitro assays with the relative potency values obtained from the in vivo uterotrophic assay. (A) MCF-7/BOS proliferation assay versus uterotrophic assay (R^2=0.85, p<0.0001, n=21, regression equation y=1.15x+0.01); (B) BGLuc ER TA versus uterotrophic assay (R^2=0.62, p<0.0001, n=16 and the regression equation is y=1.20x+0.60); (C) T47D ER-CALUX versus uterotrophic assay (R^2=0.70, p<0.0001, n=22, regression equation y=0.96x+0.28); (D) U2OS ERα-CALUX versus uterotrophic assay (R^2=0.68, p<0.0001, n=22, regression equation y=1.03x+0.45); (E) yeast estrogen bioassay versus uterotrophic assay (R^2=0.87, p<0.0001, n=21, regression equation y=1.14x+0.29); and (F) ER binding assay versus uterotrophic assay (R^2=0.75, p<0.0001, n=21, regression equation y=1.21x-1.20). The dotted diagonal line included in all figures represents potency equality. Compounds that deviating the most from the dotted diagonal line are indicated by their names.

This demonstrates that the cell proliferation endpoints has a high predictive value for the in vivo uterotrophic effect. However, the MCF-7/BOS cell proliferation assay is not very fast, i.e., the proliferative responses of the MCF-7/BOS cells can be reliably quantified only after 6 days. This might limit its practical application in the current format as a high-throughput screening tool within massive screening programs such as REACH. Moreover, crosstalk can occur due to the fact that MCF-7/BOS cells also express androgen, progesterone, glucocorticoid and retinoid receptors [21]. Thus, this assay may not provide straightforward information on the mechanism of action of the compound under investigation.

Reporter gene assays, based on stably transfected cells (either mammalian or yeast cells), provide a more specific endpoint measurement, i.e. are based on estrogen receptor-mediated activation of gene transcription. As a result, these assays can distinguish between receptor agonists and antagonists. The three mammalian cell-based reporter gene assays used in the current study, i.e., the T47D ER-CALUX, the U2OS ERα-CALUX, and the BG1Luc ER TA assay yield satisfactory correlations (R^2 = *0.62 - 0.70*) with the uterotrophic assay. The T47D ER-CALUX assay is based on a human breast carcinoma cell line that endogenously expresses both ERα and ERβ. It has been shown previously that ERα activation enhances cell proliferation in breast and uterus, [22-24] while ERβ activation counteracts the ERα-mediated stimulation of cell proliferation. [25-27] Surprisingly, the estrogenic responses obtained for the 23 compounds tested in the T47D ER-CALUX assay were essentially similar to the responses obtained in the U2OS ERα-CALUX stably expressing only ERα (R^2 = 0.95). This is most likely due to the fact that T47D cells express very low levels of ERβ, [7, 8] resulting in a minimal or even no effect via ERβ activation. A similar situation may hold for the OECD validated BG1Luc ER TA assay, which is based on the human BG1 ovarian cancer cell line that also endogenously expresses both ERs, but also with relatively low levels of ERβ. [7, 28, 29] In addition, a superinduction effect was observed for ethyl paraben and *o,p*'-DDT in both T47D ER-CALUX and U2OS ERα-CALUX assays, but not in MCF-7/BOS cell proliferation assay. It has been shown that the superinduction effect in the two CALUX assays could be ascribed to stabilization of the firefly luciferase reporter enzyme, thus increasing the bioluminescent signal during the assay. [30] In line with this, ethyl paraben and *o,p*'-DDT did not result in superinduction but rather induced a similar response as E2 in the fluorescence-based yeast estrogen bioassay. Although only 23 compounds were tested, the T47D ER-CALUX and U2OS ERα-CALUX showed a better correlation with the in vivo uterotrophic assay than the OECD validated BG1Luc ER TA assay. Due to the fact that the U2OS ERα-CALUX has also been pre-validated, [10] it is concluded that the U2OS ERα-CALUX seems the most promising human cell-based reporter gene assay to be part of an in vitro ITS for estrogenicity.

Compared with the human cell line based reporter gene assays, the yeast estrogen bioassay is less sensitive, e.g., the EC_{50} of E2 in the yeast assay is about 100 times higher than its EC_{50} in the mammalian cell-based CALUX assays. However, the yeast estrogen bioassay showed the best correlation with the uterotrophic assay (R^2 = 0.87). Although yeast cell based assays are thought to suffer from poor transport of chemicals across the yeast cell wall, previous studies demonstrated that the yeast cell wall is easily permeable for compounds with a molecular weight up to 620 or even larger molecules due to the flexibility of the

wall of living yeast cells. [31, 32] Thus, the yeast cell wall does not provide a major obstacle for low molecular weight compounds to reach the inside of the cells and activate the ER. This consideration was confirmed by the good correlation observed between the yeast estrogen bioassay and the in vivo uterotrophic assay. In addition, Kolle et al. (2010) also showed that another yeast based assay, the YES assay, has a very high concordance (87%) for predicting estrogenic effects in vivo [6] and proposed to use the YES assay among other assays in the EPA's Endocrine Disruptor Screening Program. [33] Moreover, the yeast estrogen bioassay is the only reporter gene assay used in the current study that was able to correctly predict the agonist effect of 4-OHTAM. All three mammalian cell-based reporter gene assays failed to detect the ER agonist effect of 4-OHTAM. TAM and its metabolite 4-OHTAM are not pure estrogen receptor agonists or antagonists, but act as selective estrogen receptor modulators (SERMs). They are mainly reported to act as ER-agonists on the endometrium and bone and as ER-antagonists in breast. [34-36] Several studies have shown that TAM and 4-OHTAM are also able to inhibit the effect caused by potent estrogens, e.g. EE2 or E2, in the T47D ER-CALUX, U2OS ERα-CALUX, MCF-7/BOS proliferation assay, and in the in vivo uterotrophic assay, [7-9, 19] but not in the yeast estrogen bioassay. [15, 37] Thus compared to its mammalian counterparts, the yeast estrogen bioassay failed to show the antagonistic activities of TAM and 4-OHTAM. A possible explanation for the differences observed in the different assays with the two SERMs is the absence or presence of cell-specific factors (coactivators and corepressors). It has been shown that TAM and 4-OHTAM can bind to the ligand-binding domain of ERα and cause a conformational shift of helix 12 into an adjacent coactivator binding site, which in turn prevents ERα from binding a coactivator. [38-42] The absence of the specific coactivator in different mammalian cell lines and yeast cells as compared to intact uterus tissue may therefore provide a possible explanation of the observed discrepancy. This indicates that an in vitro assay enabling to study ligand-modulated interaction of coregulators with ERα would have the potential to add relevant information to an ITS for estrogenicity testing. Such an ligand-induced coregulator binding assay has recently shown to provide results that also correlate well with the results from uterotrophic assay for a series of selected compounds. [43, 44]

The androgen testosterone (T) was used as a negative control, as it is inactive in the in vivo uterotrophic assay. However, T was slightly active in the BG1Luc ER TA assay and the yeast estrogen bioassay, and even resulted in full dose response curves in the U2OS ERα-CALUX assay and MCF-7/BOS proliferation assay. The effect of T in these assays could be due to activation of the ER, as it is known that T is able to bind ER (logRBA = -1.6). However, this can only partly explain the obtained results, as T did not elicit a full dose response in the yeast estrogen bioassay that stably expresses hERα. The full dose-response curves obtained for T in the U2OS ERα-CALUX and MCF-7/BOS proliferation assay may rather be due to the formation of estrogenic metabolites, as the aromatase enzyme or other P450 enzymes in the steroidogenesis pathway might be present in these mammalian cells, while being absent in the yeast cells. However, the T47D and BG-1 cells are also known to have aromatase activity, [7, 45] but did not give clear response when exposed to T. The reason for these unexpected observations remain to be elucidated, however, this could be due to the fact that T or the formed E2 are metabolised further in these cell lines into non-estrogenic

metabolites. On the one hand, the current study shows that presence of P450 enzymes in mammalian cell lines may lead to a false positive result as demonstrated for T in MCF-7/BOS and U2OS cell based bioassays. On the other hand, these cell lines have only a very limited metabolic capacity. Compounds that are converted into more potent metabolites in vivo mostly show much lower estrogenic potencies in these in vitro assays. For example, in the U2OS ERα-CALUX assay the organochlorine insecticide methoxychlor is about 100 times less potent than its in vivo metabolite 2,2-bis(4-hydroxyphenyl)-1,1,1-trichloroethane (HPTE). [5] This might also be the reason for the discrepancy observed for the organochloride kepone used in the present study, showing a lower logREP in all three reporter gene assay than the logRP in the uterotrophic assay. Taken together, in order to prevent misclassification of compounds it is necessary to characterize the metabolic capacities of the cell line used for estrogenicity testing. Furthermore, combination of the in vitro assays with a bioactivation step, e.g., S9 treatment, might further improve their predictivity for estrogenic potency in vivo. In addition, the estrogenic effect of a chemical can also be due to alteration of normal hormone levels. This indicates that there is a need to include in an ITS for example the H295R steroidogenesis assay that measures the potential interference of exogenous compounds with endogenous steroid hormone synthesis. In addition, as the human H295R adrenocarcinoma cell line expresses all key P450 enzymes, including aromatase, it might also be a useful model to study effects of metabolites of (steroidal) parent compounds.

Finally, the flavonone apigenin showed clear response in all the four reporter gene assays and the MCF-7/BOS proliferation assay. However, apigenin was negative in the uterotrophic assay. This is most probably due to the poor bioavailability of apigenin in rodents, as reported by Breinholt et al. (2000), showing that the urinary recovery of apigenin is only about 1% of the total administered dose in mice. [46] These results indicate that in order to better predict estrogenic property of a compound in vivo, the bioavailability of the compound should be taken into account as well.

In conclusion, the present work demonstrates that each in vitro assay has its own advantages and disadvantages. The yeast estrogen bioassay is less sensitive, but the determined effects and relative potencies are very accurate and the assay showed the best correlation with the in vivo uterotrophic assay. Compared to its mammalian counterparts, the yeast estrogen bioassay does not need expensive growth media (csFBS) nor sophisticated cell culture facilities. However, due to the limited sensitivity and because the yeast estrogen bioassay is not able to show the antagonistic properties of the two SERMs tested as shown in the previous studies, it is recommended to combine the yeast estrogen bioassay with the U2OS ERα-CALUX assay into an ITS for the detection of estrogenic activity. The main outliers identified when correlating data from the different in vitro assays and the in vivo uterotrophic assay are 4-OHTAM, T and to a lesser extent apigenin, tamoxifen and kepone. Based on the modes of action possibly underlying these discrepancies it becomes evident that to further improve the ITS and ultimately replace animal testing for (anti-)estrogenic effects, the selected bioassays have to be combined with other types of in vitro assays, including for example the H295R steroidogenesis assay and in vitro models for digestion, bioavailability and metabolism of the compounds under investigation.

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4

A low-density DNA microchip for the detection of (anti-)estrogenic compounds and their relative potencies

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Based on: Analytical Biochemistry, 435, 83-92 (2013)

ABSTRACT

In the present study, a set of 12 reference compounds was tested in a low-density DNA microchip, which contains probes for 11 different estrogen-responsive marker genes. Our results show that the seven most informative marker genes on the chip resulted in fingerprints that correctly predicted the (anti-)estrogenic activity of the model compounds, except that of the negative control testosterone. Two marker genes (i.e., myeloid leukemia factor-1 interacting protein and ubiquitin-conjugating enzyme E2C) were even capable of correctly predicting the estrogenic potency of all five estrogen receptor (ER) agonists tested and correlated well with the potencies as determined in the MCF-7/BOS proliferation assay and the in vivo uterotrophic assay. In addition, it was demonstrated that the estrogenic responses of testosterone, both in the array tube assay and in the proliferation assay, were partially due to the conversion of testosterone into 17ß-estradiol by aromatase, but also due to formation of other estrogenic metabolites, the presence and estrogenic potency of which were confirmed by GC-MS/MS analysis and a yeast-based reporter gene assay, respectively. It is concluded that low-density DNA microchip-based fingerprinting in MCF-7/BOS cells for estrogenicity marker genes provides a faster in vitro alternative to the current MCF-7/BOS cell proliferation assay (E-screen).

INTRODUCTION

Estrogens are a member of the family of steroid hormones and are primarily biosynthesized in the female ovaries. Estrogens influence the growth, differentiation and function of many target organs, such as mammary gland, uterus, ovary, vagina, as well as testis and prostate. [1-3] They also play a role in bone maintenance, the central nervous system and the cardiovascular system. [4] Estrogens mainly exert their effect through activation of the estrogen receptors (ERα and ERβ) in target cells. Upon ligand binding, a conformational change is induced and the estrogen receptor dissociates from the heat-shock proteins and becomes activated. Then, the ligand-bound activated ER dimerizes and binds to estrogen-responsive elements (ERE) on the DNA. Subsequently, coregulator proteins (i.e. coactivators and corepressors) are recruited to the promoter region of estrogen-responsive genes. [5, 6] The DNA-bound dimer activates or represses transcription of target genes, depending on the type of coregulator bound, leading to altered protein synthesis and cellular functioning. [7]

The Allen and Doisy test is the standard test for the determination of the estrogenic potency of a compound. The Allen and Doisy test is a uterotrophic assay in immature or ovariectomized rodents using uterus weight as a crucial read-out parameter. [8, 9] This in vivo test determines the overall biological effect of a given compound, including the interaction between cells and between different components of the endocrine system, thereby being able to detect complex modes of action that may occur only in the intact animal. However, this uterotrophic assay is labour-intensive and expensive; further, the use of laboratory animals raises ethical concerns. As alternatives, many in vitro assays have been developed to determine the potential estrogenic activities of compounds corresponding to the different steps in the estrogen receptor-dependent pathway. These in vitro assays were recently reviewed by Bovee and Pikkemaat, showing that every assay type has its own specific advantages and disadvantages [10]. From the cell-based assays, reporter gene assays (based on either mammalian or yeast cells) and proliferative assays (based on human cell lines) are among the most used. Several reporter gene assays have been developed and applied as screening tools to determine the estrogenic/anti-estrogenic activities of compounds. [11-13] These reporter gene assays measure the induction of a reporter gene, the expression of which is easy to quantify and is dependent on the level of activation of the estrogen receptor. Although this is very useful, these assays are based on a single estrogen-responsive cell type cultured in vitro, and do not reflect the biological complexity of multi-factorial networks regulating the physiology of native cells in a whole animal [14] (e.g., issues of bioavailability, metabolism or effects on steroidogenesis). Proliferative assays suffer the same drawbacks, although the endpoint is closer to the biological effect as determined in the in vivo uterotrophic assay. The E-screen is a proliferative assay based on the human MCF-7/BOS breast cancer cell line and is widely used as a screening tool to detect environmental and xenobiotic estrogens. [15, 16] However, the MCF-7/BOS cell proliferation assay is not very fast; i.e., the proliferative responses of the MCF-7/BOS cells can be reliably quantified only after 6 days. This test is therefore considered to be less suited for the high-throughput screening of compounds. Moreover, crosstalk can occur due to the fact that MCF-7/BOS cells also express androgen, progesterone, glucocorticoid and retinoid receptors. [10] Nevertheless, the

MCF-7/BOS proliferation assay has previously been shown to correlate best with the in vivo uterotrophic assay when compared to the proliferative responses of three other human cell lines derived from breast, endometrium and ovary. [17]

Transcriptomic fingerprinting techniques based on toxicant-specific changes of gene expression may provide an alternative tool to detect both known and unknown estrogenic compounds. Traditional methods such as northern blotting and, more recently, methods like the quantitative real-time polymerase chain reaction (qRT-PCR) are suitable, but only for analysis of a small number of candidate genes. [18] Furthermore, multiplex analysis by real-time PCR typically suffers from reduced quantification power due to inaccuracy and a relatively narrow dynamic range; therefore, optimization steps are needed. [19, 20] The introduction of the DNA microarray technology has made it possible to examine the expression of thousands of genes at the same time. Major drawbacks are related to the high costs and the time required for analysis and interpretation of the data. In contrast, low-density microarrays, containing a limited set of genes, still offer the ability to rapidly study expression changes of a substantial number of genes, and also provide a more cost-effective option to study known molecular pathways affected by exposure to a test chemical. [21] Such a low-density microchip in Array Tube format was recently developed by Alere (Jena, Germany), consisting of a custom probe microarray (the biochip) integrated into a micro reaction vial. This system has already been applied to detect zearalenone and type A trichothecenes in food matrices [21, 22] and more recently for the detection of lipophilic marine toxins. [23]

In the present study a set of 12 reference compounds was tested using a newly developed array tube, which contains probes for 11 different estrogen-responsive genes selected from a comprehensive gene expression study of estrogen-treated MCF-7/BOS cells. [24] The objective of the present study was to determine to what extent this low-density DNA microchip correctly predicts the estrogenic/anti-estrogenic activities and potencies of these 12 compounds when compared to the outcomes obtained in the MCF-7/BOS cell proliferation assay and in the in vivo uterotrophic assay.

MATERIALS AND METHODS

Chemicals

Dienestrol, 17β-estradiol (E2), zearalenone, corticosterone, tamoxifen, epidermal growth factor (EGF), dehydroepiandrosterone (DHEA), letrozole, flutamide, and β-mercaptoethanol were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). 17α-Ethinyl estradiol (EE2), progesterone, 17β-testosterone (T), 4-androstenediol, 5-androstenediol, and androstenedione were purchased from Steraloids (Newport, RI, USA). Genistein was obtained from Apin Chemicals (Abingdon, Oxon, UK); 4,4'-(octahydro-4,7-methano-5h-inden-5-ylidene)bisphenol (OMIY-bisphenol) from Acros Organics (Fisher Emergo B.V., Landsmeer, The Netherlands) and carbon tetrachloride (CCl_4) from Supelco Inc. (Bellefonte, PA, USA). The RU 58668 compound was a gift from MSD (Oss, The Netherlands). Dimethylsulfoxide (DMSO), sodium chloride, Tween 20, Tris(hydroxymethyl) aminomethane (Tris), and glycine were purchased from Merck (Darmstadt, Germany). Fetal

bovine serum (FBS), charcoal-stripped fetal bovine serum (csFBS), Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM (DMEM/F12 1:1 mixture, with L-glutamine and 15 mM HEPES) without phenol red, 20x saline-sodium phosphate EDTA (SSPE), and 20x saline-sodium citrate (SSC) were obtained from Gibco/Invitrogen (Breda, The Netherlands). Dulbecco's modified Eagle's medium (DMEM) was purchased from Lonza (Breda, The Netherlands). Acetonitrile, ethanol, methanol, acetone, isopropanol, and n-pentane were from Biosolve (Valkenswaard, The Netherlands). The derivatization reagent MSTFA++ consisted of a mixture of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) from Alltech (Anaconda, MT, US), ammonium iodide from Fluka (Zwijndrecht, the Netherlands) and dithiothreitol from Sigma-Aldrich Chemie B.V. (1000:2:4, v/w/w). Chemicals to prepare the growth media for yeast were described previously. [25]

Cell culture and treatment

MCF-7/BOS human breast cancer cells were kindly provided by Dr. Ana M. Soto (Tufts University, Boston, Massachusetts, USA) and were routinely grown at 37 °C under 5% CO_2 atmosphere in 75 cm^2 canted-neck tissue culture flasks (Greiner, Gloucestershire, UK) containing 10 mL DMEM supplemented with 10% FBS. Cells were sub-cultured at approximately 80% confluence and tested negative for mycoplasma. For exposure, MCF-7/BOS cells were plated into 6-wells plates containing 2 mL culture medium per well. When cells reached a confluence of approximately 60%, the medium was changed to assay medium (DMEM/F12 medium, without phenol red supplemented with 5% csFBS). After 24 h, the assay medium was renewed with fresh medium containing test compounds dissolved in DMSO (0.2%, v/v). Each compound concentration was tested in threefold and cells were incubated for 24 h.

RNA extraction, cDNA synthesis, amplification and biotin-labelling

Total RNA was isolated from MCF-7/BOS cells using QIAshredder and RNeasy Mini kits (Qiagen, Venlo, The Netherlands) with RNase-free DNase treatment according to the manufacturer's protocols. In short, the medium was removed and cells were washed with 1 mL PBS, and lysed with 600 µL RLT buffer containing 1% β-mercaptoethanol. The cell lysates were applied to the QIAshredder and RNeasy columns. RNA concentration and quality was determined spectroscopically (Nanodrop Technologies, Montchanin, DE, USA) and by automated electrophoresis using the BioRad Experion system (BioRad, Veenendaal, The Netherlands). Only RNA with A260/280 and A260/230 ratios above 1.8 and a quality index number above 8.0 was considered to be of sufficient quality. The specific transcripts of interest were synthesized and amplified in parallel in a linear manner, as described previously. [26] In short, multiplex cDNA synthesis was performed with 5 µg purified RNA and 1.5 µL of 0.1 µM primer set 1 (rtprimers; supplementary Table S1), which hybridizes to the 3' sequence of each chosen transcript, in an end-volume of 25 µL using the Omniscript RT reverse transcription kit (Qiagen) according to the manufacturer's instructions. Tubes were placed into a thermocycler for 30 min at 50 °C and subsequently put on ice. Primer set 2 is a mix of biotin-labelled forward primers for all selected genes (lbprimers; supplementary

Table S1). Primer set 3 is a mix of primers containing a complementary primer for each primer in primer set 1 (supplementary Table S2). They are added in excess in order to obtain linear amplification, i.e., to prevent primer set 1 from annealing to a product strand of the linear PCR. Labelling and a linear amplification were carried out by adding 1.3 µL of 3.9 µM Primer set 2 (with a 5'-biotin label) and 1.3 µL of 3.9 µM Primer set 3 to the reverse transcription reaction mixture, followed by a multiplex linear amplification reaction of 40 cycles in a thermocycler, consisting of an initial melting cycle for 15 min at 95 °C, followed by 40 cycles of 45 sec at 94 °C, annealing for 45 sec at 56 °C, and elongation for 45 sec at 76 °C.

Low-density microchip hybridisations

Biotin-labelled amplification products were heat-denatured at 95 °C for 5 min and hybridized onto the low-density DNA microchip in Array Tube format (microchips of 9 mm^2, printed with 156 oligonucleotide probes, manufactured by Alere Technologies GmbH, Jena, Germany). Each gene was represented by 2-4 oligonucleotide probes and each oligonucleotide probe was printed on the microchip in triplicate at different locations. The microchips were equilibrated at 30 °C by rinsing them twice for 5 min with 500 µL hybridization buffer (0.9 M NaCl, 60 mM sodium phosphate, 6 mM EDTA, 0.05% Triton X-100, pH 7.4). Hybridizations (60 °C for 1 h) were performed using 2 µL-16 µL biotinylated cDNA samples in 100 µL hybridization buffer to make sure the responses obtained are within the dynamic range of the assay. Next, the array tubes were washed with 500 µL of 2xSSC containing 0.1% (w/v) Triton X-100 (5 min, 30 °C), with 2xSSC (5 min, 20 °C) and finally with 0.2xSSC (5 min, 30 °C) (1xSSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0).

Probe detection and data analysis

After blocking with 2% (w/v) milk powder (Bio-Rad, Hercules, CA, USA) in 500 µL hybridization buffer containing 0.05% (w/v) Triton X-100 (15 min, 30 °C), the array tubes were incubated for 15 min at 30 °C with 100 µL of a 1:2500 dilution of a horseradish peroxidase-streptavidin conjugate (1 mg mL-1; Thermoscientific, Rockford, Canada) in hybridization buffer containing 0.05% (w/v) Triton X-100. Thereafter, the array tubes were washed with 500 µL of 2xSSC containing 0.1% (w/v) Triton X-100 (5 min, 30 °C), with 2xSSC (5 min, 20 °C) and 0.2xSSC (5 min, 20 °C). The reaction was initiated by adding 100 µL of TrueBlue Peroxidase substrate (KPL, Gaithersburg, MD, USA) and the blue precipitates were quantified by light absorption in a dedicated array tube reader ATR03 (Alere Technologies). Data analysis was conducted with the IconoClust and Partisan software version 3.5r (Alere Technologies) and all transcript levels were normalised to the reference control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative induction potency (RIP), defined as the ratio of the concentration of E2 needed to achieve 50% of maximal gene expression and the concentration of the test compounds required to achieve a similar effect, multiplied by 100, was calculated from dose–response curves for responsive genes, fitted by nonlinear regression analysis (sigmoidal dose-response curve, Graphpad Prism software version 5.04). Hereby the RIP value for E2 is thus set at 100, resulting in a logRIP of 2.0.

MCF-7/BOS cell proliferation assay

Proliferation of the human MCF-7/BOS breast cancer cells for testing the estrogenic activity of a given compound was quantified as described by Soto et al. for the E-screen. [15] Briefly, cells were plated into 24-well plates at an initial density of 25,000 cells/well in 500 µL assay medium (DMEM/F12 medium, without phenol red supplemented with 5% csFBS). After 24 h, the assay medium was renewed with fresh medium to which the compound to be tested had been added from a solution in DMSO (0.2%, final concentration). DMSO (0.2%, v/v) and E2 (300 pM) were included in each plate as solvent and positive control, respectively. Each compound concentration was tested in threefold and cells were incubated for 6 days. After 6 days, cells were trypsinized, and the number of cells was counted using a Coulter Counter (Beckman Coulter, Mijdrecht, The Netherlands). The proliferation of the cells was determined by dividing the number of cells in the sample after exposure to the compound by the number of cells in the sample after exposure to DMSO (solvent control), subsequently converting the result to a percent change by multiplying by 100. The relative proliferative potency (RPP), defined as the ratio of the concentration of E2 needed to achieve 50% of maximal cell yield and the dose of the test compounds required to achieve a similar effect, multiplied by 100, was calculated from proliferation dose–response curves, which were fitted by nonlinear regression analysis (sigmoidal dose-response curve, Graphpad Prism software version 5.04). Hereby the RPP value for E2 is thus set at 100. The median log relative potency (logRP) values based on the mouse or rat uterotrophic assay results were derived from the Endocrine Disruptor Knowledge Base (EDKB) designed and produced by the National Center for Toxicological Research (NCTR, USA). [27]

GC-MS/MS analysis

The applied GC-MS/MS analysis method is based on an established and validated method to screen for natural hormones in bovine urine. [28] Aliquots of 1 mL medium were filled up to 3 mL with Milli-Q water and 1 ng of deuterium-labelled internal standard mixture was added. Next, the samples were subjected to solid-phase extraction (SPE) using a C18 cartridge (500 mg, 3 mL, Varian Bond Elute, Harbor City, CA, USA) previously conditioned with 3 mL of methanol and 3 mL Milli-Q water. The column was washed with 3 mL water followed by 3 mL acetonitrile/water (35:65, v/v) and the free steroids were eluted with 3 mL of acetone. The eluate was collected and evaporated at 50 °C under a gentle stream of nitrogen gas until nearly dry, and reconstituted in 100 µL methanol and 2 mL Tris-buffer (0.1 M, pH 9.5). Next liquid-liquid extraction was performed with 7 mL of n-pentane, the mixture was centrifuged (5 min at 3,000 xg) and the organic layer was collected in a glass tube. This extraction procedure was repeated and the combined organic fraction was evaporated at 40 °C to dryness, under a gentle stream of nitrogen gas. The dried residues were reconstituted in 0.5 mL of ethanol, transferred into a derivatization-vial and evaporated at 50 °C under nitrogen until dryness. The dry residue was derivatized by adding 25 µL of MSTFA++ followed by incubation at 60 °C for 1 h. The derivatized mixture was evaporated at 50 °C under nitrogen until dryness and reconstituted in 25 µL iso-octane. GC-MS/MS analysis was performed on a Varian 1200L triple quadrupole mass spectrometer equipped with a CP8400 autosampler and a CP-3800

gas chromatograph (GC). The GC was equipped with a VF-17MS GC column (L = 30 m, id = 0.25 mm, df = 0.25 μm), obtained from Varian (Houten, The Netherlands). Two microliter of the derivatized samples or standard solutions were injected onto the GC column at a pulsed pressure of 30 psi. The temperature program started at 110 °C (constant for 1 min), increased 20 °C·min-1 to 240 °C and was maintained for 1.5 min. Subsequently, the temperature was increased 1 °C·min^{-1} to 244 °C followed by an increase of 25 °C·min^{-1} to 340 °C. This temperature was maintained for 2 min. The helium flow was kept constant at 1.0 mL·min^{-1} and the GC-MS/MS was operated in electron ionization (EI) mode using Multiple Reaction Monitoring (MRM) transitions as described by Blokland et al. [28]

Yeast estrogen bioassay

The yeast cytosensor expressing the human estrogen receptor α (hERα) and yeast enhanced green fluorescent protein (yEGFP) in response to estrogens was developed in-house and was described previously [25, 29]. In short, the day before running the assay, a single colony of the yeast ERα cytosensor was used to inoculate 10 mL of minimal medium supplemented with 60mg/L L-leucine (MM/L medium). This culture was grown overnight at 30 °C with vigorous orbital shaking at 225 rpm. At the late log phase, the yeast ERα cytosensor was diluted in the selective MM/L medium to an optical density (OD) value at 630 nm between 0.04 and 0.06. Subsequently, aliquots of 200 μL of the diluted yeast culture were pipetted into each well of a 96-well plate and 2 μL of test compound in DMSO was added in triplicate. Exposure was performed for 24 h at 30 °C and orbital shaking at 125 rpm. Fluorescence and OD were measured at 0 and 24 h directly in a Synergy™ HT Multi-Detection Micro-plate Reader (BioTek Instruments Inc., USA) using excitation at 485 nm and measuring emission at 530 nm. The fluorescence signal was corrected for the signal obtained with the MM/L medium containing DMSO solvent only. In order to check whether a sample was toxic for the yeast cells, densities of the yeast culture were also determined by measuring the OD at 630 nm.

RESULTS AND DISCUSSION

Detection of estrogenic activity and relative potency of a test compound

MCF7/BOS cells were exposed to 300 pM E2, 100 pM EE2, 300 pM dienestrol and varying levels of zearalenone, genistein, tamoxifen, 4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol, EGF and testosterone (i.e., at concentrations two times higher than their respective EC_{50} observed in the MCF7/BOS proliferation assay as described before [17]). For negative compounds (i.e., progesterone, corticosterone and CCl_4), cells were exposed to a concentration of 1 μM. No cytotoxicity effects were observed at the concentrations used based on LDH leakage (data not shown). After 24 h of exposure, RNA was extracted from the cells. Purified RNAs were converted to biotin-labelled complementary DNAs (cDNA) using three sets of oligonucleotide primers specifically targeting the 11 selected messenger RNA transcripts, the two constitutively expressed reference transcripts (i.e., glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin), and two poorly expressed background control transcripts (i.e., protein tyrosine phosphatase non-receptor type 21 (PTPN21) and

RAD51-associated protein 1 (RAD51AP1)). These labelled PCR products were then hybridized onto the low-density DNA microchip in the Array Tube format. The expression fingerprints were visualized by addition of a streptavidin-horseradish-peroxidase conjugate and the blue precipitate formed was quantified by light absorption using a dedicated array tube reader. Figure 1 shows an example of hybridization images of the array tubes obtained from MCF7/BOS cells exposed to a DMSO control and standards of E2 (30 and 100 pM) and EE2 (100 pM).

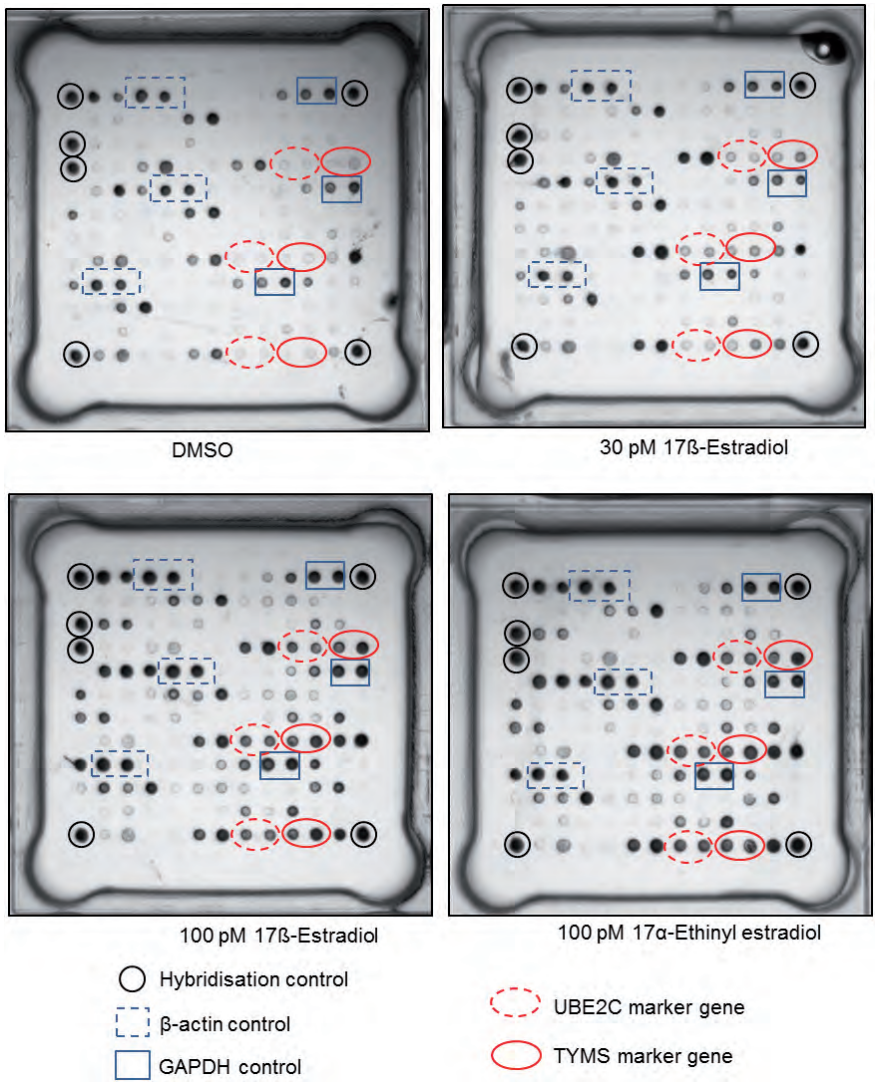

Figure 1. Hybridisation images from the array tube (microchips of 3 x 3 mm, printed with 156 oligonucleotide probes) for the detection of estrogens with MCF7/BOS breast cancer cells exposed to a DMSO solvent control, and standards of 30,100 pM 17ß-estradiol and 100 pM 17α-ethinyl estradiol.

The signal intensities of the spots on the microchip as measured by the array tube reader were analyzed using dedicated IconoClust software. As expected, the GAPDH and β-actin control were highly expressed and were not affected by any treatment (Figure 2A), whereas the expression level of the background controls PTPN21 and RAD51AP1 were all below 0.05 as determined by their spot intensities. From the 11 probes for estrogen-responsive transcripts printed on the chip, five were used for data analysis as they had been previously selected by the manufacturer as marker genes (i.e., cyclin-dependent kinase inhibitor 3 (CDKN3), myeloid leukemia factor-1 interacting protein (MLF1IP), thymidylate synthetase (TYMS), PDZ-binding kinase (PBK) and ribonucleotide reductase M2 (RRM2)). The response of these marker genes upon exposure to the 12 compounds tested is shown in Figure 2B to F. All five marker genes were clearly up-regulated by the full estrogen receptor agonists tested (E2, EE2, zearalenone and genistein). It should be noted that we observed in a previous study that dienestrol is about 3 to 10 times less potent than E2 in the MCF-7/BOS cell proliferation assay; [17] therefore, it is expected that 300 pM dienestrol would result in lower marker gene expression levels than would 300 pM E2. In contrast, EGF, tamoxifen and 4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol resulted in only moderate induction of the five selected marker genes. These compounds were classified as partial agonists in the MCF-7/BOS proliferation assay [17] because they did not result in maximum proliferation rates as high as that induced by E2. The moderate inductions of the five marker genes by these three partial agonists as measured by the array tube assay for estrogenic effects are thus in agreement with the outcomes as obtained previously in the MCF-7/BOS proliferation assay [17].

Except for testosterone, all the negative controls (i.e., progesterone, corticosterone and CCl_4) had little, if any, effect on the expression of any of the marker genes. The clear up-regulation of all five marker genes upon exposure to testosterone, however, correlates well with the proliferative response of testosterone as observed in the MCF-7/BOS cell proliferation assay. [17] It is often assumed that this proliferative response of testosterone is caused by its conversion to 17ß-estradiol by aromatase, [30, 31] but in our previous study it was shown that the proliferative response of the MCF-7/BOS cells to testosterone could only partially be explained by aromatase activity. [17] The proliferative response induced by testosterone was therefore studied in more detail here.

In addition to the five marker genes selected by the manufacturer, six other marker genes on the chip were evaluated as well. Two of those genes turned out to be at least as informative as the five previously selected marker genes. The results of these two newly selected marker genes, ubiquitin-conjugating enzyme E2C (UBE2C) and kinesin family member 14 (KIF14), are shown in Figure 2G and H. Their expression is also specifically induced by estrogens, and their levels are not only in good agreement with the overall response of the five previously selected marker genes, but Figure 2G and H also demonstrate that valuable information is overlooked when using only the standard data analysis procedure for the array tube. The UBE2C and KIF14 marker genes give additional information and, as such, have an added value to distinguish partial and full ER agonists, since UBE2C expression is highly induced by the five pure ER agonists, E2, EE2, dienestrol, zearalenone, and genistein, but also by the partial agonists tamoxifen, 4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol, and

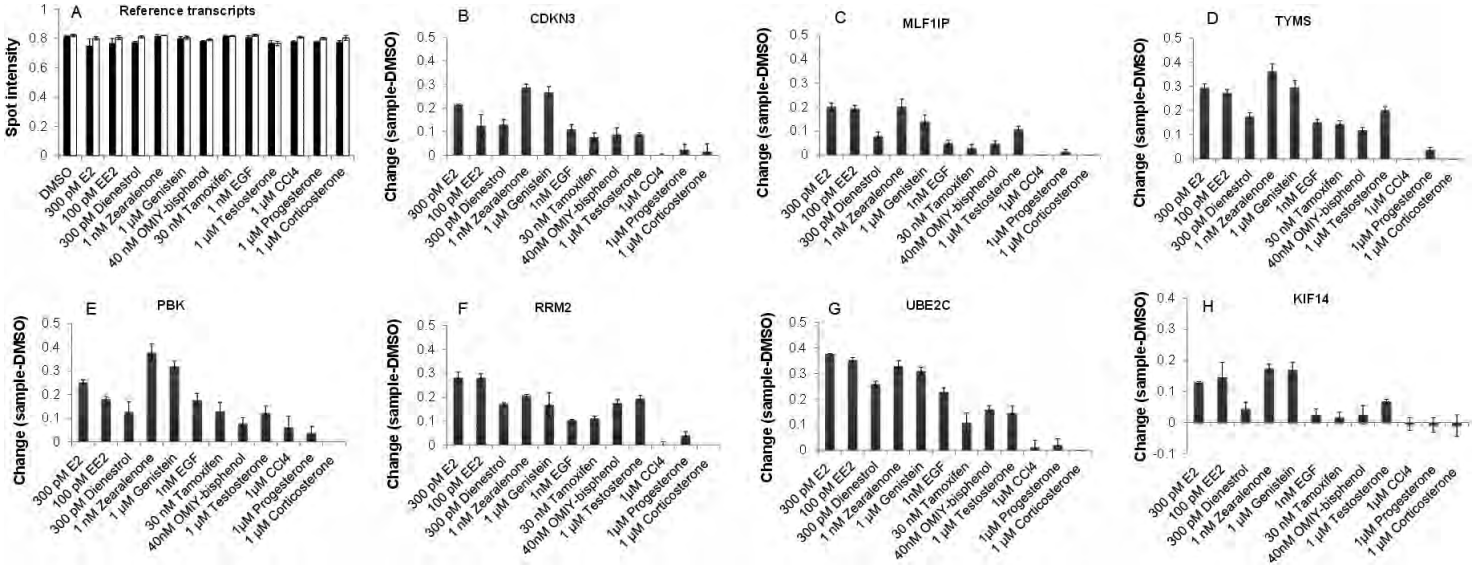

Figure 2. Marker gene expression profiles of the 12 reference compounds obtained using the array tube assay. (A) GAPDH (black column) and β-actin (white column), (B) CDKN3, (C) MLF1IP, (D) TYMS, (E) PBK, (F) RRM2, (G) UBE2C and (H) KIF14 genes. The changes of transcript levels are expressed relative to the DMSO solvent control (mean ± SEM from three individual array tubes). Abbreviations: 17ß-estradiol (E2), 17α-ethinyl estradiol (EE2), epidermal growth factor (EGF) 4,4'-(octahydro-4,7-methano-5h-inden-5-ylidene)bisphenol (OMIY- bisphenol), carbon tetrachloride (CCl_4).

EGF indicating that this marker gene is more robust than the others. KIF14, on the other hand, seems to be sensitive to pure ER agonists only, and almost insensitive for the partial agonists as well as for the negative controls CCl_4, corticosterone and progesterone. The remaining four marker genes on the chip (i.e., transforming growth factor β2 (TGFβ2), histone 1 variant H4H (HIST1H4H), G protein-coupled receptor family C group 5 member A (GPCR5A) and cyclin-dependent kinase inhibitor 2B (CDKN2B)) were also detectably up regulated by the different estrogenic compounds; however, compared to the other seven marker genes, the fold-changes in the expression levels were relatively low and less informative. To a certain extent, this may also be the consequence of the choice in the current study for a multiplex linear amplification technique, which does not suffer from saturation effects as quickly as would be the case with an exponential amplification, and therefore has a broader dynamic range. The drawback however, is that linear amplification has a higher threshold, so that targets with relatively low expression levels will not be sufficiently amplified. Given the current set-up of the assay, these four remaining genes are therefore considered to be less suited as marker genes.

In order to investigate whether the array tube could also be used to determine the relative potencies of estrogenic compounds, MCF-7/BOS cells were exposed to a concentration range of E2, EE2, dienestrol, zearalenone, genistein, and testosterone. The observed marker gene expression, expressed as fold induction relative to the DMSO control, was used to construct dose-response curves of each marker gene for these six compounds. Figure 3 shows that all six compounds, including testosterone, caused a dose-related increase in the expression of all seven marker genes selected. The relative induction potency (log RIP) as calculated for each marker gene from its dose-response curve is listed in Table 1. Note that no cytotoxicity based on LDH leakage was observed at the highest concentrations of the compounds tested. For comparison, the relative proliferative potency (log RPP) observed in the MCF-7/BOS cell proliferation assay and the relative potency based on the mouse or rat uterotrophic assay of these compounds are also shown in Table 1. From these data, it becomes clear that all marker genes correlated well with the MCF-7/BOS proliferation assay. The MLF1IP and UBE2C genes display the best correlation with the relative estrogenic potencies of the compounds as determined in the MCF-7/BOS proliferation assay (for both $R^2 = 0.96$, $p < 0.0001$) and the uterotrophic assay (for both $R^2 = 0.72$, $p < 0.0001$). The MLF1IP gene encodes for the myeloid leukemia factor-1 (MLF1)-interacting protein. This protein interacts with MLF1 and nucleophosmin-MLF1 (NPM-MLF1), which are both reported to induce apoptosis in different cell types. [32] The MLF1IP protein functions as a transcriptional repressor, preventing apoptosis by MLF1 and NPM-MLF1, and thus facilitating cell growth and proliferation. The UBE2C gene encodes for the ubiquitin-conjugating enzyme E2C and was reported to be involved in proliferation, and increased expression of UBE2C was associated with high mortality risk tumour grading and cancer progression in breast. [33] Thus, it seems logic that the expression of these genes correlates well with the proliferative response of the human breast MCF-7/BOS cells.

Detection of anti-estrogenic activity

In order to establish whether the newly developed array tube is also able to determine the ER antagonistic properties of chemicals, tamoxifen, 4,4′-(octahydro-4,7-methano-5H-inden-

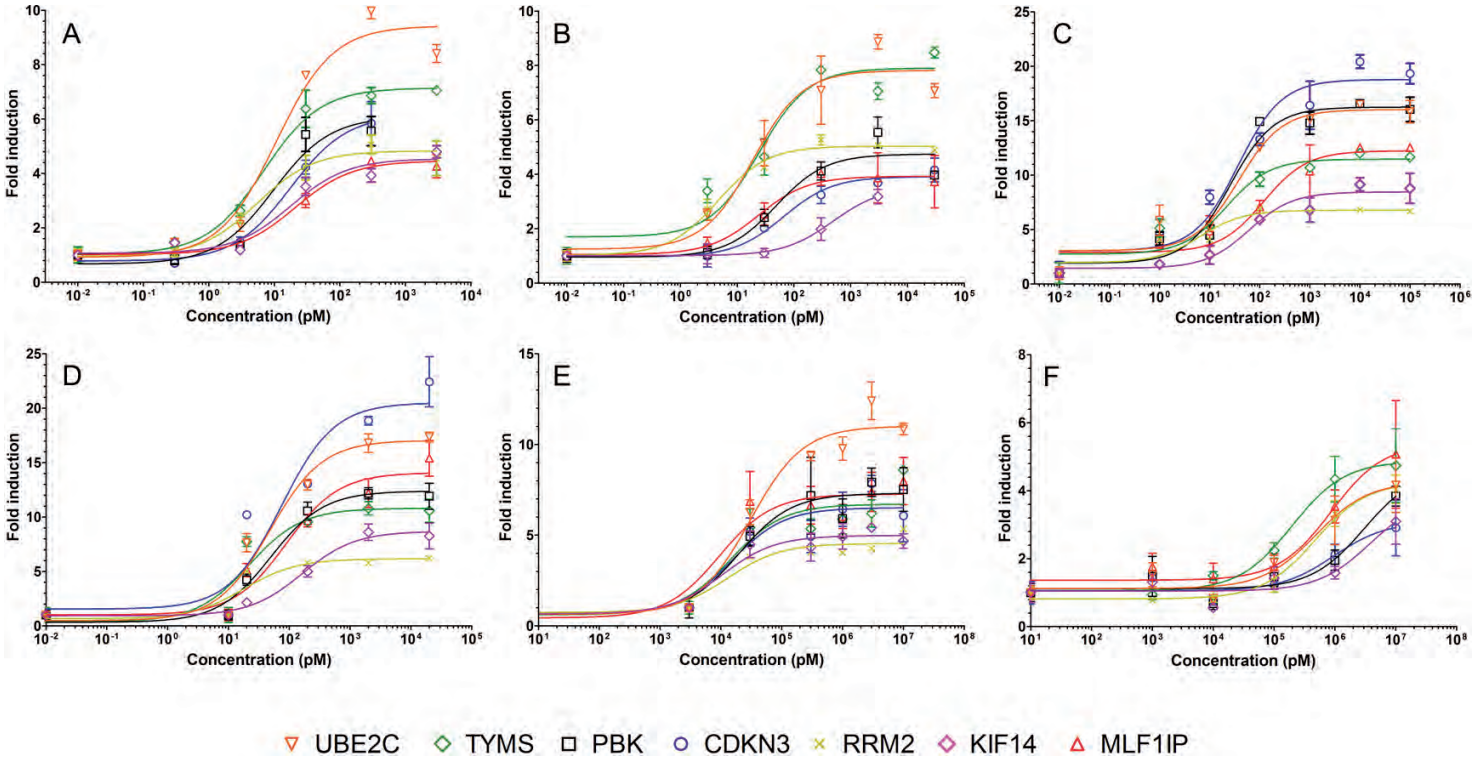

Figure 3. Dose-response curves for induction of the expression of the indicated marker genes in MCF-7/BOS cells by various estrogens and the androgen testosterone as determined using the array tube assay. MCF-7/BOS cells were treated for 24 h with different concentrations of (A) 17ß-estradiol, (B) 17α-ethinyl estradiol, (C) dienestrol, (D) zearalenone, (E) genistein and (F) testosterone. The expression of the seven selected marker genes was calculated and plotted as fold induction relative to the DMSO solvent control. Each point represents the mean of three array tube replicates ± SEM.

Table 1. Comparison of the relative potency values obtained for various compounds in the in vivo uterotrophic and proliferation assay with the relative induction potency values obtained in the array tube assay.

Compounds		CAS number	Uterotrophic assay logRP [a]	MCF-7/BOS proliferation assay logRPP [d]	Array Tube assay logRIP						
					CDKN3	MLF1IP	TYMS	PBK	RRM2	UBE2C	KIF14
Full agonists	E2	50-28-2	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
	EE2	57-63-6	3.0	2.0 [e]	1.4	1.9	1.4	1.3	2.1	1.7	0.6
	Dienestrol	84-17-3	2.4	1.4	1.7	1.2	1.5	1.6	1.3	1.4	1.5
	Zearalenone	17924-92-4	-0.7	0.7 [e]	1.4	1.4	1.5	1.3	1.5	1.4	1.0
	Genistein	446-72-0	-2.7	-2.0	-0.9	-0.6	-1.3	-1.3	-1.4	-1.5	-0.8
Partial agonists	OMIY-bisphenol	1943-97-1	0.3 [b]	-0.4	+ [f]	+	+	+	+	+	+
	Tamoxifen	10540-29-1	1.0	-0.9	+	+	+	+	+	+	+
	EGF	62253-63-8	uterotrophic effect [c]	-1.2	+	+	+	+	+	+	+
Negative controls	CCl_4	56-23-5	-5.0	-5.0	- [f]	-	-	-	-	-	-
	Corticosterone	50-22-6	-5.0	-5.0	-	-	-	-	-	-	-
	Progesterone	57-83-0	-5.0	-5.0	-	-	-	-	-	-	-
	Testosterone	58-22-0	-5.0	-2.9	+	+	+	+	+	+	+

[a] Median relative potency values based on uterotrophic assay in mouse or rat, derived from EDKB (NCTR,USA) [27]. 17ß-Estradiol is used as a reference chemical and is defined to have a relative potency of 100 (logRP=2.0). A cut-off value of -5.0 is listed for compounds showing no effect.
[b] logRP of 4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol was calculated from a minimal active dose described by Yamasaki et al. 2003. [34]
[c] EGF is able to mimic the uterotrophic effects of estrogen in the rodent. [35]
[d] Relative proliferative potency values are obtained from the ratio of the concentration of E2 needed to achieve 50% of maximal cell yield (proliferation) and the dose of the test compounds required to achieve a similar effect, and this ratio is subsequently multiplied by 100. The RPP value of E2 is thus 100, resulting in a logRPP of 2.0. A cut-off value of -5.0 is listed for compounds showing no effect.
[e] Obtained from Fang et al. 2000. [16]
[f] Compound classified as positive (+) or negative (-) for estrogenic effect.

5-ylidene)bisphenol and RU58668 were tested in combination with 300 pM E2. The selective estrogen receptor modulator (SERM) tamoxifen and 4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol are known to possess both estrogenic and anti-estrogenic activities (both in vivo and in vitro), while RU58668 is a pure ER-antagonist. All three compounds with antiestrogenic properties tested were found to reduce the expression induced by 17ß-estradiol of all seven genes in the informative marker set (Figure 4 shows two representative marker genes), demonstrating that the array tube is also useful to test the anti-estrogenic properties of compounds.

Testosterone-induced responses

As mentioned above, the atypical responses observed with testosterone, both in the array tube and the proliferation assay, can only partially be explained by the conversion of testosterone into E2 by aromatase. [17] The main cause for these estrogenic effects of testosterone remains unknown and could possibly also result from the formation of other estrogenic metabolites or from testosterone itself. To further investigate the causes of the testosterone-induced responses, MCF-7/BOS cells were treated with testosterone alone, or with testosterone in combination with the aromatase inhibitor letrozole, the pure anti-androgen flutamide, or the pure anti-estrogen RU58668, and both the proliferative and array tube responses were analyzed. The results in Figure 5 show that only the ER-antagonist RU58668 was able to inhibit the proliferative response induced by testosterone, demonstrating that this proliferative response was ER-mediated. This was strengthened by the observation that the AR antagonist flutamide had no significant effect on the proliferative response caused by testosterone, indicating that the androgen receptor was not involved. The aromatase inhibitor letrozole only partially inhibited the cell proliferation induced by testosterone, which indeed indicates that formation of E2 can only be partly responsible

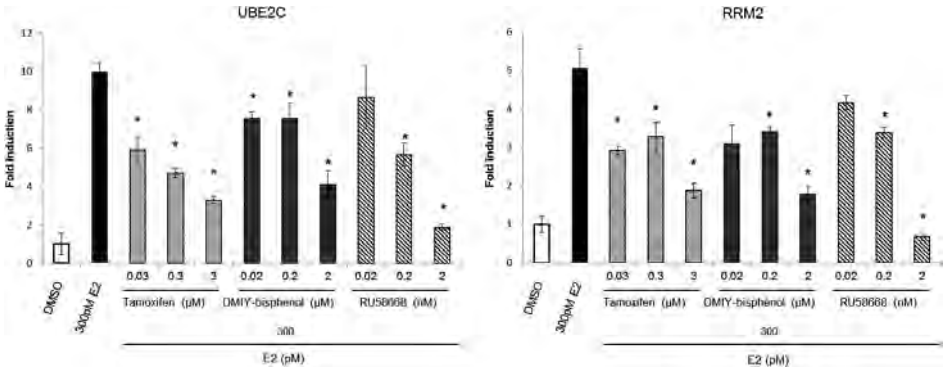

Figure 4. Expression profiles induced by anti-estrogens in MCF-7/BOS cells determined using the array tube assay. The MCF-7/BOS cells were exposed to tamoxifen, 4,4′-(octahydro-4,7-methano-5H-inden-5-ylidene)bisphenol (OMIY-bisphenol) and RU 58668 in combination with 300 pM 17ß-estradiol. The expression of the two representative marker genes UBE2C and RRM2 are shown as fold induction relative to the DMSO solvent control. Each point represents the mean of three array tube replicates ± SEM. * indicates that the expression of the gene is significantly different ($p < 0.05$) from the value of 17ß-estradiol alone.

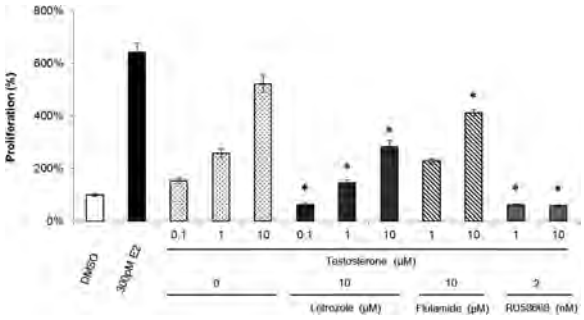

Figure 5. Proliferative response of MCF-7/BOS cells upon exposure to 17ß-estradiol and testosterone alone, and testosterone in combination with the aromatase inhibitor letrozole, anti-androgen flutamide, and anti-estrogen RU58668. Results represent at least three independent experiments and are expressed as the mean proliferative response ± SD (n=3). * indicates that the proliferative response is significantly different ($p < 0.05$) from the value of testosterone alone.

for the proliferative response induced by testosterone. This means that other estrogenic metabolites might be formed during the exposure of MCF-7/BOS cells to testosterone, or that testosterone itself is responsible for the ER-mediated cell proliferation.

As testosterone is negative in the in vivo uterotrophic assay, the second reason is highly unlikely. Therefore the medium was collected after exposure of the MCF-7/BOS cells to testosterone and analyzed by GC-MS/MS. Table 2 shows that several metabolites were formed and could be identified after 6 days of exposure. No significant amounts of the known estrogens E2 or estrone were detected in the medium (both below the detection limit of 0.05 ng mL^{-1}); however, large amounts of 4-androstenedione, 4-androstenediol, 5-androstenediol and dehydroepiandrosterone (DHEA) were detected. Subsequently, pure standards of these metabolites were tested for estrogenic activity in the yeast estrogen reporter gene assay. Androstenedione turned out to be inactive, but 4-androstenediol, 5-androstenediol and DHEA gave full dose-response curves (supplementary Figure S1). Their relative estrogenic potencies (REP), defined as the ratio between the EC_{50} of E2 and the EC_{50} of the compound, were determined and are shown in Table 2. As there is no steroid metabolism in yeast cells, these REPs really reflect the potency of the parent compound tested. Using these REPs as listed in the Table 2, the 17ß-estradiol equivalents represented by the amount of the metabolites identified in the medium after exposure of MCF-7/BOS cells to 10 µM testosterone was estimated. Table 2 shows that the E2 concentration is below the detection limit of 183 pM (0.05 ng mL^{-1}) in the medium, while the detected amount of androstenediol (4- and 5-androstenediol) is equivalent to 55-166 pM E2. Testosterone (10 µM) would give a full response in the MCF-7/BOS proliferation assay, which only occurs above - and is thus equivalent to - at least 300 pM E2 according to the dose-response curve of E2 (supplementary Figure S2). Therefore, the proliferative response induced by testosterone cannot be due only to the formation of E2, which would result <183 pM in the culture medium. Instead, it is apparently also caused by the conversion of testosterone into other estrogenic metabolites (i.e., 4-androstenediol, 5-androstenediol and DHEA), of which the total amount is equivalent to an additional 55-166 pM E2, and thus will contribute significantly to the total estrogenic response. In addition to the effects of letrozole, flutamide and RU58668

Table 2. Levels of testosterone and its metabolites in the medium of MCF-7/BOS cells as determined by GC-MS/MS, and their relative estrogenic potencies in the yeast estrogen bioassay.

	Concentration in nanograms per mililiter medium (±STDEV)					
Identified compound	**1 µM T [a] without cells**	**10 µM T without cells**	**MCF-7/BOS cells exposed to 1 µM T**	**MC-7/BOS cells exposed to 10 µM T**	**REP [b]**	**E2 equivalent [c]**
Estradiol	< 0.05	< 0.05	< 0.05	< 0.05	1.0	< 183 pM
Estrone	< 0.05	< 0.05	< 0.05	< 0.05	0.2 [d]	< 37 pM
Testosterone	258(± 24.9)	2634(± 327)	57.9 (± 5.09)	657 (± 21.1)	< 6.0E-6	< 14 pM
Androstenedione	1.26 (± 0.33)	13.04(± 0.81)	221 (± 50.9)	3393 (± 322)	no response	-
Androstenediol [e]	< 1	< 1	1.82 (± 0.12)	40.2 (± 15.6)	0.4E-3 - 1.2E-3	55-166 pM [f]
DHEA	< 0.05	< 0.05	0.55 (± 0.14)	6.19 (± 2.11)	1.0E-04	2.1 pM

[a] Testosterone
[b] The relative estrogenic potency (REP) is defined as the ratio between the EC_{50} of estradiol and the EC_{50} of the compound in the yeast estrogen bioassay.
[c] E2 equivalent was calculated based on the relative estrogenic potency using concentration of the metabolites identified in the assay medium after 6 days exposure of MCF-7/BOS cells to 10 µM testosterone.
[d] Obtained from Bovee et al. 2004. [25]
[e] Quantification of 4-androstenediol interfered with 5-androstenediol quantification in the GC-MS/MS analysis. Therefore, the total androstenediol amount was calculated.
[f] Based on REP of 5-androstenediol or 4-androstenediol.

on cell proliferation, their effect on testosterone-induced estrogen-responsive marker gene expression was also studied using the array tube assay. Figure 6 shows the effect of RU58668 on the expression of UBE2C and RRM2 marker genes, and clearly demonstrates that these two genes are up-regulated by testosterone, and that this up-regulation can be inhibited completely only by the addition of RU58668. This confirms that the effect of testosterone is caused by activation of the ER, and that metabolites formed from testosterone, including E2 as well as the other metabolites observed in the cell culture medium, may also be involved in the effect of testosterone as observed on the gene expression level. The present study shows that cell models cannot be used as a black box and that it is necessary to characterize the cell line on the metabolic level in order to prevent misclassification of compounds. In this case, testosterone would have been classified as an estrogen in both the proliferation assay and the array tube, even though the estrogenic response was mainly due to the conversion of testosterone into the estrogenic metabolites E2, 4-androstenediol, 5-androstenediol and DHEA. Taken together, this indicates that correct identification and classification of estrogens requires more than one in vitro assay or one cell type (e.g, combinations of mammalian cell lines like MCF-7/BOS and yeast-based reporter gene assays).

CONCLUSIONS

A dedicated array tube for the detection of estrogenic compounds was recently developed based on a previous global analysis of estrogen-dependent gene expression. [24] Eleven target

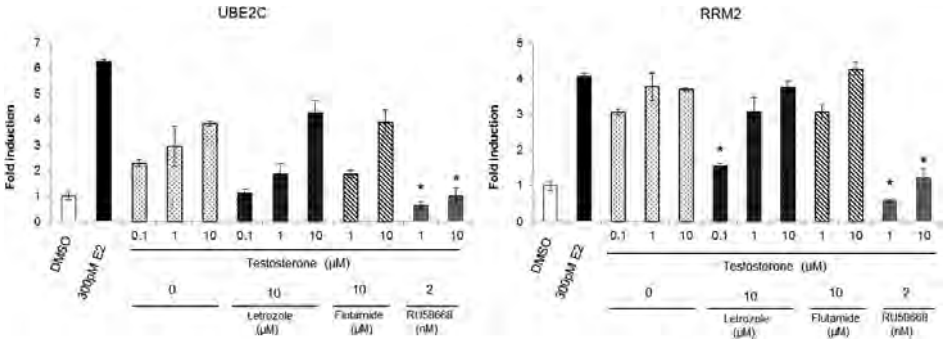

Figure 6. Marker gene expression profiles induced in MCF-7/BOS cells by 17ß-estradiol and testosterone alone, and testosterone in combination with the aromatase inhibitor letrozole, anti-androgen flutamide, and anti-estrogen RU58668, determined using the array tube assay. The expression of two representative marker genes UBE2C and RRM2 are shown as the fold induction relative to the DMSO solvent control. Each point represents the mean of three array tubes replicates ± SEM. * indicates that the expression of the gene is significantly different ($p < 0.05$) from the value of testosterone alone.

genes that were clearly up-regulated by estrogens in MCF-7/BOS cells were selected, and the DNA probes for these transcripts were printed on a low-density DNA microchip. Although linear amplification as used with the dedicated array tube is not suffering from saturation effects as quickly as an exponential amplification, it has a broad dynamic range and a higher threshold. As a consequence, genes with relatively low expression levels cannot be detected by this technique. The seven most informative marker genes on the array tube resulted in fingerprints correctly predicting the (anti-)estrogenic activities of the compounds tested. Two of these marker genes, MLF1IP and UBE2C, were even found capable of correctly predicting the estrogenic potencies of the five compounds tested. The androgen testosterone induced cell proliferation and marker gene expression in MCF-7/BOS cells, but is negative in the in vivo uterotrophic assay. It has been demonstrated that the MCF-7/BOS cells are able to convert testosterone into E2, 4-androstenediol, 5-androstenediol and DHEA, which together are considered responsible for the observed estrogenic activity of testosterone, rather than testosterone itself. As it is faster and still in vitro, the low-density DNA microchip-based analysis of marker gene fingerprints provides an attractive alternative to the current MCF-7/BOS cell proliferation assay (E-screen). Although the low-density DNA microchip-based analysis of marker gene fingerprints (3 days) is faster than the MCF-7/BOS cell proliferation assay (6 days), it is nevertheless quite laborious and requires highly skilled technicians; therefore, it is not ideal in its current format for the large-scale testing of chemicals as required by initiatives like REACH (EC 1907/2006).

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2012.12.016.

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5

A 155-plex high-throughput in vitro coregulator binding assay for (anti-)estrogenicity testing evaluated with 23 reference compounds

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Based on: ALTEX - Alternatives to Animal Experimentation, 30, 145-157 (2013)

ABSTRACT

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 for 18 compounds by the 57 coactivators on the peptide microarray 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 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.

INTRODUCTION

Estrogens exert their physiological effects mainly through activation of the estrogen receptor (ER) in target cells. [1, 2] 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. [3, 4] 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 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. [5, 6]

In general, the transcriptional coregulator family consists of coactivators, which augment the activity of receptors, and corepressors that mediate the repressive effects of receptors. [7, 8] 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 agonists-bound ERs through multiple LXXLL motifs present in these coactivator proteins (where L is leucine and X is any amino acid). [5, 9] 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 such as nuclear receptor corepressor 1 (NCOR1) and nuclear receptor corepressor 2 (NCOR2), thereby disrupting the LXXLL-binding site and preventing coactivator recruitment. [10-12]

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. [13, 14] With a view to the regulation on Registration, Evaluation, Authorization, and Restriction of Chemicals (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 gene or other downstream events such as estrogen receptor-mediated cell proliferation [15]. 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 (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 [16-18]. Proliferation assays and low-density DNA microchip-based analysis of marker gene expression have also been shown to provide valuable tools for estrogenicity testing and outcomes correlate well with the in vivo uterotrophic assay, [19, 20] but these two assays are laborious and request 3-6 days. They are therefore 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 drug development. [21-23] 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 harbouring either LXXLL (coactivators) or LXXXIXXXL (corepressors) 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. [24] 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.

MATERIALS AND METHODS

Chemicals

17ß-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) from Acros Organics (Fisher Emergo BV, Landsmeer, The Netherlands). Dimethylsulfoxide (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. [25] The PamChip® plate consists of 96 identical arrays,

each array containing 155 NR coregulator peptides harbouring either LXXLL (coactivators) or LXXXIXXXL (corepressors) 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. [26, 27] The PamChip® plate was used in combination with the glutathione S-transferase (GST)-labelled 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 (Figure 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 (20mM Tris, pH 7.5, 500mM 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 min. 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). 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 was quantified within the spot (signal) as well as that in a defined area surrounding it (background). The signal-minus-background value was subsequently used as the quantitative parameter of binding. Ligand dose-response relations were analyzed using the DRC package in R (version 2.12.0, 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 [24], 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. [28] 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 (NCTR, USA). [29]

RESULTS

Twenty-one of the 23 compounds tested were selected from the 78 compounds listed by the 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. [24] Figure 1B shows the dose-response curves of ERα-LBD binding to the 155 coregulator peptides as induced by 17ß-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 concentration (20 nM) over each pair of duplicate E2 data. This resulted 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 4,4'-(octahydro-4,7-methano-5h-inden-5-ylidene) bisphenol (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 (supplementary Figure S1 and Figure S2). Surprisingly, no binding was observed for these SERMs to the corepressors present on the peptide microarray.

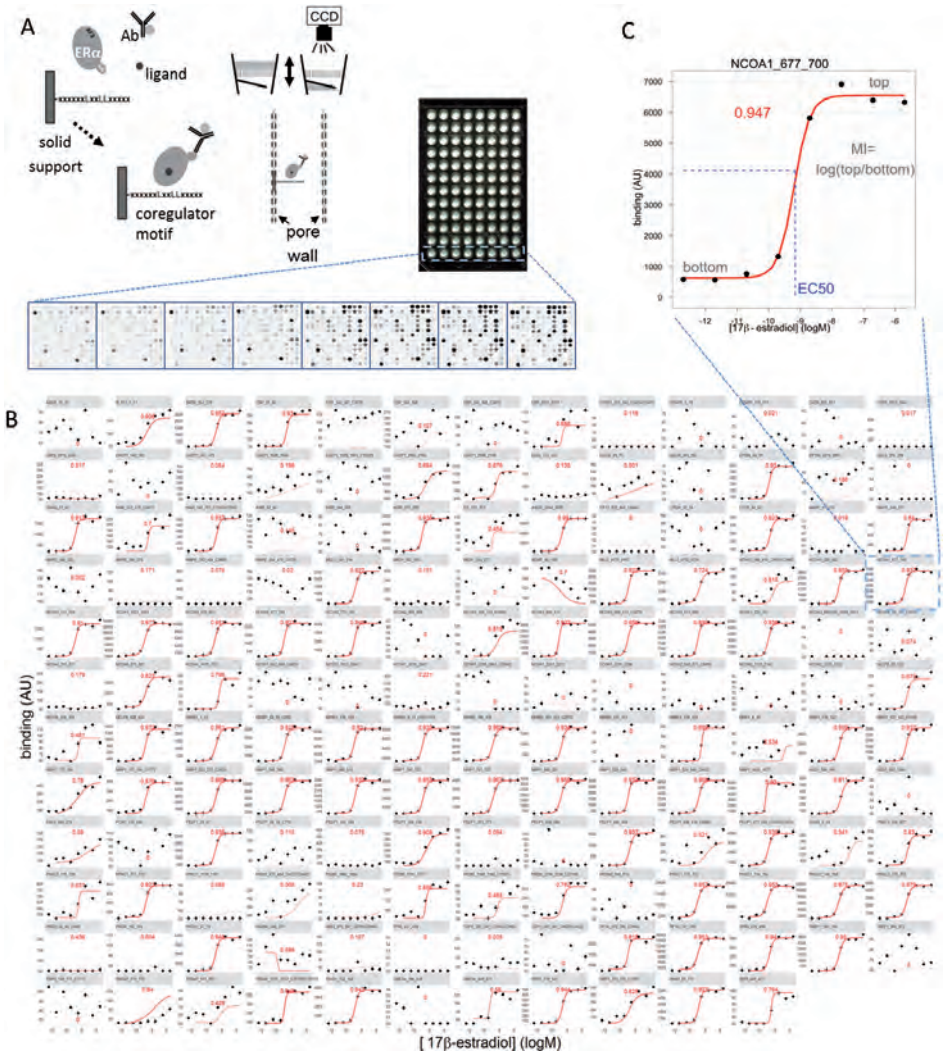

Figure 1. Analysis of ERα-LBD binding to coregulators induced by 17ß-estradiol. A. Schematic overview of peptide microarray technology. B. Dose-response curves for ERα-LBD binding to the 155 coregulator-derived receptor binding motifs induced by 17ß-estradiol. C. Enlargement of the dose-response curve for 17ß-estradiol-induced binding of ERα-LBD to coactivator NCOA1_677_700.

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

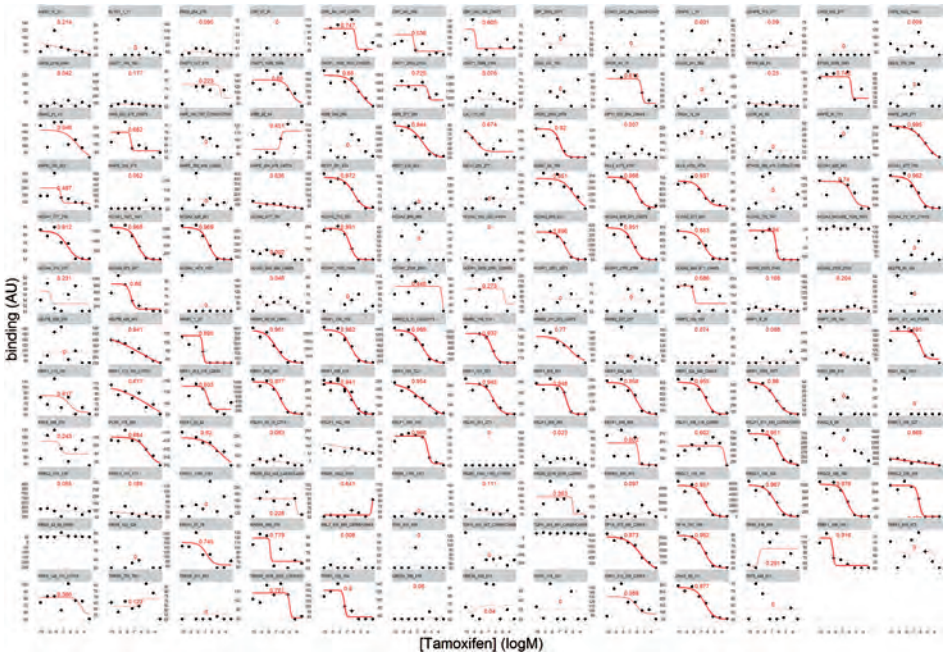

Figure 2. Analysis of ERα-LBD binding to coregulators induced by tamoxifen.

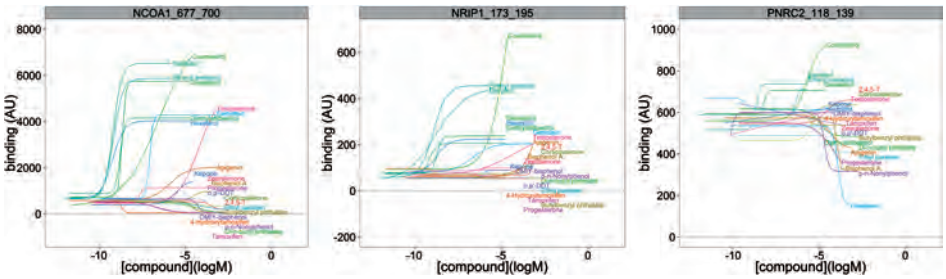

Figure 3. Dose-response curves of ERα-LBD binding to the NCOA1_677_700, NRIP1_173_195 and PNRC2_118_139 coactivator peptides for the 23 compounds.

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 (Figure 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 vitro coregulator binding assay with the in vivo uterotrophic assay, tamoxifen, 4-hydroxytamoxifen, and OMIY-bisphenol were left out (Figure 4 and Table 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 very 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 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, [30, 31] it was negative for estrogenic effects on the uterus in the uterotrophic assay. However, this is most probably due to the poor bioavailability of apigenin in rodents.[32] 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 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

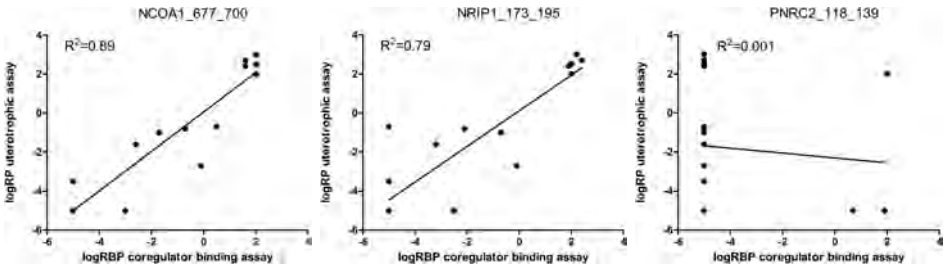

Figure 4. Comparison of the log relative potencies (logRP) measured in the in vivo uterotrophic assay with the log relative coregulator binding potency (logRBP) as determined in the coregulator binding assay for the NCOA1_677_700, NRIP1_173_195 and PNRC2_118_139 coactivators for the 18 compounds. Tamoxifen, 4-Hydroxytamoxifen, OMIY-bisphenol, apigenin and 2,4,5-T were excluded from the comparison.

Table 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 logRBA [a]	BG1Luc ER TA logREP [e]
Steroids and synthetic estrogens	E2	50-28-2	2.0	2.0
	EE2	57-63-6	2.2	1.7
	Diethylstilbestrol	56-53-1	2.1	1.2
	Dienestrol	84-17-3	2.0 [b]	NA
	meso-Hexestrol	84-16-2	2.4	1.3
	Corticosterone	50-22-6	-5.0 [c]	-5.0
	Progesterone	57-83-0	-3.5	-5.0
	Testosterone	58-22-0	-1.6	-3.2
Phytoestrogens (natural products)	Coumestrol	479-13-0	1.1	-2.6
	Genistein	446-72-0	0.2	-2.9
	Apigenin	520-36-5	0.1	-3.6
	Zearalenone	17924-92-4	1.2	NA
Phenol	OMIY-bisphenol	1943-97-1	NA [d]	NA
	p-n-Nonylphenol	104-40-5	-1.5	NA
	Bisphenol A	80-05-7	-1.5	-3.1
Organochlorines	Kepone	143-50-0	-1.5	-3.2
	o,p'-DDT	789-02-6	-1.7	-3.1
	2,4,5-T	93-76-5	-5.0	NA
Phthalates	Butylbenzyl phthalate	85-68-7	-2.7	-3.8
	Di-*n*-butyl phthalate	84-74-2	-2.6	-2.6
Paraben	Ethyl paraben	120-47-8	-3.2	-4.9
SERMs	Tamoxifen	10540-29-1	0.6	NA
	4-hydroxytamoxifen	68047-06-3	2.2	NA

[a] Logarithm of the median ER relative binding affinity values listed in the review of ICCVAM. [24]
[b] Median logRBA value derived from the EDKB (NCTR,USA). [29]
[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 ration between the EC_{50} of 17ß-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. [28] The REP value of 17ß-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). 17ß-estradiol is used as a reference chemical and is defined to have a relative potency of 100 (logRP=2.0). [29]

Uterotrophic assay logRP [f]	Coregulator binding assay logRBP [h]		
	NCOA1_677_700	NRIP1_173_195	PNRC2_118_139
2.0	2.0	2.0	2.0
3.0	2.0	2.2	-5.0
2.7	1.6	2.4	-5.0
2.4	1.6	1.9	-5.0
2.5	2.0	2.0	-5.0
-5.0	-5.0	-5.0	-5.0
-5.0	-5.0	-5.0	-5.0
-5.0	-3.0	-2.5	0.7
-0.8	-0.7	-2.1	-5.0
-2.7	-0.1	-0.1	-5.0
-5.0	-1.3	-3.8	-5.0
-0.7	0.5	-5.0	-5.0
-0.3 [g]	- [i]	-	-
-5.0	-5.0	-5.0	-5.0
-1.6	-2.6	-3.2	-5.0
-1.0	-1.7	-0.7	-5.0
-3.5	-5.0	-5.0	-5.0
NA	-5.0	-5.0	-5.0
-5.0	-5.0	-5.0	-5.0
-5.0	-5.0	-5.0	-5.0
-5.0	-5.0	-5.0	1.9
1.0	-	-	-
1.0	-	-	-

[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. [33]

[h] Relative binding potency values are obtained from the ratio of the concentration of 17ß-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 17ß-estradiol is thus 100, resulting in a logRBP of 2.0.

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

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).

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 labelled 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

coactivator 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. [10, 12, 34-36] 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 is also 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, [37] 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. [19, 33, 38] 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. [19] 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 the 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 17ß-estradiol by aromatase, [19] partially due to formation of other estrogenic metabolites [20] 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 the very high concentrations, [39] 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 [24]. In our coregulator binding assay, this

Table 2. Correlation ofthe 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 17ß-estradiol-induced binding of ERα-LBD as reflected by a goodness-of-fit value of 0.9 or higher.

			Coefficient of determination (R^2)		
Coregulator binding assay Peptide ID [a]	**Motif**	**Uniprot Accession**	**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

Table 2. *Continued*.

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_488_510	LxxLL501		0.83	0.80	0.89
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. [25]

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, [40, 41] 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. [39] 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. [42] 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. [43] 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. 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 effect. [44-46] 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, [47, 48] but did not show an effect in the current coregulator binding assay. This is probably due to the low 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 assay (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 PamChip® plate peptide microarray in the presence of E2. [25] Twenty-one of these 25 coactivators (highlighted in Table 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 are generally 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 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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SUPPLEMENTARY FIGURE

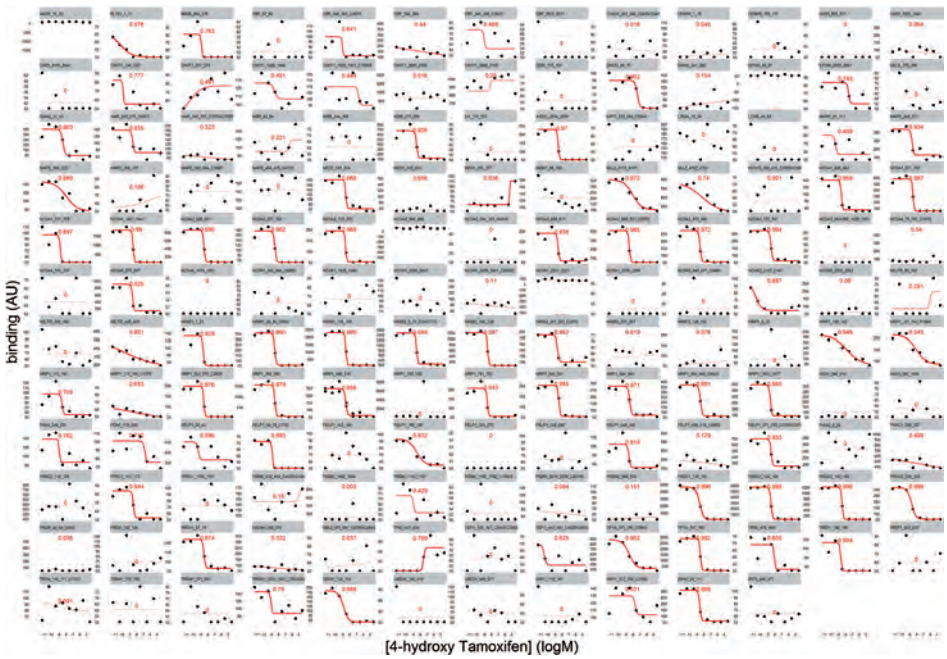

Figure S1. Analysis of ERα-LBD binding to coregulators induced by 4-Hydroxytamoxifen.

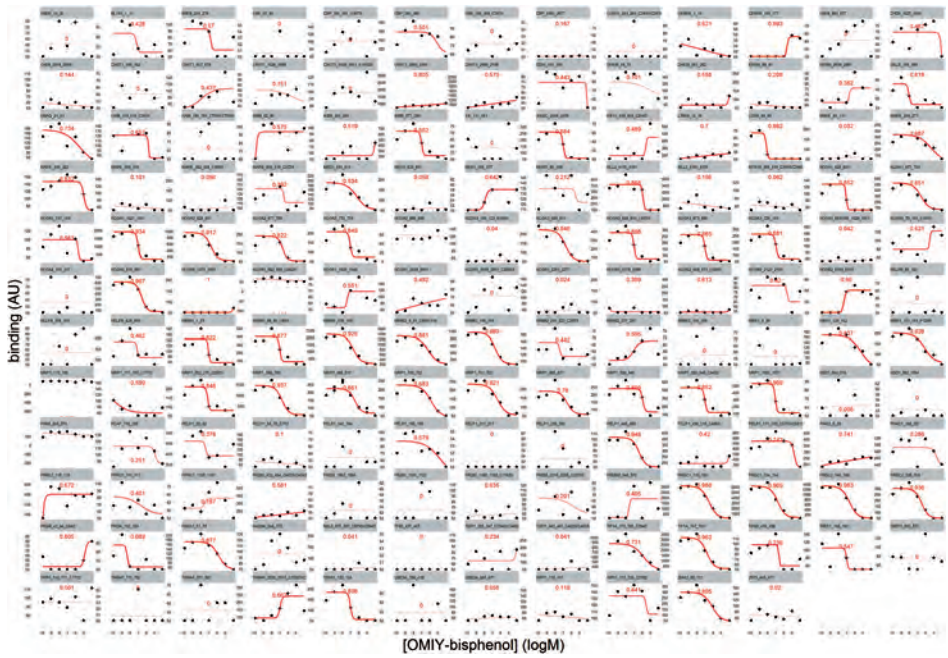

Figure S2. Analysis of ERα-LBD binding to coregulators induced by OMIY-bisphenol.

6

Robust array-based coregulator binding assay predicting ERα agonist potency and generating binding profiles reflecting ligand structure

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Based on: Chemical Research in Toxicology, 26, 336-346 (2013)

ABSTRACT

Testing chemicals for their endocrine-disrupting potential, including interference with estrogen receptor (ER) signaling, is an important aspect of chemical safety testing. Because of the practical drawbacks of animal testing, the development of in vitro alternatives for the uterotrophic assay and other in vivo (anti)estrogenicity tests has high priority. It was previously demonstrated that an in vitro assay that profiles ligand-induced binding of ERα to a microarray of coregulator-derived peptides might be a valuable candidate for a panel of in vitro assays aiming at an ultimate replacement of the uterotrophic assay. In the present study, the reproducibility and robustness of this coregulator binding assay was determined by measuring the binding profiles of 14 model compounds that are recommended by the Office of Prevention, Pesticides and Toxic Substances for testing laboratory proficiency in estrogen receptor transactivation assays. With a median coefficient of variation of 5.0% and excellent correlation (R^2 = 0.993) between duplicate measurements, the reproducibility of the ERα-coregulator binding assay was better than the reproducibility of other commonly used in vitro ER functional assays. In addition, the coregulator binding assay is correctly predicting the estrogenicity for 13 out of 14 compounds tested. When the potency of the ER-agonists to induce ERα-coregulator binding was compared to their ER binding affinity, their ranking was similar, and the correlation between the EC_{50} values was excellent (R^2 = 0.96), as was the correlation with their potency in a transactivation assay (R^2 = 0.94). Moreover, when the ERα-coregulator binding profiles were hierarchically clustered using Euclidian cluster distance, the structurally related compounds were found to cluster together, whereas the steroid test compounds having an aromatic A-ring were separated from those with a cyclohexene A-ring. We concluded that this assay is capable of distinguishing ERα agonists and antagonists and that it even reflects the structural similarity of ERα agonists, indicating a potential to achieve identification and classification of ERα endocrine disruptors with high fidelity.

INTRODUCTION

The estrogen receptor (ER) is a member of the nuclear receptor (NR) family, a class of gene transcription regulators which are activated upon binding of a low molecular weight ligand. The major endogenous agonist activating the ER is 17β-estradiol (E2), which regulates the expression of genes involved in the reproductive system and bone development, especially in females, but also in males.[1, 2] Two types of ER can be found in mammalians, ERα and ERβ. The ERα type is mainly expressed in the sex organs, while ERβ is the dominating type in the bone and brain.[3, 4] Transcription activation by the ER is dependent on binding of agonistic ligands to the binding site in the ligand-binding domain (LBD) of the ER polypeptide chain. The conformational change upon agonist binding results in uncovering of the DNA-binding domain and of the cofactor-binding motif within the LBD.[5] Two functional classes of cofactors or coregulators have been found to interact with the ER and other nuclear receptors, i.e., coactivators and corepressors, respectively, stimulating and inhibiting the basal transcription complex formed on promoter sequences.[6, 7] Coactivators and corepressors interact with nuclear receptors through their specific (iso)leucine-rich amino acid sequence motifs, LXXLL and LXXXIXXX(I/L), for activators and repressors, respectively.[5, 6]

Investigating chemicals for their endocrine-disrupting potential, including interference with estrogen receptor (ER) signaling, is an important aspect of chemical safety testing. Because of the severe drawbacks of in vivo testing, e.g., ethical objections and its labor and resource intensiveness, the development of in vitro alternatives for in vivo (anti)estrogenicity tests, such as the in vivo uterotrophic assay, has high priority. Recently, an ERα-coregulator binding assay in PamChip plate format has been developed that enables one to measure the interaction of the ERα-LBD with these coregulator binding motifs. The PamChip plate consists of 96 identical arrays, each array holding 155 different NR-binding coregulator peptides immobilized on a porous metal oxide carrier.[8, 9] First, purified LBD from ERα, labeled with glutathione-S-transferase (GST) or histidine (His), is preincubated with the test compound. Subsequently, the peptide microarray is incubated with this mixture and the binding of the labeled ERα-LBD to the coregulator binding motifs as induced by the test compound is quantified. In a previous study, it was demonstrated that the binding of tagged ERα-LBD to the peptide microarray correctly predicted the estrogenicity of the reference compounds tested and that the determined estrogenic potencies of the compounds also correlated well with their in vivo potency in the uterotrophic assay.[10] Moreover, that study also indicated that the ERα-coregulator binding assay was capable of identifying ER antagonistic properties, and the obtained binding profiles for the selective estrogen receptor modulators (SERMs) tamoxifen, 4-hydroxytamoxifen, and 4,4'-(octahydro-4,7-methano-5h-inden-5-ylidene) bisphenol suggested that their antagonistic properties are more likely to result from inhibition of the binding of ERα to coactivators than from increased binding to corepressors. Altogether, it was concluded that this ERα-coregulator binding assay is a valuable candidate to be part of a panel of in vitro assays for screening and prioritization of chemicals, and thus would contribute to reduce and ultimately replace animal testing for (anti)estrogenic effects.[10]

In this study, the reproducibility and robustness of the PamChip plate in combination with the ERα-LBD-His was investigated by determining the coregulator binding profiles of

14 model compounds (Table 1). This set of compounds included 10 proficiency compounds recommended by the Office of Prevention, Pesticides and Toxic Substances (OPPTS) for testing laboratory proficiency in carrying out ER-controlled reporter gene transactivation assays,[11] i.e., seven compounds with varying (anti)estrogenic potential, hexestrol, 17α-ethinyl estradiol, diethylstilbestrol, estrone, genistein, 1,3,5-tris(4-hydroxyphenyl)benzene (1,3,5-TB), and butyl paraben; and three nonestrogenic compounds, atrazine, dibutyl phthalate, and corticosterone. The remaining 4 compounds were the reference compounds: 17β-estradiol (E2; strong estrogen), 17α-estradiol (weak estrogen), 17α-methyltestosterone (very weak ER agonist), and corticosterone (negative compound). The obtained binding profiles were also used to determine whether the ERα-coregulator binding assay correctly predicted the estrogenicity and potency of these compounds and furthermore to investigate the relationship between the coregulator binding profile and the structure of the compound under investigation.

MATERIALS AND METHODS

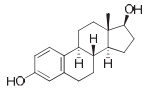
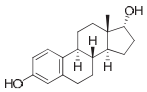
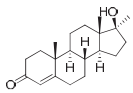
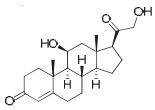
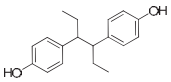
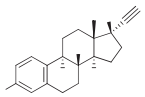
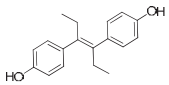
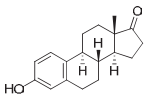
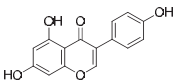
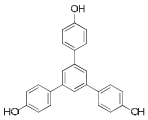
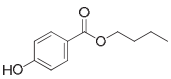
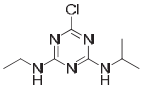
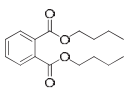
Chemicals

17β-Estradiol (E2) was provided by MSD (Oss, The Netherlands) and another batch of 17β-estradiol (referred to as from source 2), as well as diethylstilbestrol, hexestrol, estrone, 17α-estradiol, butyl paraben, 1,3,5-tris(4-hydroxyphenyl)benzene (1,3,5-TB), atrazine, and corticosterone were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). 17α-Ethinyl estradiol and 17α-methyltestosterone were purchased from Steraloids (Newport, RI, USA). Genistein was obtained from Apin Chemicals (Abingdon, Oxon, UK) and dibutyl phthalate from TCI Europe N.V. (Zwijndrecht, Belgium). Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany).

Nuclear receptor-coregulator interaction profiling

Ligand-modulated interaction of ERα-LBD-His with coregulators was assessed using a PamChip plate (PamGene International B.V., 's-Hertogenbosch, The Netherlands) containing 96 identical peptide microarrays (Figure 1a) as described previously.[12, 13] The peptide microarray was used in combination with a polyhistidine-tagged human ERα-LBD (ERα-LBD-His; amino acids 302-552, partially purified from *E.coli*) to screen a dilution series of a set of 14 compounds. The peptide microarray was incubated with the test solution containing ERα-LBD-His in the absence or presence of ligand by pumping the sample up and down the three-dimensional metal oxide carrier (Figure 1b). In short, assay mixtures were prepared on ice in a master 96-well plate and contained ERα-LBD-His (optimal assay concentration of a crude lysate containing the conjugate was empirically determined and estimated to lie between 1 and 10 nM), 25 nM of an Alexa Fluor 488-conjugated polyhistidine antibody (penta-His Alexa Fluor 488 conjugate, Qiagen no. 35310), and ligand at the indicated concentration in reaction buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% BSA, 0.05% Tween-20). All assays were performed in a fully automated microarray processing platform (PamStation96, PamGene International B.V.) at 20 °C applying two cycles per min. Initial blocking was performed by incubating the peptide microarray plate

Table 1. Chemical properties of the model compounds as recommended by the Office of Prevention, Pesticides and Toxic Substances (OPPTS) of the United States Environmental Protection Agency for testing estrogen receptor-mediated transcriptional activation (OPPTS guideline 890.1300).

Chemical name	CAS No.	Chemical Classification	Functional Classification	Structure
17β-Estradiol	50-28-2	Steroid; phenolic;estrene	Natural hormone	
17α-Estradiol	57-91-0	Steroid; phenolic;estrene	Natural hormone stereoisomer	
17α-Methyl-testosterone	58-18-4	Steroid; nonphenolic; androstene	Pharmaceutical	
Corticosterone [a]	50-22-6	Steroid; nonphenolic	Pharmaceutical	
Hexestrol	84-16-2	Diphenylalkane; bisphenol; phenol	Pharmaceutical	
17α-Ethinyl estradiol	57-63-6	Steroid, phenolic	Pharmaceutical	
Diethylstilbestrol	56-53-1	Stilbene; benzylidene; diphenylalkene	Pharmaceutical	
Estrone	53-16-7	Steroid, phenolic; estrene	Pharmaceutical	
Genistein	446-72-0	Flavanoid; isoflavone; phenol	Natural product	
1,3,5-Tris (4-hydroxyphenyl) benzene	15797-52-1	Triphenyl	Chemical	
Butyl paraben	94-26-8	Paraben; organic acid	Pharmaceutical	
Atrazine	1912-24-9	Aromatic amine; Triazine; Arylamine	Pesticide	
Dibutyl phthalate	84-74-2	Phthalate	Plasticizer	

[a] Corticosterone is both on the list of reference chemicals and on the list of proficiency compounds recommended by OPPTS.

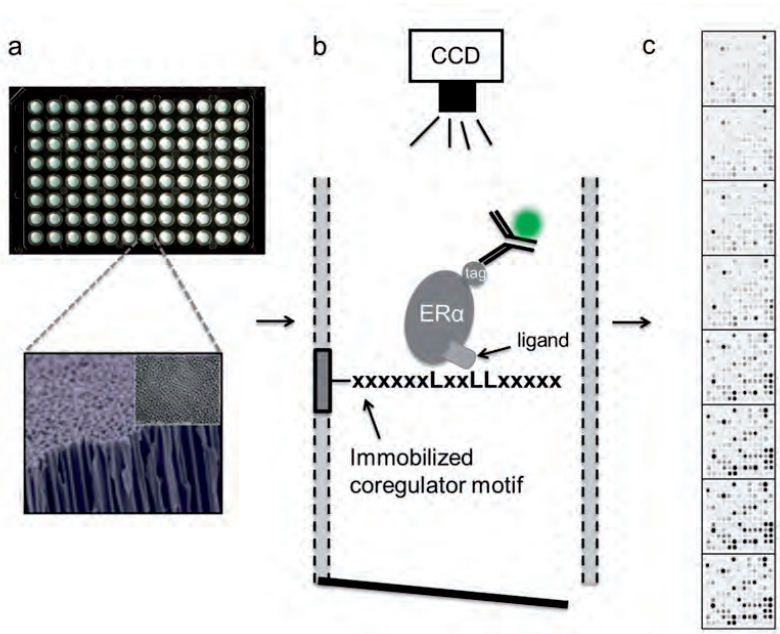


Figure 1. Schematic overview of the PamChip peptide microarray for nuclear receptor-coregulator interaction profiling. a) PamChip-96 design. The PamChip plate consists of 96 identical arrays, each array containing 155 NR coregulator peptides (coactivators and corepressors) immobilized on a porous metal oxide carrier; b) Ligand-modulated interaction of coregulators with ERα-LBD. Depending on the presence of ligand, the ERα-LBD will bind to coregulator-derived peptides, which can be detected by fluorescent antibodies against the ERα-LBD; c) Images obtained with an increasing series of concentrations of a ligand.

for 20 cycles with 25 µL of blocking buffer (TBS with 1% BSA, 0.01%, Tween-20, and 0.3% skimmed milk). Subsequently, the blocking buffer was removed, and 25 µL of the assay mix was transferred to each well/array on the plate and incubated for 80 cycles (~40 min). For each compound, 7–11 concentrations with 3-fold serial dilution in DMSO (final concentration 2%) were tested in two technical replicates (arrays). The tested compounds were distributed over four plate runs using 2% DMSO as the negative control and 50 µM E2 as the positive control on each plate (each measured in 4 technical replicates). After removal of the unbound receptor by washing each array with 25 µL of TBS, .tiff images were obtained by a CCD camera, which is part of the PamStation96 platform (Figure 1c).

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 was quantified within the spot (signal) as well as in a defined area surrounding it (background). The signal-minus-background value was subsequently used as the quantitative parameter of binding, and the average

over the duplicate measurements was calculated for each test compound concentration. Ligand dose–response curve fitting and hierarchical clustering (Euclidean distance, average linkage) were performed using the drc and stats packages in R (version 2.12.0, www.r-project.org). A sigmoidal, 4-parameter Hill (logistic) model (response = $((A - D)/(1 + ((\text{concentration}/C)^B))) + D$, with parameters A = response minimum, B = Hill slope, C = EC_{50}, and D = response maximum) 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.

The estrogenicity data used for comparisons with the presented ERα-coregulator binding data were ER binding data collected by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), [14] and ERα-controlled reporter gene transactivation data obtained by the Japanese Chemicals Evaluation and Research Institute (CERI) in the stably transfected hERα-HeLa-9903 human reporter cell line [15] prescribed by the OPPTS guideline 890.1300 [11] from which also our compound set was derived. The reporter gene assay using the hERα-HeLa-9903 human cell line is one of the ER transactivation assays that has been accepted for testing ER agonist activity and is part of the Test Guideline (TG) 455 from the Organisation for Economic Co-operation and Development (OECD).[16] 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 (NCTR, USA).[17]

RESULTS

Reproducibility and robustness of the ERα-coregulator binding assay

In a preceding study, it was demonstrated that ERα-coregulator interaction profiling, using the PamChip technology, would make a valuable contribution to an in vitro battery for estrogenicity testing.[10] With a view to practical application of such a coregulator binding assay, for example, in a regulatory toxicology setting, the present study assesses the technical characteristics of the assay, such as the reproducibility (by the same operator in the same laboratory) and robustness. The 14 compounds listed in Table 1 were tested in 7–11 concentrations for their capacity to modulate coregulator binding by the ERα-LBD. Each concentration was tested on duplicate arrays on the same plate. The entire sample set was distributed over 4 PamChip plates holding 96 arrays each, with 50 μM E2 as the positive control and 2% DMSO as a negative control on each plate, comprising 363 ERα-coregulator peptide array measurements in total. As an example, Figure 2a shows the induction of ERα-LBD-His binding to a peptide representing the NR-binding motif within amino acid 677–700 of the well-known nuclear receptor coactivator 1 (NCOA1_677_700) by E2, 17α-estradiol, 17α-methyltestosterone, and corticosterone, i.e., single measurements of all tested concentrations of these four compounds on 1 of the 155 coregulator peptides on the array. The lowest concentration of the potent E2 that resulted in a detectable binding of ERα-LBD-His to NCOA1_677_700 was 0.26 nM, a half maximal binding level (the EC_{50}) was reached at approximately 1.0 nM, and binding was saturated above 6.4 nM. As

expected, the less potent agonists 17α-estradiol and 17α-methyltestosterone reached the half maximal binding level at higher concentrations, and the maximal binding levels were lower than obtained for E2. All experiments together, represented a thousand-fold signal dynamic range, from a minimum of 40 to a maximum of 40000 measured arbitrary units. The coefficient of variation (CV) was calculated over each pair of duplicate data, and the median of the CVs per plate is shown in Table 2, ranging from 4.7 to 6.4% at the highest on plate 1. All duplicate data together are plotted against each other in Figure 3, showing the excellent reproducibility of the measurements, which is generally increasing with signal strength, resulting in a CV between duplicates around 5.0% (median value over the four plates) and a correlation (R^2) between the duplicates over all plates of 0.993 ($p < 0.0001$).

Determination of the estrogenic potency of the test compounds

Binding of the ERα-LBD-His to two coregulator binding motifs, NCOA1_677_700 and nuclear receptor-interacting protein 1 (NRIP1_805_831, binding motif within amino acids 805 – 831 of NRIP1), as a function of concentration is shown for the three positive reference compounds E2, 17α-estradiol, and 17α-methyltestosterone, the negative control corticosterone (Figure 2a and d), and for the 10 proficiency compounds recommended by the OPPTS. The proficiency compounds include 7 positive compounds (Figure 2b and e) and 3 negatives (Figure 2c and f). The selection of these coregulators was based on a previous study where the potency of tested compounds to induce the binding of ERα-LBD-GST to these coregulators was demonstrated to correlate well ($R^2 \geq 0.88$) with their in vivo estrogenic potency in the uterotrophic assay.[10] The ERα-LBD-His binding curves obtained for these two coregulators in the present study demonstrate that binding is negative for all nonestrogens in the OPPTS test set and positive for all known estrogens, except for butyl paraben. The strong ER-agonists E2, 17α-ethinyl estradiol, diethylstilbestrol, and hexestrol induced increased binding already at low concentrations (below 0.5 nM), and the moderate ER-agonists estrone and 17α-estradiol started to induce increased binding around 2 nM, while for the weak agonist genistein and very weak agonist 17α-methyltestosterone increased binding was observed to take off at a still higher concentration, around 200 nM and above 1 μM, respectively. In general, the maximal binding induced by a test compound decreased with decreasing ER agonist potency of the compound. Binding was reduced below baseline in the case of the very weak ER antagonist 1,3,5-tris(4-hydroxyphenyl)benzene (1,3,5-TB; Figure 2b and e). Because of its antagonistic coregulator binding behavior, it was not appropriate to include 1,3,5-TB in the comparisons described below regarding the ER-agonistic potency of the test compounds.

Table 2. Median CV over all points in the signal range (40 - 40.000) and correlation (R^2) between the duplicates listed per plate.

Plate number	1	2	3	4
Median CV(%)	6.4%	4.7%	4.7%	4.9%
R^2	0.9651	0.9936	0.9926	0.9961

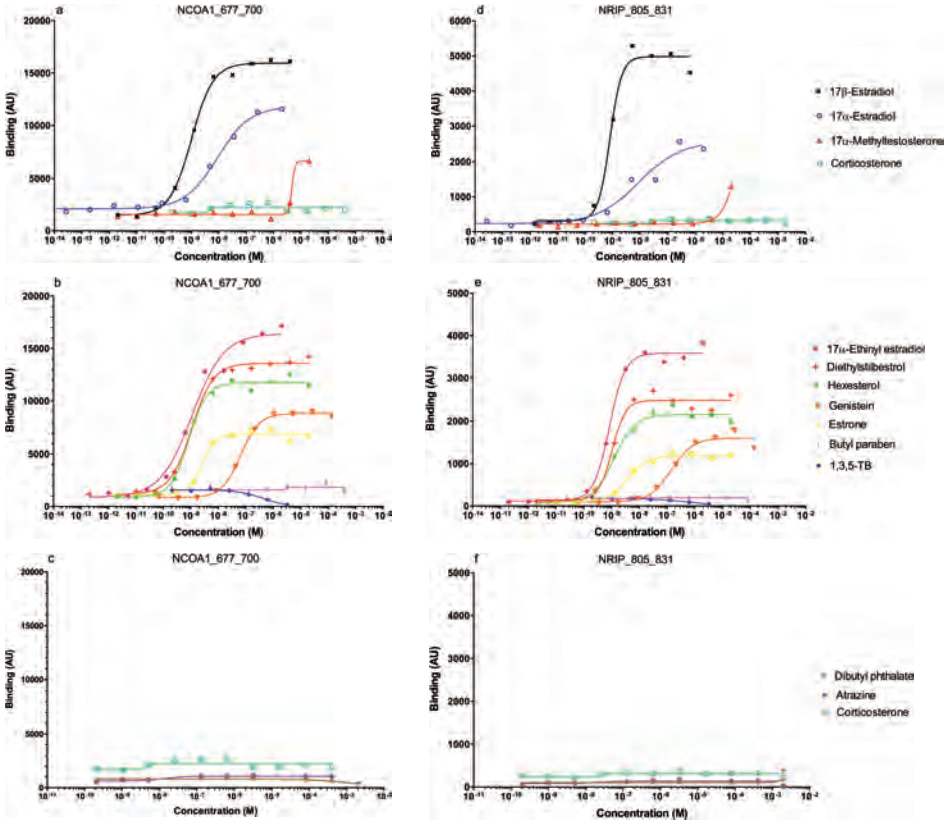


Figure 2. Binding of ERα-LBD-His to the coregulator binding motif peptides NCOA1_677_700 (a-c) and NRIP1_805_831 (d-f) as function of the concentration of the indicated compounds measured in the ERα-coregulator binding assay. Curve-fitting was performed using the 4-parameter Hill (logistic) equation allowing to calculate the concentration resulting in 50% of the maximum coregulator binding (EC_{50} values) based on the best fit. Subsequently an overall EC_{50} over all coregulators was obtained by calculating the median of the EC_{50} values from the individual curves, as shown in this figure, over all those coregulators showing a goodness-of-fit above 0.9. a and d) Reference compounds; b and e) proficiency compounds classified as positive, or (c and f) negative for ERα-mediated transcription activation by the OPPTS 890.1300 guideline.

For each test compound, dose–response data were obtained for all 155 coregulator-derived NR-binding motifs on the peptide microarray. The resulting dose–response curves allowed us to determine for each compound and each coregulator peptide an EC_{50} for inducing ERα binding to that peptide. To obtain an overall measure for the potency as an inducer of ERα-coregulator binding, the median of the EC_{50} values was determined, over all those coregulator peptides showing a comparatively good fit of the applied dose–response model (goodness-of-fit of the Hill equation above 0.9). Relative binding potencies (RBPs) compared to E2 were calculated on the basis of these median EC_{50} values (Table 3). For the positively tested estrogenic compounds, the number of coregulator peptides meeting this requirement varied

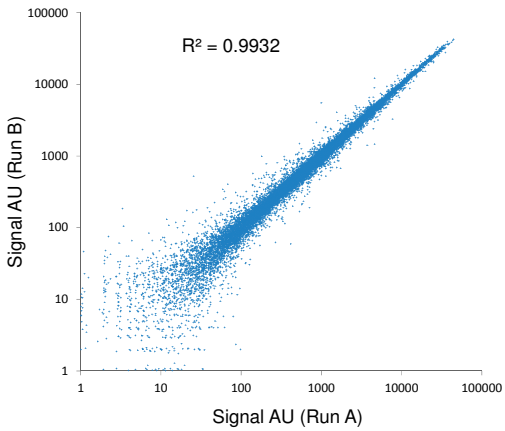


Figure 3. Technical variability of the PamChip ERα -coregulator binding assay. For each concentration of each compound duplicate assays were run on the same coregulator binding motif array plate. The signals (in arbitrary units, AU) of the two duplicate runs are plotted against each other for all samples tested. The average coefficient of variation (CV) between duplicate measurements was 5.0%. To achieve a more even distribution of the data we used a logarithmic scaling of the plot axes. The coefficient of determination (R^2 = 0.993, p < 0.0001) across all duplicates and plates was however calculated using the original binding values.

from 28 (for 17α-methyltestosterone) to 57 (for hexestrol). The relative potencies in the ERα-coregulator binding assay were then correlated with the relative affinities for ER binding as listed in the review of ICCVAM,[14] and the relative potency for ERα-controlled transcription activation insofar as available from CERI,[15] and were also compared to the estrogenicity in vivo as observed in the uterotrophic assay [17] (Table 3). Only one compound was misclassified in the ERα-coregulator binding assay, i.e., butyl paraben was a false negative as compared to the in vivo uterotrophic assay. The ER binding data from ICCVAM, however, included three misclassifications, i.e., two false positives (dibutyl phthalate and atrazine) and one false negative (17α-methyltestosterone). When leaving out the compounds misclassified by ICCVAM (this would introduce unjustified negative bias since for misclassified compounds a bad correlation with another estrogenicity assay is to be expected), the ERα-coregulator binding and ER binding assay resulted in the same potency ranking for 8 out of the 9 agonists tested and the correlation between the relative potency values for coregulator and ER binding was excellent (R^2 = 0.96, p < 0.0001, n=9), as is shown in Figure 4. Although ERα transactivation is one step further downstream along the signal transduction pathway than ERα-coregulator binding, the correlation between the ERα-coregulator binding and ERα transactivation assay was found to be excellent as well (Figure 5, R^2 = 0.94, p < 0.0001, n=11; 1,3,5-TB and butyl paraben were excluded from this comparison since no data were available for these two compounds in the transactivation assay). In contrast to the situation for ER binding, no discrepancies appear to exist between the transactivation data from CERI and estrogenicity in vivo. Altogether, these findings imply that, in terms of misclassification with respect to in vivo estrogenicity, ERα-coregulator binding performs better than ligand binding and was found to be comparable to hERα-HeLa-9903 transactivation assay.

ERα-coregulator binding profiles reflect compound structure similarity

For each test compound and each of the 155 coregulator–nuclear receptor binding motifs represented by the peptide microarray, the modulation index (MI) was calculated as the

Table 3. Estrogenic properties of the OPPTS test compounds in uterotrophic assay, ERα-coregulator binding assay, estrogen receptor binding assay and HeLa-9903 transactivation assay.

Chemical name	Uterotrophic assay [a]	ERα-coregulator binding Median EC_{50} (M)	ERα-coregulator binding log (Median RBP) [e]	ER binding log (Median RBA) [g]	ERα transactivation EC_{50} (M) [j]	ERα transactivation logREP [k]
17β-Estradiol	POS [b]	1.5E-09	2.0	2.0	8.2E-12	2.0
17α-Estradiol	POS	9.7E-09	1.2	1.0	6.0E-10	0.1
17α-MT	POS	7.6E-06	-1.7	-5.0	4.1E-6	-3.7
Corticosterone	NEG [b]	NEG	-5.0 [f]	-5.0	NEG	-5.0
Hexestrol	POS	1.0E-09	2.2	2.4	NA	1.5 [l]
EE2	POS	1.2E-09	2.1	2.2	5.7E-12	2.2
DES	POS	1.5E-09	2.0	2.1	2.4E-11	1.5
Estrone	POS	3.7E-09	1.6	1.6	4.9E-10	0.2
Genistein	POS	9.6E-08	0.2	0.2	2.5E-08	-1.5
1,3,5-TB	NA [c]	- [d]	-	-0.4 [h]	NA	NA
Butyl paraben	POS	NEG	-5.0	-3.1 [i]	NA	NA
Atrazine	NEG	NEG	-5.0	-3.5	NEG	-5.0
Dibutyl phthalate	NEG	NEG	-5.0	-2.6	NEG	-5.0
Corticosterone	NEG	NEG	-5.0	-5.0	NEG	-5.0

[a] Data reported by CERI *[15]* and National Center for Toxicological Research (NCTR, USA) *[17]*. [b] POS / NEG = classified as positive / negative for estrogenic effect. [c] Data not available. [d] Compound only showing antagonist binding effect, therefore the EC_{50} and relative potency values cannot be determined. [e] Relative binding potency values are calculated as the ratio of the concentration of E2 needed to achieve 50% of maximal ERα-LBD to coregulator binding (median EC_{50}) and the concentration of the test compound required to achieve a similar effect, and this ratio is subsequently multiplied by 100. The RBP value of E2 is thus 100, resulting in a log (RBP) of 2.0. [f] Compounds which were found inactive for a particular effect were assigned an arbitrary low cut-off value of -5.0 for the log (relative potency). [g] Logarithm of the median relative affinity value for ER binding as reported by ICCVAM *[14]*. [h] Obtained from Mizutani et al. 2012 *[41]* [i] Obtained from Blair et al. 2000 *[27]* [j] Data reported by CERI *[15]*, obtained using the human cell line hERα-HeLa-9903 as prescribed by the OPPTS guideline 890.1300. [k] Relative estrogenic potency is defined as the ration between the EC_{50} of E2 and the EC_{50} of the compound, and this ratio is subsequently multiplied by 100. The REP value of E2 is thus 100, resulting in a logREP of 2.0. [l] Based on the relative estrogenic potency reported by CERI *[15]*. Abbreviations: 17α-MT, 17α-Methyltestosterone; EE2, 17α-Ethinyl estradiol; DES, Diethylstilbestrol;

log-transformed ratio of receptor binding at a saturating compound concentration over that in the absence of ligand. The MIs for the 155 binding motif peptides constitute an ERα-coregulator binding profile for each compound tested. Unsupervised hierarchical clustering of these MI values over the various compounds and over all coregulator peptides on the array (Figure 6a) revealed that compounds with structural similarity tend to cluster together (Figure 6b). For instance, the structurally related compounds 17β-estradiol and 17α-estradiol, as well as hexestrol and diethylstilbestrol, and the two compounds with a butyl-4-benzoate moiety, i.e., butyl paraben and dibutyl phthalate,

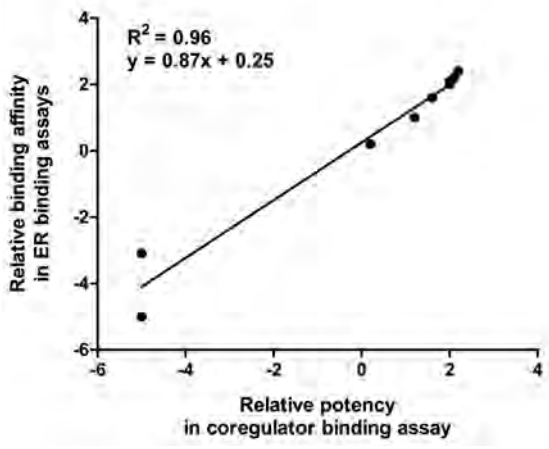


Figure 4. Correlation between the relative affinity for binding to the ER according to ICCVAM [14] and the relative potency to induce coregulator binding observed in this study, calculated over all coregulators showing a goodness-of-fit of the 4-parameter Hill equation above 0.9. The two data sets compared were both plotted as the logarithm of the relative value as compared to E2 expressed as a percentage. A cut-off value of -5.0 was arbitrarily assigned to inactive compounds (see Table 3). Coefficient of determination: R^2 = 0.96 ($p < 0.0001$, n = 9); correlation equation, y = 0.87x + 0.25.

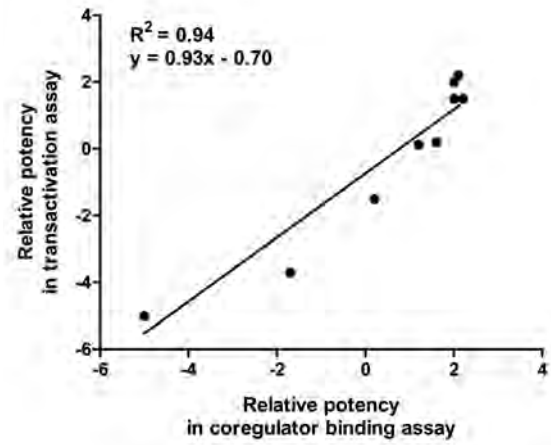


Figure 5. Correlation between the relative potency compared to E2 for ERα-mediated transcription activation, as reported by CERI [15], and for inducing ERα-coregulator binding, calculated over all coregulators showing a goodness-of-fit of the 4-parameter Hill equation above 0.9. Both relative potencies were expressed as the log(percentage of E2). A cut-off value of -5.0 was arbitrarily assigned to inactive compounds (see Table 3). R^2 = 0.94 ($p < 0.0001$, n = 11); correlation equation, y = 0.93x - 0.70.

cluster next to each other, while the steroids with an aromatic A-ring (17β-estradiol, 17α-ethinyl estradiol, 17α-estradiol, and estrone) are separated from those with a cyclohexene A-ring (corticosterone and 17α-methyltestosterone) (Figure 6b). As expected, the closest correlation is obtained between two sources of the same compound (17β-estradiol_1 and 17β-estradiol_2 in Figure 6b).

DISCUSSION

Endocrine disruption, including interference with ER signaling, is an important endpoint, also with a view to identifying a potential risk for reproductive toxicity, and is therefore an important aspect of chemical safety testing. The uterotrophic assay in premature or ovariectomized rodents measures increase in uterus weight. This is a strictly estrogen-dependent in vivo response and is considered the gold standard to identify compounds

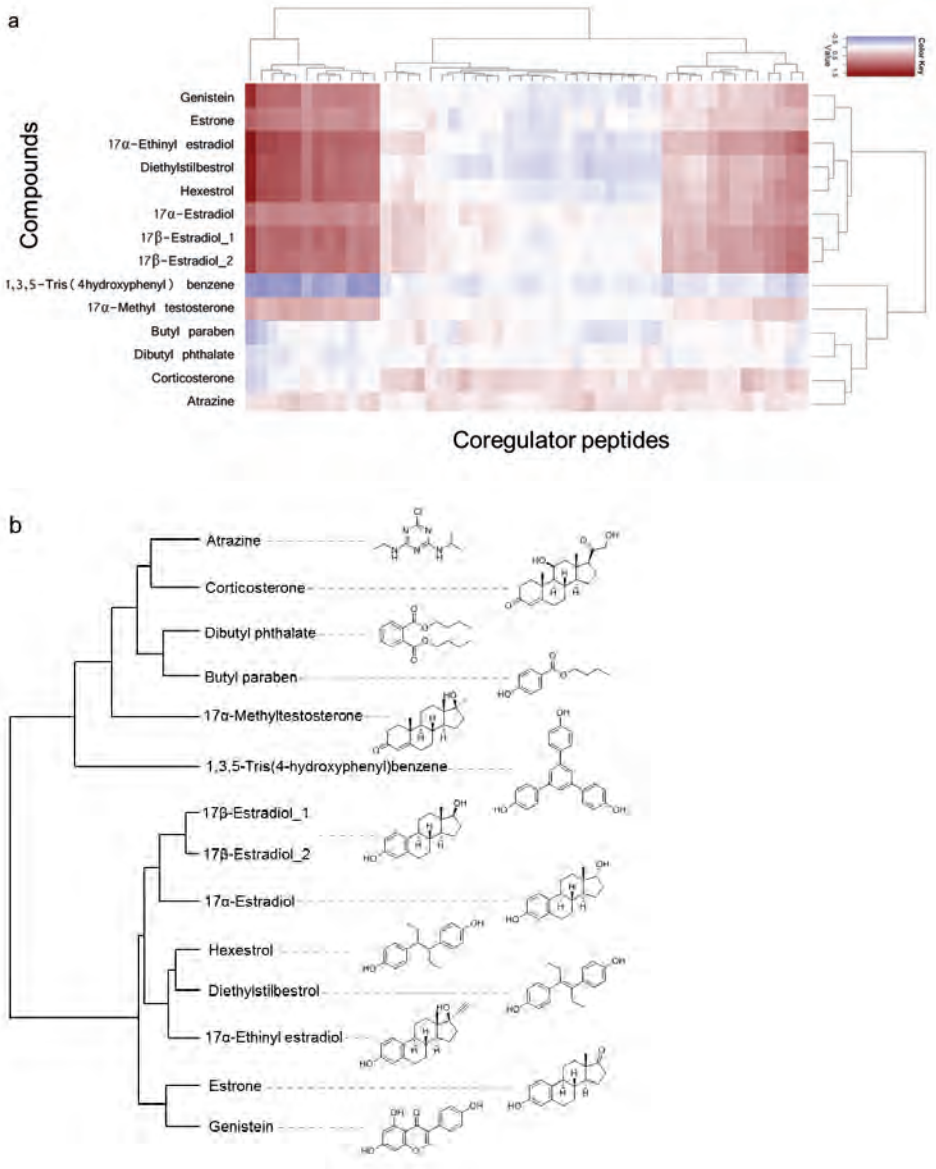


Figure 6. Heatmap of the unsupervised hierarchically clustered values for ERα-coregulator binding across compounds and coregulators shows structural similarity of compounds. a) Two-dimensional clustering heatmap; b) clustering pattern of compounds.

with estrogenic or antiestrogenic activity, the latter being tested in general by measuring the potential to inhibit the effect of an uterotrophic dose of the reference estrogen 17α-ethinyl estradiol.[18, 19] However, being a rodent-based in vivo assay, application of the uterotrophic assay has severe drawbacks because of the labor- and resource-intensive nature of animal

testing, not to mention the associated ethical issues and the resulting political and public resistance. Altogether, these issues hamper its practical application within massive screening programs such as the REACH initiative recently launched by the European Union. Therefore, the development of in vitro alternatives for in vivo (anti)estrogenicity tests, such as the uterotrophic assay, has a high priority. In a previous study,[10] it was reported that coregulator binding to ERα could be measured in vitro, using a microarray of peptides representing nuclear receptor binding motifs from various coregulators. ERα-coregulator binding measured using this array-based method appeared to be a very good predictor of estrogenic potency in vivo as observed in the uterotrophic assay (R^2 values for the in vitro–in vivo correlation were higher than 0.85 for 30 of the coregulator–nuclear receptor binding motifs tested). The present study was undertaken to establish whether the ERα-coregulator binding assay, using the PamChip plate in combination with ERα-LBD-His, gives reproducible results. In addition, the measured potencies (RBP values) of the current test panel of compounds to induce ERα-coregulator binding were compared to the relative affinities for ER binding as reported by ICCVAM,[14] and the relative potency for ERα-controlled transcription activation from CERI,[15] and were also compared to the estrogenicity in vivo as observed in the uterotrophic assay [17] (Table 3). Moreover, binding profiles were used to perform an unsupervised hierarchical clustering of the MI values over the various compounds and over all coregulator peptides on the array in order to determine whether the compounds with structural similarity cluster together.

With a median coefficient of variation of 5.0% and an excellent correlation ($R^2 = 0.993$) between duplicate measurements, the reproducibility of the ERα-coregulator binding assay was better than other commonly used in vitro ER functional assays. For example, for ER transactivation assays, such as the ER-CALUX, the coefficient of variation (CV) between replicate EC_{50} measurements of E2 was reported [20] to amount up to 32.7%, and in a more extensive reproducibility study with the ERα-CALUX the CV averaged over all compounds measured was 19.8% for EC_{50} determinations carried out in the same lab. [21] For the yeast estrogen screen (YES) assay,[22] another ER-controlled reporter gene transactivation assay using a recombinant yeast strain, the variability observed when measuring a saturating E2 concentration (6.96×10^9 M), expressed as the CV, amounted up to 7.4%. However, this relatively low CV was only achieved after sophisticated optimization of the protocol,[23] whereas studies with other types of yeast estrogen assays reported mean CVs of 18.0%[24] and 18.6%.[25] A variability in the same range as for the transactivation assays was reported for classical ERα ligand binding assays, e.g., a CV of 11% in a study performed by Kase et al. in 2009.[26] In addition to its high reproducibility, the present study also shows that the ERα-coregulator binding assay is correctly predicting the estrogenicity for 13 out of 14 compounds tested, as only the ER-agonistic properties of butyl paraben were not represented in the obtained binding profiles on the PamChip plate. However, this is probably due to the tested concentration range (20 nM–400 μM), which was too low compared to the reported IC_{50} (105 μM) for ER binding,[27] resulting in poor fitting of the applied dose–response model to the data, and as a consequence, classification of the response of butyl paraben as negative according to the data quality criterion applied. When the potency to induce ERα-coregulator

binding of the ER-agonists was calculated over the entire coregulator binding profile of a compound and compared to the potency as observed for in vitro ER binding, the potency ranking of 8 out of 9 ER-agonists tested was exactly the same for binding to ER (butyl paraben is missed in the PamChip plate assay). A similar comparison was performed with the potency to induce ERα transactivation, and again, the ranking of the compounds was found to be essentially similar for ERα-coregulator binding and ERα transactivation, with only minor nearest neighbor swaps among the strong estrogens tested. The correlation of coregulator binding with both ERα binding and ERα-mediated transcription activation was excellent (in both cases $R^2 \geq 0.94$). This correlation was obtained when the three misclassifications as to estrogenicity in vivo within the ER binding data from ICCVAM were left out,[14] i.e., two false positives (dibutyl phthalate and atrazine) and one false negative (17α-methyltestosterone). In addition to the ERα transactivation data from the CERI report [15] used in Table 3 of this study, the ICCVAM report also contains ER transactivation data, including a median EC_{50} calculated over the entire range of reporter gene studies covered by the report. Even though these EC_{50} values were based not only on the hERα-HeLa-9903 human cell line recommended by OPPTS,[11] but also on other mammalian cell lines, there were no inconsistencies (not shown) with our ERα-coregulator data, except for 17α-estradiol. The latter compound was reported by ICCVAM to be slightly more potent than E2, but this was not confirmed by CERI, and therefore, it is unclear whether 17α-estradiol presents a true exception. Taken together, this study provides additional evidence that the ERα-coregulator binding assay reliably reflects ERα agonist potency, which is the crucial factor for being uterotrophic and estrogenic in vivo. As shown before,[10] the PamChip plate assay also reflected inhibition of coregulator binding by the weak ER antagonist 1,3,5-TB (Figure 2b and 2e). This is consistent with the inhibitory effect observed with ER antagonists in previous studies using other ER–coregulator peptide binding assays,[28-30] and is an advantage over the classical ligand binding assay.

During recent years, our understanding of the dynamics of NR–mediated transactivation of gene transcription has increased substantially, leading to the perception that allosteric effects within the NR-coregulator complex are a major determinant of the complex formation process. Besides the ligand bound to the ligand-binding pocket of the NR,[31, 32] a number of other effectors have been reported to induce conformational changes in protein components or interfere with molecular interactions within the complex. These include cofactor proteins recruited to the complex,[33] as well as the response element nucleotide sequence interacting with the DNA-binding domain,[34] and even other small molecules,[35] which may also have affinity for other sites within a NR than the ligand-binding domain.[36] These allosteric effects have consequences for binding affinity and dynamics of the various components within the NR–coregulator complex, which ultimately determines target gene expression. For example, differences in the coregulator complement expressed in a certain cell type will not only have a direct effect on the composition of the ER–coregulator complex assembled on the estrogen response element (ERE) but also may influence the binding affinity of other coregulators. This may have consequences for the binding affinity for certain response elements as compared to others, dependent on nucleotide sequence context, and thereby for the target gene set of which the expression level is altered. Altogether, this points to a much

more complex role for coregulators of ERα and other NRs than just a connector to the RNA polymerase complex but rather indicates that NR coregulators provide an extra level of gene expression regulation, enabling subtle allosteric mechanisms of fine-tuning. Therefore, the possible relationship between ERα agonist structure and the induced coregulator binding profile was investigated in the present study by clustering the coregulator binding profiles in both dimensions (test compounds and coregulator peptides), applying hierarchical clustering with Euclidian distance as the cluster distance metric. The structurally related compounds, such as hexestrol and diethylstilbestrol, were found to cluster together, whereas the steroid test compounds having an aromatic A-ring were separated from those with a cyclohexene A-ring. Our observation that the coregulator binding profile induced by an ERα ligand reflects information about the molecular structure of the inducer, confirms that the molecular structure of an ERα ligand affects coregulator-binding affinities. This may be explained by the allosteric mechanisms discussed, implying that the actual conformation of the NR-LBD, imposed upon ligand binding, is an important determinant of coregulator binding. Using other techniques, mostly based on fluorescent microsphere-bound peptides to monitor interaction with ER receptors, it has been shown before that the ER-coactivator binding profile is dependent on the type of ligand bound.[29, 37-40] However, most studies involve only very few ER ligands and therefore can only reveal incidental associations between ligand molecular structure and the peptide binding profile induced. An exception is the study by Iannone et al. (2004),[38] which investigated 405 ERα-binding compounds and reported peptide binding profile similarity within relatively large clusters of compounds. However, the structures of the cluster members remain mostly unspecified, except for the structurally related compounds raloxifene, lasofoxifene, and levormeloxifene. However, E2 and diethylstilbestrol, which have a clearly different molecular structure, display a similar peptide binding profile in the study of Iannone et al. (2004), whereas these two compounds show a separate peptide binding profile in our study (Figure 6b), as was to be expected. Our study extends these preliminary observations to a larger group of ER ligands, including both closely and more distantly related molecular structures, and thereby decisively substantiates the relationship between the coactivator binding profile induced and ligand molecular structure. Moreover, our observations only involve known coregulator NR box-derived peptides, not random peptides,[38, 40] of which the mechanistic relevance is less obvious.

In conclusion, the results of the present study demonstrate that the high reproducibility of the coregulator binding assay in combination with a high multiplicity level (155 coregulator peptides for 96 samples tested in one plate run) and short processing time (less than 2h), present important benefits, enhancing its practical applicability as a high-throughput estrogenicity assay within current chemical safety screening programs. Moreover, the results of the present study illustrate the biological relevance of the outcomes of the ERα-coregulator binding assay, which even reflects the structural similarity of ERα agonists. Thus, the ERα-coregulator binding assay is a promising assay that has the potential to achieve identification and classification of ERα endocrine disruptors with high fidelity for those disruptors affecting the ERα directly. Further validation with a larger set of test compounds will be the next step toward implementation in practice.

ACKNOWLEDGEMENTS

We thank Diana Melchers, PamGene International B.V., 's-Hertogenbosch, The Netherlands, for her technical support with the PamChip peptide microarray experiment.

ABBREVIATIONS

ER, estrogen receptor; NR, nuclear receptor; LBD, ligand-binding domain; GST, glutathione-*S*-transferase; His, histidine; SERM, selective estrogen receptor modulator; OPPTS, Office of Prevention, Pesticides and Toxic Substances; ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; OECD, Organisation for Economic Co-operation and Development (OECD); CERI, Chemicals Evaluation and Research Institute, Japan; EDKB, Endocrine Disruptor Knowledge Base; E2, 17β-estradiol; 1,3,5-TB, 1,3,5-tris(4-hydroxyphenyl)benzene; DMSO, dimethylsulfoxide; NCOA1, nuclear receptor coactivator 1; NRIP1, nuclear receptor-interacting protein 1; MI, modulation index; CALUX, chemical-activated luciferase gene expression; ERE, estrogen response element; CV, coefficient of variation; YES, yeast estrogen screen

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7

Evaluation of an extended test panel for in vitro estrogenicity testing

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ABSTRACT

In the present study the previously established integrated testing strategy (ITS) for in vitro estrogenicity testing was extended with additional in vitro assays in order to broaden its sensitivity to different modes of estrogenic action than estrogen receptor (ER) binding. To this end an additional series of 10 estrogenic compounds with modes of action in part different from ER binding were tested in the previously defined ITS, i.e., in a yeast estrogen reporter gene assay, a U2OS estrogen receptor (ER) α CALUX reporter gene assay and a cell-free coregulator binding assay. Results show that the two reporter gene assays in the ITS accurately predict the estrogenicity of the model compounds and revealed 100% concordance with the in vivo uterotrophic assay. Moreover, the coregulator binding profiles provided new mechanistic insights into estrogen receptor signaling of the model compounds tested. Moreover, by adding androgen reporter gene assays as well as the H295R steroidogenesis assay to the ITS, several model compounds also showed potent antiandrogenic properties and effects on steroidogenesis that might potentiate the estrogenic effects in vivo. By covering these additional mechanisms of estrogen-related endocrine disruption, this extended ITS will go beyond in vivo estrogenicity testing by the uterotrophic assay. The extended ITS presented in this study may allow easy high-throughput screening and prioritization of chemicals, thereby contributing to refinement, reduction and to some extent even a replacement of current animal testing for (anti)estrogenic effects.

INTRODUCTION

With a view to the regulation on Registration, Evaluation and Authorisation of Chemicals (REACH) in the European Union and the need for reducing, refining and replacing (3Rs) the use of experimental animals for safety testing, there is a clear need to develop high-throughput in vitro methods to efficiently screen chemicals and prioritize them for further testing. Due to the increasing need for new methods to be developed and proposed for validation in the European Union, the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) has been formally established in 2011. In the US, the Environmental Protection Agency (EPA) is in the process of establishing in vitro testing programs and strategies to predict potential toxicity and to develop a cost-effective approach to prioritize toxicity testing of chemicals. For example, one of the leading programs is ToxCast, which is currently evaluating over 1,000 chemicals in more than 600 in vitro assays resulting in hazard identification. [1]

Testing chemicals for their endocrine-disrupting potential, including interference with estrogen receptor signaling, is an important factor to be taken into account when assessing the safety of currently used chemicals. Presently, the standard test for disruption of normal estrogen function is the Allen and Doisy test, a uterotrophic assay in immature or ovariectomised rodents with uterus weight as the crucial read-out parameter. [2, 3] Due to the high costs, ethical objections and labour intensiveness of the in vivo uterotrophic assay, the development of an in vitro battery for prediction of in vivo (anti)estrogenicity has high priority. We have previously demonstrated that combination of a yeast or U2OS estrogen receptor α (ERα) reporter gene assay with a cell-free coregulator binding assay that enables characterisation of ligand-modulated interaction of coregulators with ERα, would have the potential to form an adequate integrated testing strategy (ITS) for in vitro estrogenicity testing. These three assays were selected from a large battery of in vitro tests based on the fact that their outcomes correlated best with results from the in vivo uterotrophic assay [4, 5] for a set of reference compounds selected from the ICCVAM list of compounds defined for validation of in vitro tests for estrogenicity. [6] However, the uterotrophic assay has its limits for estrogenicity testing, for example because it does not include estrogenic effects on the hypothalamic-pituitary-gonadal (HPG) axis or male organs, since the assay uses either female ovariectomised or immature rodents, i.e. animals without endogenous estrogen production. Additional in vivo assays are needed to achieve more comprehensive estrogenicity screening, and provide data on multiple mechanisms and organs, e.g. the female and male pubertal assays (OPPTS 890.1450 and OPPTS 890.1500, respectively). Indeed, the shortcoming of the uterotrophic assay has been demonstrated by the fact that several compounds failed to show an (anti)estrogenic effect in the uterotrophic assay while eliciting (anti)estrogenic effects in other in vivo tests, e.g. atrazine and carbon tetrachloride (CCl_4). Atrazine delayed puberty and sexual development in both male and female rodents [7, 8] and CCl_4 is estrogenic as it decreases the metabolic inactivation of endogenous estradiol. [9, 10] In addition, antiandrogenic compounds elicit in vivo effects by the androgen receptor (AR) that can hardly or not be distinguished from an estrogenic effect by an estrogen via the ER and some compounds even have combined modes of action, e.g. several

estrogenic compounds like 17α-ethinyl estradiol (EE2) and diethylstilbestrol (DES) are also antiandrogenic, and in this way enhance the overall 'estrogenic' effect in vivo. [11, 12] The previously established ITS correlates well with the in vivo uterothropic assay, but is expected to suffer from the same drawbacks as the in vivo uterotrophic assay, as the selected in vitro bioassays only measure effects directly mediated by ERα, and are not able to detect chemicals that elicit their estrogenic effects through indirect mechanisms, such as, alteration of hormone biosynthesis, hormone metabolism and transport, and mixed estrogenic/anti-androgenic effects. This emphasises the need to extend the previously established in vitro ITS, by including androgen reporter gene assays and the H295R steroidogenesis assay. Such an extended ITS would have the clear advantage that it would outcompete the uterotrophic assay with respect to the multitude of mechanisms of estrogen signaling disruption that can be detected. Thereby, such an extended ITS would allow to further reduce or eliminate the need for testing certain endocrine disruption effects in animal models.

The aim of the present study was to demonstrate whether the extension of the previously established ITS for in vitro estrogenicity testing by two androgen reporter gene assays as well as the H295R steroidogenesis assay goes beyond replacement of the in vivo uterotrophic assay, i.e., is able to detect (anti)estrogenic and antiandrogenic effects in intact animals as observed in the female and male pubertal assays and the Hershberger assay (OECD 441). Ten compounds, with (expected) specific modes of action, were tested, i.e., equilin, mestranol, diethylstilbestrol monomethyl ether (DES-ME), bisphenol A (BPA), bisphenol B (BPB), bisphenol C1 (BPC1), bisphenol C2 (BPC2), butyl paraben, atrazine and vinclozolin. Equilin was included because it is structurally related to the well-known ERα agonist estrone. Mestranol and DES-ME were included because they are structural analogues of the previously tested EE2 and DES that have been shown to be able to activate the ER and inactivate the AR. Bisphenol A (BPA) was included since this compound is shown to be weakly estrogenic in several in vitro models, [13-15] whereas controversy exists about the relevance of these effects for the in vivo situation. [16] Some studies suggest that BPA has a greater in vivo potency than would be predicted based on the results obtained in vitro, indicating that certain test animal species might be more sensitive to BPA than the in vitro models commonly used, [17] while other studies suggest the opposite. [18, 19] Since it is also reported that some BPA analogues might even be more potent than BPA, [20] bisphenol B (BPB), bisphenol C1 (BPC1) and bisphenol C2 (BPC2) were included in order to investigate this phenolic compound group in more detail. The herbicide atrazine and the pesticide vinclozolin do not show any direct ER (ant)agonistic effects but have been shown to exert estrogenic effects through mechanisms other than ER binding. [21-24] The endogenous estrogen 17β-estradiol (E2) was chosen as a reference compound and was used to derive relative estrogenic potencies of the ten newly selected test compounds.

MATERIALS AND METHODS

Chemicals

Atrazine, bisphenol A (BPA), bisphenol B, bisphenol C1, bisphenol C2, butyl paraben, equilin, 17ß-estradiol (E2), flutamide, mestranol, tamoxifen, vinclozolin, 4-dimethyl-aminopyridine,

2-methyl-6-nitrobenzoic anhydride, picolinic acid, triethylamine, tetrahydrofuran, Dulbecco's modified Eagle medium/Ham's F-12 nutrient mix (DMEM/F12), $NaHCO_3$ and PBS were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Diethylstilbestrol monomethyl ether (DES-ME) was purchased from LGC Standards GmbH (Wesel, Germany). Ammonia, acetic acid, formic acid and dimethyl sulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany). Methanol, acetonitrile, acetone and ethanol were from Biosolve (Valkenswaard, The Netherlands). Pregnenolone, 17α-OH-pregnenolone, progesterone, 17α-OH-progesterone, dehydroepiandrosterone (DHEA), androstenedione, testosterone, estrone, estradiol, 11-deoxycorticosterone, corticosterone, 11-deoxycortisol, cortisol and dihydrotestosterone (DHT) were obtained from Steraloids (Newport, RI, USA) and the deuterium-labelled internal steroid standards were from CDN isotopes (Point-Claire, Canada) and ITS+ premix and NuSerum from BD Biosciences (Bedford, MA, USA). Chemicals to prepare the growth media for yeast were described previously [25]. Milli-Q water was obtained using a Purelab Ultra system from Elga (Bucks, UK).

CALUX bioassays

The ERα and AR agonist or antagonist potencies of a test compound were determined in the ERα- and AR-CALUX bioassays, respectively, as described previously. [26, 27] In short, CALUX cells were plated in 96-well plates with phenol red-free DMEM/F12 (1:1) mixture supplemented with dextran-coated charcoal-stripped FCS. One day later, the medium was refreshed and cells were incubated with the compounds to be tested (final DMSO concentration 2%). Each test compound at each concentration was tested in triplicate. After 24 h exposure, the medium was removed, cells were lysed in Triton lysis buffer and measured for luciferase activity. To test for antagonism, CALUX cells were incubated with test compound in combination with a non-saturating level of agonist (around the EC_{50} value of the reference compound), i.e., 10^{-11} M E2 in the ERα-CALUX bioassay, and 2×10^{-10} M DHT in the AR-CALUX bioassay.

Yeast estrogen and androgen bioassays

The estrogenic, anti-estrogenic, androgenic and anti-androgenic properties of the compounds were tested as described previously. [11, 28] In short, cultures of the yeast estrogen and androgen biosensor were grown overnight at 30 °C with vigorous orbital shaking. At the late log phase, the culture was diluted in selective MM/L medium till an OD value at 630 nm between 0.04 and 0.06 was reached. To expose the yeast cells, 200 µL aliquots of the diluted culture were pipetted into each well of a 96-well plate and 2 µL of a stock solution in DMSO was added when testing the agonistic properties of the compounds (final DMSO concentration 2%). To test for antagonism, yeast cells were incubated with test compound in combination with a non-saturating level of agonist (around the EC_{50} value of the reference compound), i.e., 10^{-9} M E2 in the yeast estrogen bioassay, and 7×10^{-9} M T in the yeast androgen bioassay. DMSO (negative control), and E2 or T (positive controls) were included in each experiment, and each sample concentration was assayed in triplicate. Exposure was performed for 24 h at 30 °C and orbital shaking at 125 rpm. Fluorescence and

OD were measured at 0 and 24 h directly in a SynergyTM HT Multi-Detection Microplate Reader (BioTek Instruments Inc., USA) using excitation at 485 nm and emission at 530 nm. Normally, the cell density at 630 nm increases from 0.05 at 0 h to about 0.9 at 24 h. If the OD at 24 h was below 0.7, concentrations of the test compound were considered to cause cytotoxicity and the corresponding data were rejected for analysis.

ERα coregulator binding assay

Ligand-modulated interaction of ERα-LBD-His with coregulators was assessed using a PamChip® plate (PamGene International B.V., 's-Hertogenbosch, The Netherlands) containing 96 identical peptide microarrays as described previously. [4, 29-31] The peptide microarray was incubated with the test solution containing ERα-LBD-His in the absence or presence of ligand by pumping the sample up and down the three-dimensional metal oxide carrier. In short, assay mixtures were prepared on ice in a master 96-well plate and contained ERα-LBD-His (optimal assay concentration of a crude lysate containing the conjugate was empirically determined, and estimated to lie between 1 and 10 nM), 25 nM of an Alexa Fluor 488-conjugated polyhistidine antibody (penta-His Alexa Fluor 488 conjugate, Qiagen no. 35310), and ligand at the indicated concentration in reaction buffer (20mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% BSA, 0.05% Tween-20). All assays were performed in a fully automated microarray processing platform (PamStation®96, PamGene International B.V.) at 20 °C applying two incubation cycles per min. For each compound, 8 concentrations added from 50 fold concentrated stock solutions with fivefold serial dilutions in DMSO were tested in singular (final DMSO concentration 2%). After removal of the unbound receptor by washing each array with 25 µL Tris-buffered saline, tiff images were obtained by the CCD camera which is part of the PamStation®96 platform. The tested compounds were distributed over one plate run using 2% DMSO as the negative control and 50 µM E2 as the positive control (each control measured in 4 technical replicates).

Enhanced H295R steroidogenesis assay

Human H295R adrenocarcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured according to the protocol described in the OECD test guideline 456. Cells were routinely grown at 37 °C under 5% CO_2 atmosphere in 75 cm^2 culture flasks containing 12 mL DMEM/F12 culture medium supplemented with 1.2 g/L $NaHCO_3$, 1% ITS+ premix and 2.5% NuSerum. For sub-culturing, the H295R cells were washed three times with PBS, detached by trypsine/EDTA (0.25/0.05 v/v-% in HBSS) and seeded in a 1:3 ratio. For testing, 1 mL cell suspension containing 2×10^5 to 3×10^5 cells was seeded in each well of the 24-well plate. After 24 h, the medium was refreshed and compounds dissolved in DMSO (1µL) were added. Exposures were performed in triplicate and the final concentration of the solvent carrier DMSO was 0.1%. After 48 h of exposure, the medium was stored at -80 °C for steroid hormone analysis. Effects on viability and cytotoxicity were evaluated by the live/dead viability/cytotoxicity kit (Molecular probes, Eugene, OR, USA) using the protocol described by the OECD (OECD TG 456). After washing the cells with PBS twice, a calcein and ethidium

bromide solution was added to the cells. After 1 h, fluorescence was measured (excitation/emission at 530/645 nm and excitation/emission at 485/530 nm) using a SynergyTM HT multi-detection microplate reader (BioTek Instruments Inc., USA). Exposures showing a decrease in cell viability were excluded from hormone analysis.

For hormone analysis, 900 µL H295R medium aliquots were adjusted to 1 mL with 18 µL of deuterium labelled internal steroid standard mix and Milli-Q water. Similarly, a standard steroid curve was prepared by spiking 900 µL of supplemented DMEM-F12 medium with a mix of steroid standards, resulting in final concentrations of 10, 25, 50, 100, 250, 500, 1000, 2500, 5000, 10000 and 25000 pg/mL. Next, both standards and samples were subjected to solid-phase extraction (SPE) using OASIS HLB cartridges (Waters, 60 mg) in 96-wells format, previously conditioned with 1 mL methanol and 1 mL Milli-Q water. Washing was carried out with subsequently 1 mL Milli-Q water, 1 mL of methanol/water/acetic acid (55:43:2, v/v/v-%), 1 mL of methanol/water/25% ammonia (30:62:8, v/v/v-%), 1 mL MillQ water and 1 mL acetonitrile/water (35/65, v/v-%). The free steroids were eluted with 1 mL acetone and this eluate was evaporated to dryness at 45 °C under nitrogen. The derivatising reagent was prepared freshly before use by mixing 1 mg 4-dimethyl-aminopyridine, 5 mg 2-methyl-6-nitrobenzoic anhydride and 3 mg picolinic acid in 1 mL tetrahydrofuran, after which 10 µL of triethylamine was added. Picolinoyl derivatisation was achieved by incubating the dried sample extracts with 35 µL of derivatisation reagent for 45 min at room temperature. The reaction was terminated by adding 50 µL of a 5% ammonia solution after which the samples were analysed directly by Ultra Performance Liquid Chromatography tandem Mass Spectrometry (UPLC-MS/MS) according to the method described by Rijk et al. (manuscript in preparation). Steroid hormone concentrations in the samples were calculated using the steroid standard reference line, constructed by plotting the peak area ratios versus the spiked concentration.

Data analysis

For the CALUX bioassays, luciferase activity per well was measured as relative light units (RLU). Fold induction was calculated by dividing the mean value of light units from exposed and non-exposed (DMSO control) wells. For the yeast bioassays, the fluorescence signals were corrected for the signal obtained with the MM/L medium containing DMSO solvent only. The relative estrogenic potency (REP), defined as the ratio of the concentration of E2 needed to achieve 50% of maximal response (EC_{50}) and the concentration of the test compounds required to achieve a similar effect multiplied by 100, was calculated from fitted dose–response curves (four parameter sigmoidal dose-response curve, Graphpad Prism software version 5.04). The REP value for E2 is, hereby, set at 100 for both the CALUX and the yeast ERα reporter gene assays. The relative transactivation activity (RTA) of each compound tested was calculated as the ratio of maximal luciferase or yEGFP reporter gene induction values of each compound and the maximal induction value of reference compound E2. The antiandrogenic activity was characterized by the IC_{50}, i.e. the concentration that inhibited the response of DHT or T by 50%.

For the coregulator binding assay, image analysis was performed using BioNavigator software (PamGene International B.V.) as described previously. [4, 29] In short, the boundaries

of a spot are determined and the median fluorescent signal was quantified within the spot (signal) as well as in a defined area surrounding it (background). The signal-minus-background value was subsequently used as the quantitative parameter of binding, and the average over the duplicate measurements was calculated for each test compound concentration. Ligand dose-response curve fitting and hierarchical clustering (Euclidean distance, average linkage) were performed using the drc and stats packages in R (version 2.12.0, www.r-project.org). A sigmoidal, 4-parameter Hill (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.

Fold changes in steroids levels in the H295R steroidogenesis assay were calculated by comparing the mean steroid levels of the DMSO solvent control (SC) versus the mean steroid levels in medium of H295R cells exposed to the compound under investigation. A one-tailed Student's t-test was used to test for significance. For comparison of the presented in vitro 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 (NCTR, USA). [32]

RESULTS

Estrogenic and antiestrogenic activities

Ten compounds, selected based on their different modes of disruption of normal estrogen action, were tested in the U2OS ERα-CALUX and the yeast estrogen bioassay for estrogenic and antiestrogenic activities. Figure 1 shows typical dose-response curves for several of the compounds as obtained in these two assays. The EC_{50} and relative transcriptional activity (RTA) value of each compound was calculated from the fitted dose-response curve and are shown in Table 1. The reference compound estradiol (E2) was the most potent ER agonist and showed an EC_{50} of 8.8×10^{-12} M and 8.7×10^{-10} M in the U2OS ERα-CALUX and the yeast estrogen bioassay, respectively. Equilin, mestranol and DES ME were about 2-30 times less potent compared to E2 and the obtained RTA values were between 80% and 105% (E2 being 100%). All four phenolic compounds showed full agonistic responses in the two reporter gene assays (Figure 1 and Table 1), with BPC1 being the most potent compound and BPA being the weakest compound in both assays. Butyl paraben was negative under the concentration range tested in a previous study using the ERα-coregulator binding assay [29]. However, when tested at higher concentrations this compound was clearly positive in the yeast and U2OS cell based estrogen bioassays and induced a much higher maximal response (RTA =256%) than E2 in the U2OS ERα-CALUX. The fungicide vinclozolin was slightly active (RTA < 10%) in both cell based estrogen bioassays, showing only a response at the highest concentration tested, and therefore the EC_{50} value could not be calculated reliably. The herbicide atrazine was not active in these two reporter gene assays. In addition to the agonist activity, all the compounds were tested in combination with E2 for potential antagonist activities. However, no ER-antagonism was observed for any of the compounds tested (data not shown).

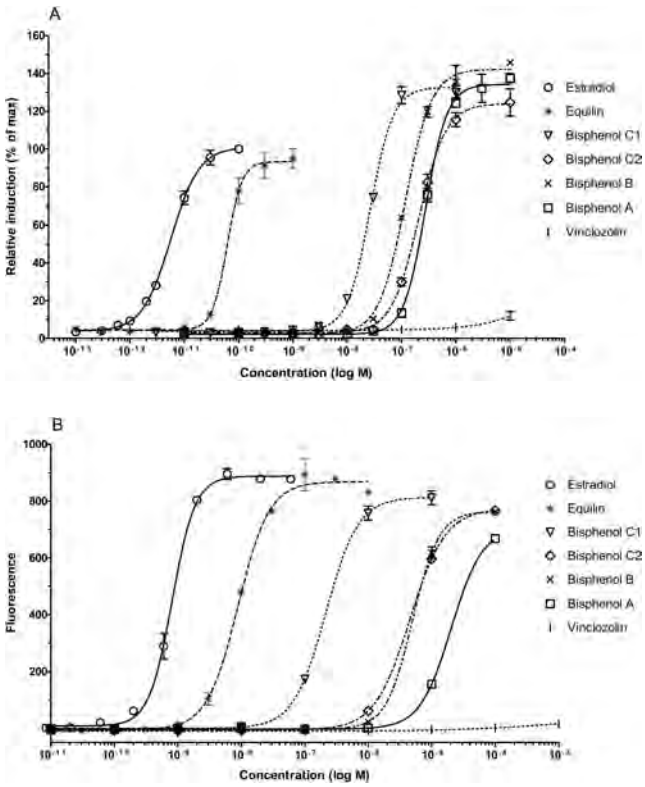

Figure 1. Dose-response curves of the indicated test compounds in the ERα-CALUX (A) and yeast estrogen bioassay (B). The response is displayed as the mean with SD of a triplicate measurement.

Coregulator binding

The 10 compounds were also tested in the coregulator binding assay to evaluate their capacity to modulate ERα-LBD binding to NR-coregulator motifs. As an example, Figure 2 shows the induction of ERα-LBD-His binding to a peptide representing the NR-binding motif within amino acid 677-700 of the well-known nuclear receptor coactivator 1 (NCOA1_677_700) by several compounds tested, i.e. single measurements of 8 concentrations per compound on 1 of the 155 coregulator peptides on the array. The lowest concentration of E2 resulting in a detectable binding of ERα-LBD-His to NCOA1_677_700 was 1.3×10^{-9} M, and a half maximal binding level (EC_{50}) was reached at approximately 6.4×10^{-9} M. Equilin, mestranol and DES-ME also induced binding of ERα-LBD to NCOA1_677_700, while the bisphenol compounds displayed antagonist binding curves on NCOA1_677_700 when compared to the solvent control DMSO and the known ER-agonists (Figure 2).

Dose response curves were generated for all 155 coregulator-derived NR-binding motifs for each compound in order to obtain an overall measure for the potency as an inducer of ERα-coregulator binding. These dose-response curves allowed to determine for each coregulator

Table 1. Estrogenic activities of the test compounds in the in vivo uterotrophic bioassay, U2OS ERα-CALUX assay, yeast estrogen bioassay and ERα coregulator binding assay.

			U2OS ERα-CALUX bioassay			Yeast estrogen bioassay			ERα coregulator binding assay	
Compound	**CAS nr.**	**Uterotrophic assay logRP[a]**	**EC_{50} (M)**	**logREP**	**RTA (%)**	**EC_{50} (M)**	**logREP**	**RTA (%)**	**Median EC_{50} [e] / IC_{50} [f] (M)**	**logRBP**
Estradiol	50-28-2	2.0	8.8×10^{-12}	2.0	100	8.7×10^{-10}	2.0	100	7.5×10^{-9} [e]	2.0
Equilin	474-86-2	1.7	6.5×10^{-11}	1.1	95	8.7×10^{-09}	1.0	105	3.4×10^{-8} [e]	1.3
Mestranol	72-33-3	2.0	3.2×10^{-10}	0.4	84	7.9×10^{-09}	1.0	88	5.1×10^{-7} [e]	0.2
DES-ME	7773-60-6	1.9	2.4×10^{-10}	0.6	88	1.8×10^{-09}	1.7	94	1.7×10^{-7} [e]	0.6
Bisphenol A	80-05-7	-1.6	2.7×10^{-07}	-2.5	136	2.0×10^{-05}	-2.4	76	-	-5.0
Bisphenol B	77-40-7	+ [b]	1.2×10^{-07}	-2.1	144	5.0×10^{-06}	-1.8	87	3.2×10^{-6} [f]	na
Bisphenol C1	14868-03-2	NA [c]	2.7×10^{-08}	-1.5	133	2.2×10^{-07}	-0.4	93	2.7×10^{-8} [f]	na
Bisphenol C2	79-97-0	NA	2.1×10^{-07}	-2.4	125	4.5×10^{-06}	-1.6	87	7.4×10^{-4} [f]	na
Butyl paraben	94-26-8	+	2.9×10^{-06}	-3.5	256	5.1×10^{-06}	-1.8	86	-	-5.0
Atrazine	1912-24-9	-5.0 [d]	- [b]	-5.0	1	-	-5.0	0	-	-5.0
Vinclozolin	50471-44-8	-5.0	-	-5.0	8	-	-5.0	4	-	-5.0

[a] Median relative potency values based on the uterotrophic assay in mouse or rat, derived from EDKB (NCTR,USA) [32]. Estradiol is used as a reference chemical and is defined to have a relative potency of 100 (logRP=2.0).
[b] + = positive; - = negative
[c] NA =not available; na = not applicable
[d] A cut-off value of -5.0 is listed for compounds showing no effect.

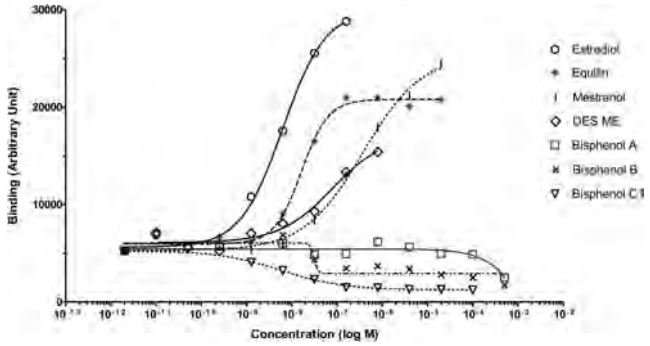

Figure 2. Binding of ERα-LBD-His to the coregulator binding motif peptide NCOA1_677_700 as function of the concentration of the indicated compounds measured in the ERα-coregulator binding assay.

peptide an EC_{50} for inducing ERα binding by a compound to that peptide. The median EC_{50} value of each compound was determined over 48 coregulator peptides that show a comparatively good fit of the applied dose-response model (goodness-of-fit of the four parameter Hill equation above 0.9) and are shown in Table 1. The selection of these coregulators was based on a previous study where the potency of compounds to induce binding of ERα-LBD-GST to these coregulators was demonstrated to correlate well ($R^2 \geq 0.80$) with their in vivo determined estrogenic potency in the uterotrophic assay. [4] Equilin, mestranol and DES-ME resulted in coregulator binding profiles similar to that of E2 and the calculated median EC_{50} values were in the nanomolar range. BPA showed a slightly bell-shaped dose-response binding profile, i.e. inducing low level binding to coactivators at low concentrations and inhibitory effects at high concentrations (not shown). In contrast, BPB, BPC1 and BPC2 strongly inhibited binding of ERα-LBD to almost all coactivator peptides on the peptide microarray, however, no increased binding was observed for any of the compounds on the corepressors present on the peptide microarray (see supplementary Figure 1 for BPC1 as an example). To provide an overall measure of the antagonistic binding potency for the bisphenols, the median IC_{50} values were calculated and are shown in Table 1. Vinclozolin showed no statistically significant altered binding signals compared to the DMSO solvent control. Butyl paraben was negative under the concentration range (20 nM–400 μM) tested in a previous study. However, higher concentrations of butyl paraben tested in the current study lead to precipitation in the assay buffer and resulted in an overall negative binding profile. Atrazine was also tested in our previous study (negative) and the results for the coregulator binding assay were taken from that study. For each test compound and each of the 155 coregulator-nuclear receptor binding motifs represented by the peptide microarray, the modulation index (MI) was calculated as the log-transformed ratio of receptor binding at a saturating compound concentration over that in the absence of ligand. The MIs for the 155 binding motif peptides constitute an ERα-coregulator binding profile for each compound tested. Unsupervised hierarchical clustering of these MI values over the various compounds over all coregulator peptides on the array revealed that compounds with structural similarity tend to cluster together (Figure 3).

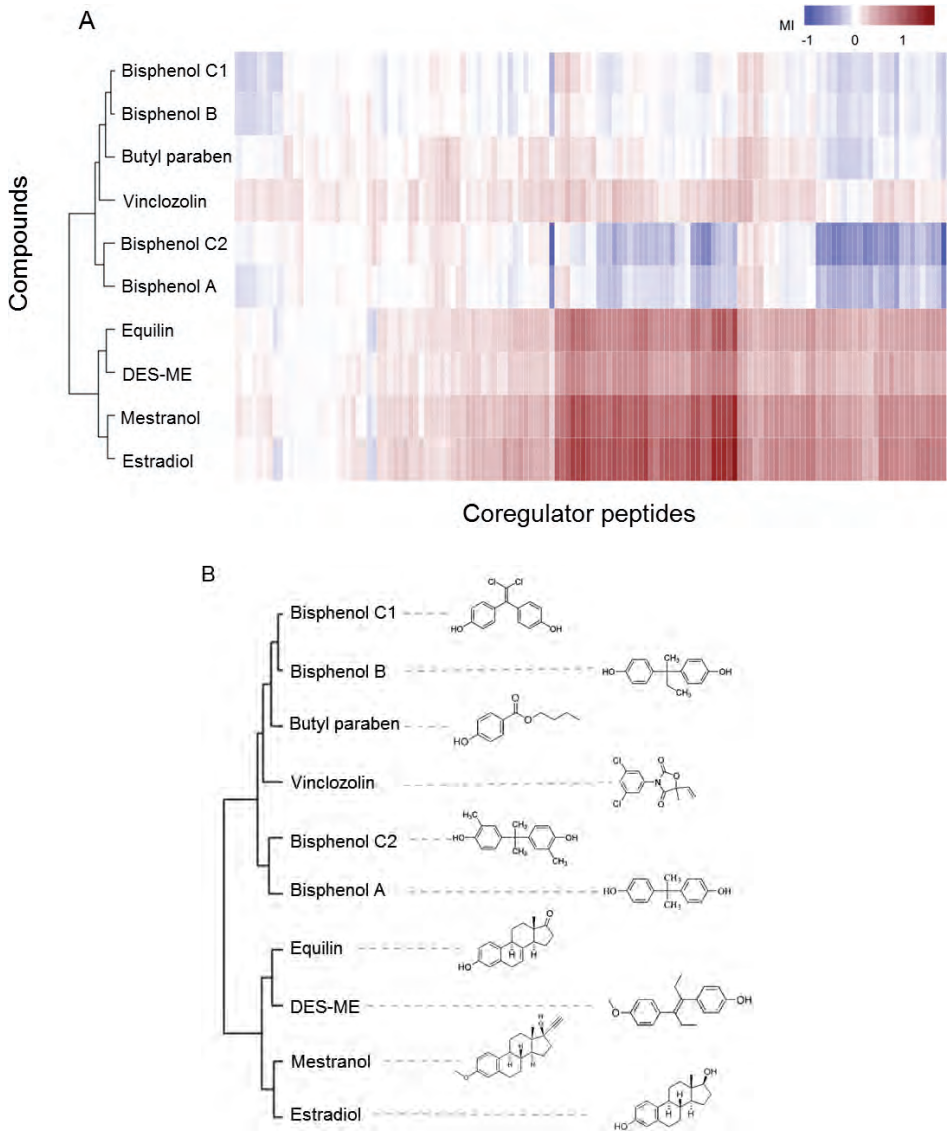

Figure 3. Heatmap of the unsupervised hierarchically clustered values for ERα-coregulator binding across compounds and coregulators shows structural similarity of compounds. A) Two-dimensional clustering heatmap; B) Clustering pattern of compounds.

Comparison with the in vivo uterotrophic assay

To compare relative estrogenic potencies of positively tested compounds with their uterotrophic effects in vivo, the relative estrogenic potencies (REP) or relative binding potencies (RBPs) of these compounds were calculated on the basis of the obtained EC_{50} values (Table 1). The estrogenic potencies obtained from the yeast estrogen bioassay and

the U2OS ERα-CALUX assay showed very good correlation with the outcomes of the in vivo uterotrophic assay, i.e., in both case $R^2 = 0.99$ ($p < 0.0001$, n=6). Because no estrogenic potency data were available for butyl paraben, BPB, BPC1 and BPC2, these compounds were excluded from correlation analysis. Furthermore, both reporter gene assays showed 100% (8/8) concordance with the in vivo uterotrophic assay, with no misclassification of compounds. In addition, the U2OS ERα-CALUX assay and the yeast estrogen bioassay resulted in similar potency ranking of the 10 compounds and the correlation between the relative potency values obtained from these two assays was excellent ($R^2 = 0.93$, $p < 0.0001$).

In the ERα coregulator binding assay butyl paraben was classified as negative. BPA, BPB, BPC1 and BPC2 showed only antagonistic binding profiles and can therefore not be compared with the agonistic effects as observed in the uterotrophic assay or in the reporter gene assays. This assay gave a lower correlation ($R^2 = 0.86$) and lower concordance (83%; 5/6) with the in vivo uterotrophic assay. However, the potency ranking of equilin, mestranol and DES-ME in the ERα coregulator binding assay was the same as in the U2OS ERα-CALUX assay.

Antiandrogenic activities

No androgen agonism was observed for any of the compounds tested (data not shown). The potential antiandrogenic activity of the test compound was studied by co-exposure of each compound with DHT or testosterone in the AR-CALUX assay or the yeast androgen bioassay, respectively. Figure 4 shows the antagonistic effects of several of the compounds tested. The potent androgen antagonist flutamide was used as positive control in both assays. Surprisingly, BPC1 and vinclozolin showed even more potent antiandrogenic activities than flutamide in both reporter gene assays (Figure 4 and Table 2). Although less potent, also

Table 2. Antiandrogenic activities of the test compounds in the U2OS AR-CALUX and yeast androgen bioassay.

Compound	U2OS AR-CALUX assay IC_{50} (M)	Yeast androgen bioassay IC_{50} (M)
Equilin	+ [a]	+
Mestranol	+	+
DES-ME	+	+
Bisphenol A	1.5×10^{-06}	8.1×10^{-05}
Bisphenol B	9.3×10^{-07}	- [a]
Bisphenol C1	4.3×10^{-08}	2.4×10^{-06}
Bisphenol C2	6.3×10^{-07}	2.2×10^{-05}
Butyl paraben	7.1×10^{-06}	2.0×10^{-06}
Atrazine	4.7×10^{-05}	+
Vinclozolin	4.8×10^{-08}	1.9×10^{-06}
Flutamide [b]	5.1×10^{-07}	1.5×10^{-05}

[a] + = positive; - = negative [b] Flutamide was used as a positive control.

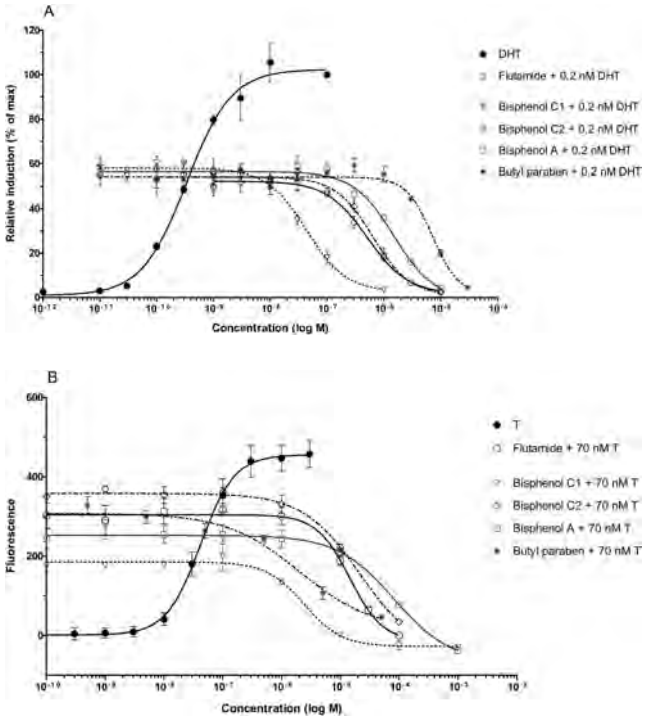

Figure 4. Antagonistic effect of several compounds, including the reference antiandrogen flutamide, measured in the AR-CALUX (A) and the yeast androgen bioassay (B). Signals are displayed as the mean with SD of triplicate measurements.

BPA and BPC2 inhibited the response induced by the potent androgens (DHT or T) to the baseline level. Equilin, mestranol and DES-ME showed weak antiandrogenic activities at relative high concentrations. However, due to the poor fitting of the applied dose-response model to the data, IC_{50} values for these compounds could not be reliably calculated.

Modulation of steroidogenesis

Effects on steroidogenesis were evaluated using the H295R steroidogenesis assay. The effects of 7 of the model compounds were assessed by measuring levels of 13 steroids in medium of H295R cells, and for each steroid hormone a dose response curve was constructed. Figure 5 shows for example the BPA dose-dependent changes in hormone levels in medium of H295R cells being exposed for 48 h. Exposing H295R cells to increasing concentrations of BPA resulted in increased levels of pregnenolone, progesterone and estradiol, while levels of 17α-OH-progesterone, 11-deoxycortisol, cortisol, androstenedione and testosterone were dose-dependently decreased by BPA. Levels of the other steroids were not significantly affected by BPA. Among the 8 steroids modulated by BPA, androstenedione and testosterone were the two most sensitive endpoints with a lowest observed effective concentration (LOEC) of 0.03 and 0.1 μM BPA, respectively.

A summary of the results of all compounds tested in the H295R steroidogenesis assay is presented in Table 3, showing the LOEC together with the maximal fold change of induction or inhibition. The hormone profile caused by BPB was similar to that caused by BPA, showing a dose dependent decrease in androgens (androstenedione and testosterone) and glucocorticoids (11-deoxycortisol and cortisol) and an increase in pregnenolone, progesterone, and less pronounced, in estrone. BPC1 resulted in a dose-dependent increase in levels of pregnenolone, progesterone, 17α-OH-progesterone and estradiol with an LOEC of 10, 0.3, 0.3 and 10 μM respectively. With the exception of testosterone and estrone, levels of all other steroids were decreased by BPC1. BPC2 resulted in a dose-dependent decrease in the levels of most steroid hormones, while progesterone and estradiol were up-regulated, but only at the highest nontoxic test-concentration of BPC2 (10 μM). The highest concentration of BPC2 (30 μM) was cytotoxic for the H295R cells (data not shown). Results of equilin, mestranol and DES-ME were not included, as these compounds did not significantly affect the hormone levels (data not shown). In general it is observed that all four bisphenolic compounds show a dose-dependent decrease in androgens, while levels of estrogens are increased after exposure. Exposure of H295R cells to the fungicide vinclozolin resulted in a maximum 1.99 fold increase in estradiol (LOEC of 10 μM), while levels of progesterone, 17α-OH-progesterone, androstenedione, testosterone, 11-deoxycorticosterone and 11-deoxycortisol were down-regulated by vinclozolin.

DISCUSSION

Our previous studies have shown that the yeast estrogen bioassay, the U2OS ERα-CALUX reporter gene assay and the ERα coregulator binding assay all revealed a good correlation with the in vivo uterotrophic assay based on a set of 23 compounds (most of which were selected from the ICCVAM list of compounds defined for validation of in vitro tests for estrogenicity testing). [4] It was advised to include these assays in an ITS for estrogenicity testing and prioritization of chemicals, aiming at refinement, reduction and ultimately replacement of the current animal testing for (anti)estrogenic effects, as these three high-throughput in vitro assays were also shown to be reproducible, fast and robust. The present study with 10 extra compounds with specific modes of action was set up in order to further validate the previously established ITS and to demonstrate the added value of including in vitro tests for AR-antagonism and interference with steroidogenesis. Among these compounds, five of the them (i.e., equilin, DES-ME, BPB, BPC1 and BPC2) have not been tested before in any of the three in vitro assays in the ITS and the other five compounds (i.e., BPA, mestranol, butyl paraben, vinclozolin and atrazine) have been tested only in some of the assays of our previously proposed ITS or by other groups using the same assays. The present results show that the U2OS ERα-CALUX and yeast estrogen bioassays both revealed 100% concordance with the in vivo uterotrophic assay, i.e., no misclassification of any compound for which uterotrophic potency data are available. In addition, these two reporter gene assays resulted in similar potency ranking of all the 10 compounds tested. In the ERα coregulator binding assay, only butyl paraben was misclassified as negative. Overall, these results demonstrated that the previously established ITS enables accurate prediction of the estrogenic properties

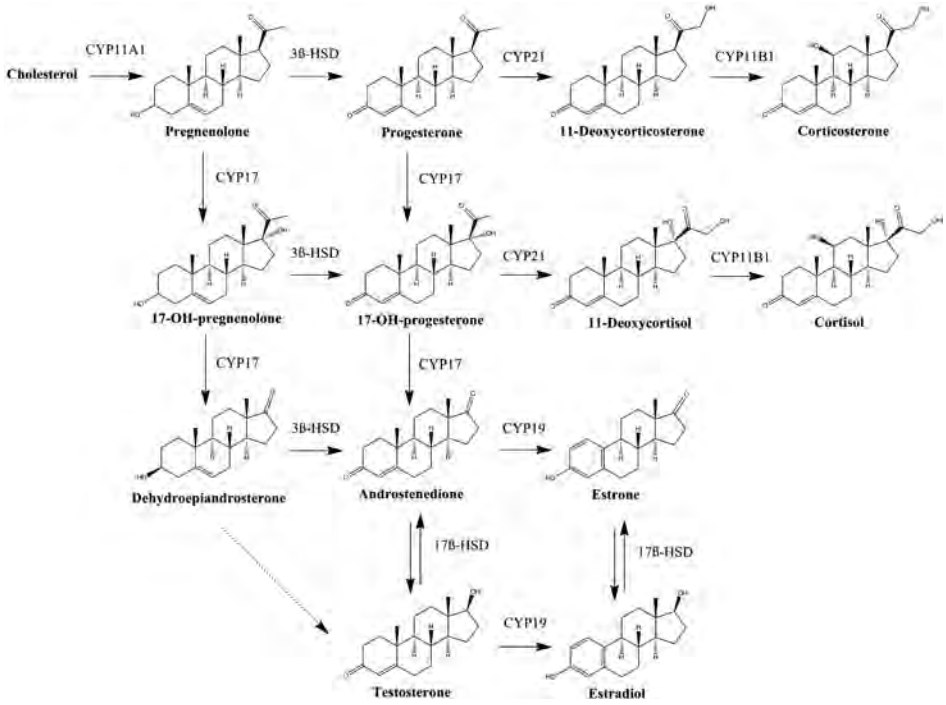

Figure 5. (A) Steroid biosynthesis pathway.

in vivo as observed in the uterotrophic assay. However, quantitative discrepancies do exist between the results obtained in vitro and in vivo, as well as between the results obtained by the different in vitro assays. Mestranol and DES-ME were about 20 to 300 times less potent than E2 in the ITS, while these two compounds showed a similar estrogenic potency as E2 in the in vivo uterotrophic assay. It has been shown that mestranol and DES-ME bind to the ER with high affinity [33] and act as ER agonists in the in vivo uterotrophic assay. [32] As expected both compounds elicited clear ER-agonistic properties in the ITS, but with lower potencies compared to in vivo. However, mestranol and DES-ME are structural analogues of EE2 and DES, respectively, both known ER-agonists that are also antiandrogenic (AR-antagonists) and in this way enhance the overall 'estrogenic' effect in vivo. [11, 12] For example, it is known that estrogens and antiandrogens inhibit prostate cancer cell proliferation in vitro [34, 35] and cause feminization in fish. [36, 37] Estrogens and antiandrogens are also used for the treatment of prostate cancer. [38] Thus, compounds that possess both estrogenic and antiandrogenic activity may induce stronger estrogenic effects in vivo than what would be expected based on their estrogenic activity alone. [39] In the present study, both mestranol and DES-ME were shown to act as AR-antagonist too, but this is likely not the only explanation for the observed in vitro/in vivo discrepancy in potency. Differences in metabolism provide a better explanation, as it is known that mestranol is demethylated in vivo into the more potent ER-agonist EE2. [40, 41]

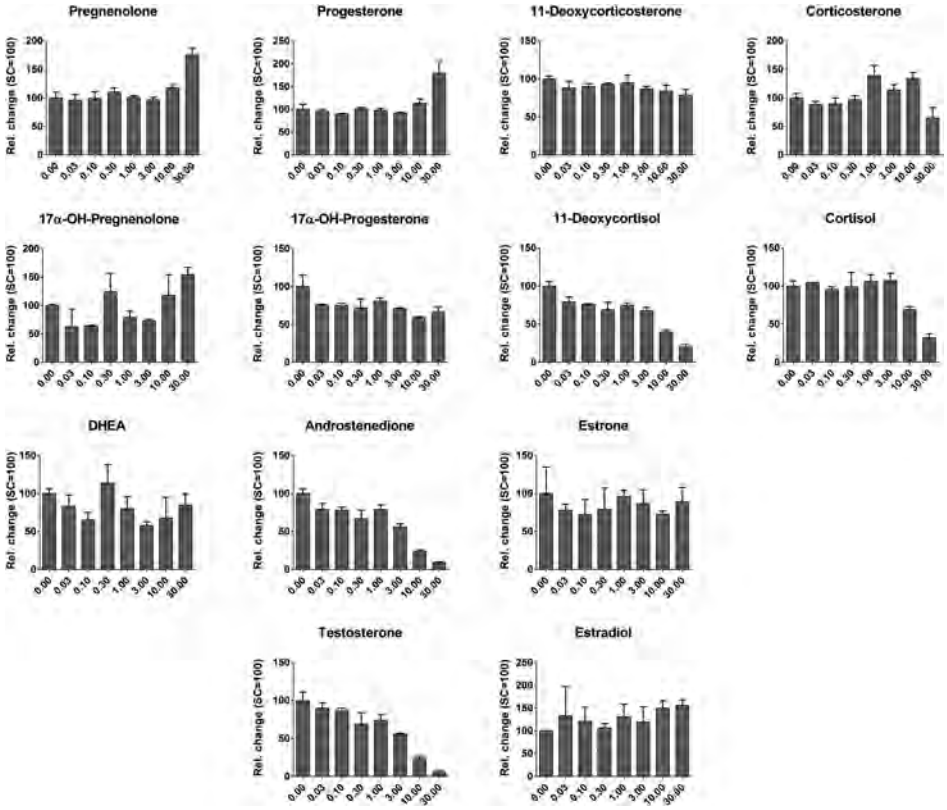

Figure 5. (*continued*). (B) Changes in hormone levels in medium of H295R cells exposed to BPA. Changes in hormone levels are expressed relative to the DMSO solvent control (mean ± SD, n=3). Statistical significance: * = p<0.05: ** = p<0.01 and *** p<0.001.

The observed in vitro/in vivo discrepancy for mestranol could thus be due to the lack of metabolism in the in vitro systems used in the current ITS. A similar explanation might be valid for DES-ME, as this compound can be metabolised in vivo into the more potent ER-agonist DES. [42] Thus, despite the fact that the ERα-CALUX assay is based on a mammalian cell line, this assay did not perform better in predicting the estrogenic potency of mestranol or DES-ME when compared to the in vitro systems that are lacking mammalian steroid metabolism, i.e., the yeast based reporter gene assay or the cell-free coregulator binding assay. Taken together, this strengthened the idea that mammalian cell lines, at least the U2OS cell line on which the ERα-CALUX is based, are generally quite limited in their metabolic capacity, and therefore, the combination of the ITS with a bioactivation step might further improve its predictive capacity for estrogenic potency in vivo.

Numerous studies have shown that BPA exhibits (weak) estrogenic activity [13, 20, 43] and strong antiandrogenic activity in vitro. [44-46] In our study, all three BPA analogues showed stronger estrogenic activities than BPA, and BPC1 was even 10 to 100 times more potent than

Table 3. Effects on steroid hormone production in exposed H295R cells.

	BPA		BPB		BPC1		BPC2		Vinclozolin		Butyl paraben[e]		Atrazine[e]	
	LOEC[a] (μM)	Max[b] change	LOEC (μM)	Max change	LOEC (μM)	Max change	LOEC (μM)	Max change	LOEC (μM)	Max change	LOEC (μM)	Max change	LOEC (μM)	Max change
Pregnenolone	10	1.76	10	1.97	10	1.70	3	0.38	NE	-	-	-	-	-
17α-OH-pregnenolone	Eq.[c]	-	NE	-	0.1	0.25	0.3	0.17	NE	-	-	-	-	-
Progesterone	30	1.80	3	2.41	0.3	6.35	10	1.30	3	0.66	-	-	-	-
17α-OH-progesterone	3	0.71	1	1.69	0.3	5.98	Eq.	-	3	0.53	-	-	-	-
DHEA	Eq.	-	10	0.56	1	0.15	Eq.	-	Eq.	-	-	-	-	-
Androstenedione	0.03	0.09	3	0.19	10	0.13	3	0.09	10	0.72	-	-	-	-
Testosterone	0.1	0.08	3	0.16	Eq.	-	0.1	0.10	3	0.70	1	<2.00	100	<2.00
Estrone	NE[d]	-	1	1.34	NE	-	NE	-	NE	-	-	-	-	-
Estradiol	10	1.56	NE	-	10	1.86	10	1.86	10	1.99	1-10	2.0-4.0	10	2.0-20
11-Deoxycorticosterone	NE	-	30	0.82	3	0.15	1	0.39	3	0.49	-	-	-	-
11-Deoxycortisol	10	0.23	3	0.36	10	0.04	0.30	0.14	1	0.53	-	-	-	-
Corticosterone	Eq.	-	Eq.	-	10	0.32	Eq.	-	NE	-	-	-	-	-
Cortisol	10	0.32	10	0.29	1	0.04	3	0.20	NE	-	-	-	-	-

[a] Lowest observed effective concentration (LOEC in μM), which is defined as the lowest concentration statistically significant different (p <0.05) from the solvent control average.
[b] Maximum fold change (up or down) of the average response observed at any concentration significantly different from the average solvent control level.
[c] Equivocal results.
[d] NE: No effect observed at the highest non-cytotoxic concentration tested.
[e] Data extracted from [58].

BPA in both reporter gene assays. In addition to the estrogenic effects, unique antagonistic coregulator binding characteristics were observed in the ERα coregulator binding assay for the bisphenols. The coregulator binding profile obtained for BPC1 was essentially the same as that of the selective estrogen receptor modulator (SERM) tamoxifen reported in our previous study. [4] Interestingly, no estrogen antagonism was observed for the bisphenols when tested in combination with E2 in the two reporter gene assays. However, a recent study of Delfosse et al. shows that both tamoxifen and BPC1 prevent NOCA1 binding to ERα LBD. [47] As the coregulator binding assays use ERα-LBD that contains the activation function 2 (AF-2; localized within the C-terminal of LBD), our study reveals that BPB, BPC1 and BPC2 act as general AF-2 antagonists, similar as tamoxifen. Additionally, the estrogen reporter gene assays used in the present study stably express full-length ERα. This further suggests that the ERα activation function 1 (AF-1; localized within the N-terminal A/B domain) is needed for the estrogenic activities of the bisphenols. Moreover, BPC1 and BPC2 showed significant inhibitory effects on the transcriptional activity induced by DHT in or T in the AR-CALUX and the yeast androgen bioassay, respectively. The IC_{50} values of BPC1 and BPC2 were 2 to 30 times lower than that of BPA and were comparable with the potent AR antagonist flutamide, demonstrating the strong antiandrogenic activities of BPC1 and BPC2. Furthermore, the bisphenols elicited strong disruptions on the hormone synthesis in the H295R cells, in general resulting in decreased levels of androgens and elevated levels of estrogens. Together these properties of the bisphenol compounds, i.e. ER-agonist, AR-antagonist and decreasing androgen levels and elevating estrogen levels in the H295R assay, possibly direct these compounds to be stronger estrogens in vivo than predicted by the in vitro ER-agonist properties alone. BPA was shown to be weakly estrogenic in several in vitro models, [13-15] and some studies suggest that BPA has also a greater in vivo potency than would be predicted based on the results obtained in vitro. Thus the combined actions as determined in vitro in the present study might explain the in vivo potency of BPA more accurately. BPC1 is apparently the most potent bisphenol analogue that possesses estrogenic and antiandrogenic properties and at the same time affects steroidogenesis into the direction of decreased androgen levels and elevated estrogen levels. Furthermore, BPA and its analogues are widely used as raw material in the production of polycarbonate plastics and epoxy resins, and are found to contaminate a broad range of end products. [48-51] Given the complex biological activities of BPC1 and the on-going debate on endocrine disrupting chemicals and BPA in particular, further in vivo testing of BPC1 in animal models should have high priority, as our data suggest that the use of BPA analogues might lead to a higher risk than BPA itself. Similar concerns were published by Grignard et al. (2012), showing that bisphenol S (BPS), used as a BPA substitute in the production of plastic baby bottles, has a comparable estrogenic potency as BPA in in vitro transcriptional activation assays. [13]

It has been shown that atrazine does not to bind to the ER or AR [33, 52] and, consistently, failed to induce ER- or AR-dependent transcription in several reporter gene assays. [50, 53-55] Atrazine is also unable to stimulate estrogen-dependent MCF-7 cell proliferation in the E-screen. [56, 57] These findings are in agreement with the outcomes of the present study, i.e. demonstrating that atrazine has no affinity for the ER and only showed weak antiandrogenic

activities in the AR reporter gene assays. However, atrazine showed clear effects in the H295R steroidogenesis assay, resulting in elevated levels of estradiol. [58] Thus, the results from the current study indicate that further testing of atrazine in the in vivo uterotrophic assay or Hershberger assay is not needed, as atrazine did not show clear effects via the ER or AR and only affected the steroidogenesis. Therefore atrazine should rather be tested in an in vitro model or a panel of models replacing the male and female pubertal assays. Indeed, this was demonstrated by the fact that atrazine failed to show estrogenic or androgenic activities in the uterotrophic assay or Hershberger assay, [59, 60] respectively, but delayed puberty and sexual development in both male and female rodents in pubertal assays. [7, 8] The fungicide vinclozolin does not bind to the ER and was also unable to induce uterotrophic effects in vivo, [33, 61] but has been reported to have antiandrogenic effects in vitro and vivo. [62, 63] In the present study, vinclozolin did not show any estrogenic effects, but elicited strong antiandrogenic activity. It also resulted in increased estrogen levels and decreased androgens levels in the H295R cells, suggesting that vinclozolin may induce aromatase activity.

Taken together, the extended ITS, including the H295R steroidogenesis assay and an androgen reporter gene assay, would allow to reduce or eliminate the need for testing certain endocrine disruption effects in animal models as illustrated for atrazine and vinclozolin. However, the examples of mestranol and DES-ME show that the current ITS has to be combined with additional types of in vitro assays, including in vitro models for digestion, bioavailability and metabolism of the compounds under investigation.

In conclusion, the ITS consisting the U2OS ERα-CALUX, yeast estrogen bioassay and ERα coregulator binding assay enables an accurate prediction of the estrogenic effects in vivo and provides mechanistic insights. By including the H295R steroidogenesis assay as well as an androgen reporter gene assay, the extended ITS was demonstrated to go beyond in vivo estrogenicity testing by the uterotrophic assay, as it can detect possible (anti)androgenic effects and effects on steroidogenesis that are not covered by the in vivo uterotrophic assay. The extended ITS presented in this study may therefore allow easy high-throughput screening and prioritization of chemicals, thereby contributing to refinement, reduction and to some extent even a replacement of current animal testing for (anti)estrogenic effects.

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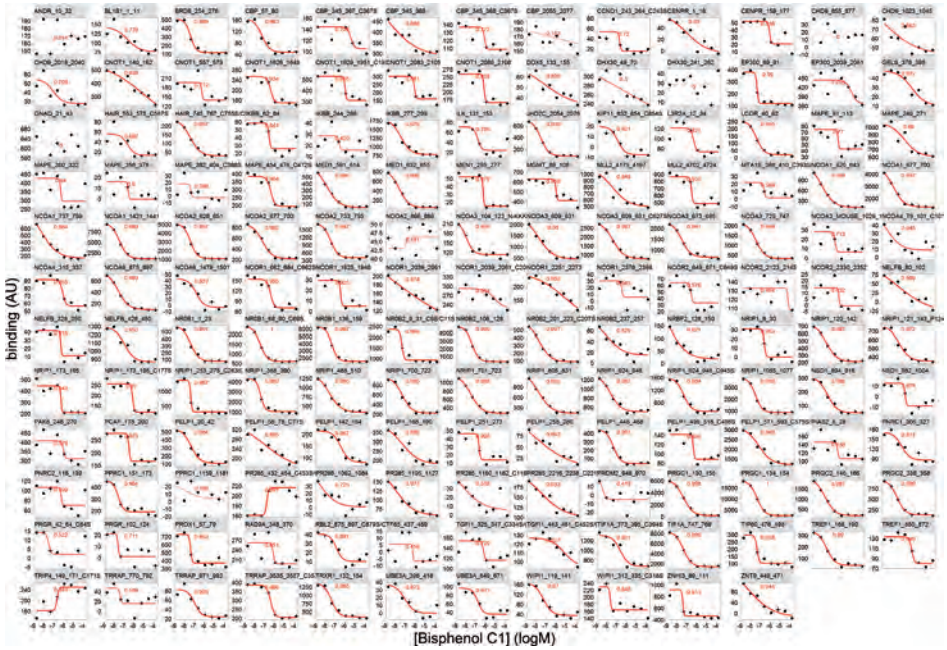
SUPPLEMENTARY FIGURE



Supplementary Figure 1. Analysis of ERα-LBD binding to the 155 coregulators on the peptide microarray as induced by BPC1.

8

General discussion and future perspectives

GENERAL DISCUSSION

In the present thesis, results obtained from a series of in vitro assays covering a broad range of endpoints and representing different steps of the estrogen-signaling pathway, i.e., receptor binding, receptor-coregulator binding, reporter-gene transcriptional activation, targeted gene expression, and cell proliferation were compared with outcomes from the in vivo uterotrophic assay, in order to define an integrated testing strategy (ITS) for in vitro estrogenicity testing. The selected in vitro assays mainly focus on measuring effects directly mediated by estrogen receptor (ER)α. This is because binding and activation of ERα is implicated as a key molecular initiating event (MIE) in estrogenicity-related adverse endpoints. [1] Moreover, activation of ERα is the driving force behind the uterus growth response in the uterotrophic assay, and accordingly, ERα knockout animals have an irresponsive uterus. [2] Most of the test compounds used, were selected from the ICCVAM defined list for validation of in vitro tests for estrogenicity testing and represent a diverse range of chemicals, i.e., natural steroids, synthetic steroids, flavonoids, phenols, organochlorines, and phthalates. [3] In addition, published results from ER binding assays and two OECD-validated reporter gene assays (i.e., ERα-HeLa-9903 and BG1Luc ER TA) were also compared to the results obtained in the current thesis and with the data from the in vivo uterotrophic assay.

In general, results showed that all in vitro assays revealed a reasonable to good correlation with the in vivo uterotrophic assay (R^2 = 0.62-0.89). However, in order to define a test panel which allows easy high-throughput screening and prioritization of chemicals, the selection of the in vitro assays cannot be based only on the in vitro-in vivo correlation, since other aspects such as fastness, simplicity, reproducibility and adaptability for high-throughput applications should also be taken into account. Table 1 summarizes some of these properties of the in vitro assays used in the present thesis.

Based on 12 reference compounds, data obtained in the MCF-7/BOS proliferation assay correlate better (R^2 = 0.85) with the data from the in vivo uterotrophic assay than the proliferative responses of three other human cell lines, i.e., another cell line derived from breast (T47D), one derived from the endometrium (ECC-1) and one from the ovary (BG-1) (Chapter 2). When tested with a larger set of 23 compounds, the obtained correlation of the MCF-7/BOS proliferation assay with the uterotrophic assay showed an identical correlation (R^2 = 0.85) (Chapter 3). This demonstrates that the proliferation endpoints has a high predictive value for the in vivo uterotrophic effect. However, the MCF-7/BOS cell proliferation assay is not very fast, i.e., the proliferative responses of the MCF-7/BOS cells can be reliably quantified only after 6 days. This might limit its practical application in the current format as a high-throughput screening tool within massive screening programs such as REACH. Moreover, crosstalk can occur due to the fact that MCF-7/BOS cells also express androgen, progesterone, glucocorticoid and retinoid receptors. [4] Thus, this assay may not provide straightforward information on the mechanism of action of the compound under investigation. Therefore, a newly developed low-density microarray assay, based on marker gene expression in estrogen-treated MCF-7/BOS cells, was evaluated in chapter 4. These microarrays allowed to rapidly study expression changes of a substantial number of genes, and also provided a more cost-effective option to study known molecular pathways

Table 1. Summary of the characteristics of the in vitro estrogenicity assays studied in the present thesis.

Thesis chapter	Assay name	In vivo correlation (R^2)	Time required	ER-dependent endpoint measured	Comments
Chapter 2,3	MCF-7/BOS proliferation	0.85	6 days	Cell proliferation	Simple, but crosstalk with other nuclear receptor may occur
Chapter 3, 7	Yeast estrogen bioassay	0.87	1 day	Transcription activation	Simple and robust; validated for screening calf urine, animal feed and water samples, but less sensitive compared to mammalian cells
Chapter 3	T47D ER-CALUX	0.70	3 days	Transcription activation	Sensitive, expresses endogenous ERα and ERβ
Chapter 3, 7	U2OS ERα-CALUX	0.68	3 days	Transcription activation	Sensitive; prevalidated; constitutively expresses ERα
Chapter 4	DNA microchip	0.72	3-4 days	Marker gene expression	Labor-intensive, expensive
Chapter 5, 6, 7	Coregulator binding assay	0.89	< 2 hours	Ligand-induced ERα binding to coregulators	Cell-free method; highest reproducibility, but requires to use PamStation platform and expensive PamChip arrays
Chapter 3 [a]	ER binding assays	0.75	2 days	Receptor binding	Validated by EPA; only measures the affinity of a ligand binding to ER; animals are needed as a source of ERs
Chapter 3 [a]	BG1Luc ER TA	0.62	3 days	Transcription activation	Validated by OECD (TG 457) for testing both ER agonist and antagonist activities
Chapter 6 [a]	ERα-HeLa-9903	-	3 days	Transcription activation	Validated by OECD (TG 455) for testing only ER agonist activity

[a] Literature data were used, no experimental data were generated in the present thesis study.

affected by exposure to an estrogenic test chemical. Eleven target genes that were clearly up-regulated by estrogens were selected, and the DNA probes for these transcripts were printed on a low-density DNA microchip. The seven most informative marker genes on the DNA microchip resulted in fingerprints correctly predicting the estrogenic activities of the compounds tested. Two of these marker genes, MLF1IP and UBE2C, were even found capable of correctly predicting the in vivo estrogenic potencies of the five ER-agonists tested and resulted in a satisfactory correlation with the in vivo uterotrophic assay (R^2 = 0.72). Although the low-density DNA microchip-based analysis of marker gene fingerprints takes only 3 days and is faster than the MCF-7/BOS cell proliferation assay, which takes 6 days, it is nevertheless quite laborious and requires skilled technicians; therefore, this assay is also not ideal in its current format for the large-scale testing of chemicals.

In vitro ER competitive binding assays have been well established and extensively used to investigate ER-ligand interactions. These assays measure the displacement of a receptor-bound probe molecule, e.g., ^{3}H-labeled estradiol, by a test compound and subsequently determine the relative binding affinity of the test compound for the ER. The ER binding assays are rapid

and easy to perform, but only the strength of the binding of a compound to the receptor is determined and not the activation or inhibition of activation of the receptor. Thus, compounds with high binding affinities to the ER might show lower estrogenic activities in cell-based and in vivo assays. This was clearly demonstrated in chapter 3, by the fact that several compounds showed high binding affinities to the ER, but were inactive in the in vivo uterotrophic assay. In addition, conventional ER binding 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 (EPA) Endocrine Disruptor Screening Program Tier 1 screening battery, still requires the use of animals as a source of ERs. These ER binding assays are therefore considered to be less suited for an in vitro high-throughput screening approach to test compounds for estrogenic activity. It was therefore decided to investigate the performance of three existing in vitro reporter gene assays for estrogenicity testing that are known to be fast and suited for high-throughput purposes.

The three reporter gene assays used in chapter 3 are based on stably transfected cells, either mammalian (T47D ER-CALUX assay and U2OS ERα-CALUX assay) or yeast cells (yeast estrogen bioassay). These assays measure the induction of a reporter gene, the expression of which is easy to quantify, and is put under transcriptional control of one or more EREs and therefore depends on the level of activation of the estrogen receptor. As shown in Table 1, compared to its mammalian counterparts, the yeast estrogen bioassay is faster, easier to perform and does not need expensive growth media (e.g., charcoal-stripped fetal bovine serum) nor sophisticated cell culture facilities or an expensive substrate (e.g., luciferine in the luciferase based CALUX assays). Moreover, the results of the yeast estrogen bioassay showed a good and better correlation ($R^2 = 0.87$) with the outcomes of the in vivo uterotrophic assay than those obtained with the mammalian cell-based CALUX assays. Although yeast cell-based assays are thought to suffer from poor transport of chemicals across the yeast cell wall, previous studies demonstrated that the yeast cell wall is easily permeable for compounds with a molecular weight up to 620 daltons, or even larger molecules, due to the flexibility of the wall of living yeast cells. [5, 6] Thus, the yeast cell wall does not provide a major obstacle for low molecular weight compounds to reach the inside of the cells and activate the ER. This consideration was confirmed by the good correlation observed between the yeast estrogen bioassay and the in vivo uterotrophic assay. In addition, Kolle et al. (2010) compared experimental data from a yeast estrogen screen (YES) assay with the literature data from the uterotrophic assay and also showed that the YES assay has a high degree of qualitative concordance (the rate agreement of the results among assays; 87%) with estrogenic effects in vivo. [7] The human cell-based T47D ER-CALUX and the U2OS ERα-CALUX assay showed a relatively lower correlation with the uterotrophic assay (R^2 of 0.70 and 0.68, respectively); however, this was mainly due to 4-hydroxytamoxifen being an outlier. When this compound was excluded from the comparison, the R^2-value of the correlation between the uterotrophic assay and the two human cell-based reporter gene assays increased to around 0.85. Moreover, these human cancer cell line-based reporter gene assays are generally more sensitive than the yeast-based reporter gene assays, which is an advantage for screening samples with low concentrations of estrogenic compounds such as usually found in food or environmental

samples. For prioritization and testing of pure chemicals, all these in vitro assays are sensitive enough to measure potent to weak estrogenic compounds.

From Table 1 it becomes clear that the coregulator binding assay (chapter 5, 6 & 7) is the fastest assay and showed the best correlation with the in vivo uterotrophic assay. The coregulator binding assay uses the PamChip® plate holding 96 identical peptide probe arrays, thus allowing the ligand-modulated interaction of 155 different NR-binding coregulator peptides with ERα to be measured for 96 samples in one plate run. It was shown in chapters 5 and 6 that the coregulator binding assay is capable to distinguish ERα agonists from antagonists and is even reflecting structural similarity of ERα agonists, indicating its potential to achieve identification and classification of ERα endocrine disruptors with high fidelity. In addition, ERα LBD–coregulator binding profiles induced by a series of bisphenols provided new mechanistic insights into estrogen receptor signaling by these compounds. The antagonistic binding profiles demonstrated that bisphenol A, B, C1 and C2 might act as general ERα activation function 2 (AF-2) antagonists, similar to the selective estrogen receptor modulator tamoxifen (chapter 7). Moreover, the coregulator binding assay is a cell-free method; therefore no cell culturing facilities are needed. However, this assay requires the use of a dedicated PamStation platform and multiple PamChip arrays for generating dose-response curves of test compounds. The total costs of this assay are therefore rather high when compared to the consumables needed for the cell-based assays described in the present thesis. For further practical application of the coregulator binding assay, a more cost-effective screening protocol is needed. This may be achieved, for example, by testing multiple doses of a compound on a single array. However, the feasibility of such an approach should be further explored.

Reproducibility and robustness are also important aspects to take into account when selecting in vitro assays for an ITS. With a median coefficient of variation of 5.0 % and excellent correlation (R^2 = 0.993) between duplicate measurements, the reproducibility of the coregulator binding assay was better than the reproducibility of other commonly used in vitro ER functional assays. The yeast cell-based reporter gene assay also showed good results on reproducibility in an inter-laboratory study. [8] In addition, yeast-based reporter gene assays are extremely robust and can be used for screening of complex biological samples without extensive clean-ups. This was demonstrated by the validation studies of the yeast estrogen bioassay for screening of estrogenic activity in calf urine, [9] animal feed [10] and water samples. [11] The ERα-CALUX has been prevalidated as a test for ER agonist and antagonist activity of compounds. Although the ERα-CALUX had problems in the initial phase of the validation studies with transferability, these issues were solved by isolation of a novel cell clone of the ERα-CALUX line with greatly improved stability and luciferase levels. [12] Thus far, the coregulator binding assay, the yeast estrogen bioassay, and the ERα-CALUX bioassay showed a clear advantage over the T47D ER-CALUX bioassay, the MCF-7/BOS cell proliferation assay and the low-density DNA microchip, and no real validation studies have been carried out yet to demonstrate the reproducibility of the later three bioassays, as was done for the first three bioassays.

When compared to the published results, as shown in Table 1, the coregulator binding assay and the yeast estrogen bioassay showed a better correlation with the in vivo uterotrophic assay than the OECD validated BG1Luc ER TA assay (based on the results

of the 23 compounds tested). The T47D ER-CALUX and the U2OS ERα-CALUX assay showed relatively lower correlation with the uterotrophic assay, however, their results were found to be slightly better than those from the BG1Luc ER TA assay. Further evaluation of the performance of the in vitro assays used in the present thesis with the OECD-validated BG1Luc ER TA assay, and formal validation might be the next steps towards the practical application of these in vitro bioassays. At the moment, validation of an in vitro alternative method is a lengthy process and may take about 5 years. [13] However, this process can be speeded up through the so called "catch-up" or "Me-too" validations, i.e., by comparison of the results to the validated method using defined "performance standards" or "performance criteria". [14] To date, several in vitro reconstructed human epidermal models have been successfully validated in "catch-up" validation studies and are implemented into the OECD in vitro skin corrosion Test Guidelines (e.g., TG 430 and TG 431). [15, 16] Therefore, the coregulator binding assay, the U2OS ERα-CALUX bioassay and the yeast estrogen bioassay could be validated through the "catch-up" validation by testing the complete set of 78 reference compounds defined by ICCVAM for validation of in vitro ER binding and transcription activation test methods.

The results from the comparisons made in the present thesis provide useful information to select the most suitable in vitro assays for estrogenicity testing. However, the complementary nature of each assay should also be taken into account when defining an ITS. Therefore, a multiple linear regression analysis was performed to demonstrate whether linear combinations of certain in vitro assays can lead to a higher prediction value for the outcomes as observed in the in vivo uterotrophic assay. Results showed that upon combining all the in vitro data sets the correlation with the in vivo uterotrophic assay was improved to an R^2 of 0.97, which is higher than the R^2 obtained for any of the in vitro assay alone. The yeast estrogen bioassay showed the most significant influence on the prediction value for the in vivo uterotrophic outcomes. In addition, combination of the yeast estrogen bioassay with either the coregulator binding assay or the MCF-7/BOS cell proliferation assay resulted in the highest correlations, i.e., R^2 of 0.93 and 0.90, respectively for a combination of two assays. Combinations of any other two in vitro assays resulted in lower correlations. Combination of all the reporter gene assays resulted in an R^2 of 0.87 with the in vivo uterotrophic assay which is the same as the R^2 obtained when using the yeast estrogen bioassay alone. These findings indicate that combination of in vitro assays measuring the same endpoint, e.g., transcription activation may not further improve the predictivity of the in vitro test panel. Taken all these aspects together, i.e., predictivity, fastness, simplicity, reproducibility, and complementary nature of the selected in vitro assays, the yeast estrogen bioassay or the U2OS ERα-CALUX assay together with the coregulator binding assay appears to be the most promising combination of assays to be included in the ITS. This was further demonstrated in chapter 7, showing that the yeast estrogen bioassay and the U2OS ERα-CALUX assay accurately predict the estrogenicity of a series of newly tested model compounds and revealed 100% concordance with the in vivo uterotrophic assay.

Although good in vitro - in vivo correlations were obtained in the studies described in this thesis, discrepancies do exist, and this is due, at least to some extent, to the multiple

biological effects of estrogens. The three in vitro assays that were recommended to be part of the ITS for estrogenicity testing, i.e., the coregulator binding assay, the yeast estrogen bioassay and the U2OS ERα-CALUX assay, only measure effects directly mediated by ERα and are therefore not able to detect chemicals that elicit their estrogenic effects through indirect mechanisms, e.g., alteration of hormone biosynthesis, metabolism or transport. In addition, the antiandrogenic compounds elicit in vivo effects by the androgen receptor (AR) that can hardly or not be distinguished from an estrogenic effect by an estrogen via the ER, and some compounds even have combined modes of action, e.g., several estrogenic compounds like 17α-ethinyl estradiol (EE2) and diethylstilbestrol (DES) are also antiandrogenic, and in this way may enhance the overall 'estrogenic' effect in vivo. [17, 18] This emphasises the need to extend the ITS initially established based on ERα responsive assays, by including androgen reporter gene assays and the H295R steroidogenesis assay. This was clearly demonstrated in chapter 7 which presents data on several model compounds that possess strong estrogenic activities and/or antiandrogenic activities, or effects on steroidogenesis, or combinations of those at the same time. These findings provided valuable information on the modes of action of the test compound, and this can help in the prioritization of chemicals for further screening in selected animal models.

Thus, the in vitro assays used in the present thesis provide valuable insights on mechanisms of action of the compounds under investigation. However, these in vitro assays are restricted in their capacity to reflect the absorption, metabolism, distribution, and excretion (AMDE) processes of the intact animal. Poor metabolic capacity is one of the most frequently cited limitations of in vitro assays compared to in vivo assays. This was also demonstrated in the present thesis since, for example, tamoxifen is less potent than its metabolite 4-hydroxytamoxifen in in vitro proliferation assays, while these two compounds showed the same estrogenic potency in the in vivo uterotrophic assay. The same applies for the organochlorine insecticide methoxychlor: This compound is about 100 time less potent than its in vivo metabolite 2,2-bis(4-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) in the U2OS ERα-CALUX assay, indicating the low metabolic capacity of the U2OS cells. In addition, steroid compounds can also be deactivated in vivo. In this case in vitro assays may lead to overestimation of the estrogenic potency of the test compound. To overcome these issues, several in vitro and ex vivo methods are currently being applied to study the metabolism of compounds. These methods range from the use of simple enzymatic reactions and cell fractions to the use of more complicated cell lines, primary cell cultures and tissue slices. Enzymatic hydrolysis and deconjugation methods are effective and easy to perform. Rijk et al. (2011), for example, reported that enzymatic deconjugation of genistin resulted in a significant increase in estrogenic activity due to the formation of the more potent genistein. [19] However, such enzymatic methods are suited for activation of only certain groups of compounds, e.g., hormone esters and glycoside derivatives. Liver cell lines, for example human HepG2 and HepaRG, primary hepatocytes and liver slices provide a more complete set of biotransformation enzymes including cytochromes P450 and Phase II enzymes and are therefore considered as more complete and representative models for in vivo metabolism. Due to the elaborated preparation procedures or limited availability of liver tissues, the latter

two methods are not suitable for high-throughput screening of chemicals. Liver S9 fractions are easier to prepare, contain major biotransformation enzyme activities, and can be used in combination with in vitro assays for endocrine disruption testing. [20-23] Although S9 is very useful, the major potential drawback is that S9 liver fractions need cofactors and it may not be known on forehand what cofactor is needed for the chemical under investigation. [24] In addition, co-incubation of liver S9 can lead to toxicity in mammalian cell-based reporter gene assays, in these cases the chemical should be pre-incubated with S9 first, extracted (mother compounds and metabolites formed) and then tested in the in vitro assay. A better option might be to combine the S9 with the more robust yeast-based reporter gene assays or with cell-free assays such as the coregulator binding assay. Another advantage of using combinations of S9 with in vitro assays that lack metabolism, such as the yeast based reporter gene assays and the coregulator binding assay, is that any change in estrogenic activity is most likely due the S9 treatment and thus due to metabolism, which allows important mechanistic information to be obtained. Although the mammalian reporter cells described in this thesis have a very limited metabolic capacity, compounds can be converted to more potent metabolites and lead to a false positive result. This was demonstrated in chapters 2, 3 and 4, as testosterone induced a full dose response in the MCF-7/BOS proliferation assay, and it was shown that this was mainly due to the conversion of testosterone into the estrogenic metabolites E2, 4-androstenediol and 5-androstenediol. Therefore, cell models cannot be used as a black box, and it is necessary to characterize the cell line on the metabolic level in order to prevent misclassification of compounds.

In addition to the use of in vitro tools for metabolism, methods that address in vivo digestion and absorption are also needed to further improve the in vitro prediction of in vivo effects. One of the in vitro models for digestion is the human gastrointestinal digestion model, which involves subsequent incubations of a test compound in saliva, and in gastric, duodenal and bile juice. Among others, this model has been applied for studying the bioavailability of compounds such as heavy metals, [25] mycotoxins [26] and silver nanoparticles. [27] The Caco-2 cell line, derived from a human colorectal carcinoma, is the most commonly used and well-established in vitro model for the prediction of intestinal absorption and transport of compounds. [28, 29] In this model, chemicals are typically added to the apical side of Caco-2 cell monolayers, and both their transport and metabolism can be measured upon a defined incubation time at the apical or basolateral side. Although the gastrointestinal digestion and the intestinal absorption models are routinely used in toxicology and pharmaceutical research, the utility of combining these and other ADME models with existing in vitro assays for high-throughput screening of estrogenicity should be further explored. In addition, the data obtained on absorption and metabolism can also contribute to building physiologically based kinetic (PBK) models that allow conversion of in vitro concentration-response curves to in vivo dose-response curves, in order to improve the impact and applicability of in vitro methods in risk assessment practice. [30, 31]

FUTURE PERSPECTIVES AND CONCLUSIONS

The studies presented in this thesis provide further insight on developing an integrated testing strategy (ITS) based on existing and newly developed in vitro assays for estrogenicity. Although the proposed in vitro test panel cannot directly replace the in vivo uterotrophic assay, i.e., should be expanded with in vitro models for digestion, metabolism and absorption, it could be incorporated as part of a weight-of-evidence approach to reduce or eliminate the need for testing endocrine disruption effects in animal models. To this end, the information obtained from different in vitro endpoints should all be taken into account in reaching and supporting a conclusion concerning the endocrine disruption potential of a chemical. To date, most of the attention to endocrine disruption has focused on estrogenic and androgenic effects. [32, 33] Therefore, our knowledge on disruption of other hormone signaling pathways either directly through nuclear receptors (e.g., thyroid receptor, progesterone receptor and glucocorticoid receptor) or through indirect mechanisms (e.g., steroidogenesis) should be broadened.

The current thesis can be used as a departure point towards defining an in vitro ITS for androgenic activity, thyroid hormone activity, progestagenic activity, or glucocorticoid activity. Similar as the in vivo uterotrophic assay, the golden standard for evaluating androgen agonists or antagonists, i.e., the Hershberger assay, is also based on the changes in tissue weight, more specifically, on five androgen-dependent tissues (i.e., ventral prostate, seminal vesicle, levator ani-bulbocavernosus muscle, paired Cowper's glands and the glans penis) in castrated peripubertal male rat. [34] Various in vitro bioassays have been developed and applied for screening androgenic properties of chemicals, such as androgen receptor (AR) binding assays, [35] yeast androgen reporter gene assays [36, 37] and several mammalian reporter gene assays. [38-41] As the Hershberger assay is already validated [34] and a substantial amount of chemicals have been tested, [42-44] the in vitro – in vivo correlation approach, as used in the current thesis, could also be applied in order to investigate the predictivity of the existing in vitro assays for in vivo (anti) androgenic effects and to identify discrepancies between in vitro data and results from the in vivo Hershberger assay. Only a few studies explored the correlation between in vitro assays and the Hershberger assay. Sonneveld et al. (2004), for example, reported that the results obtained with the human U2OS AR-CALUX reporter gene assay resulted in a correlation coefficient of $R^2 = 0.46$ with the Hershberger assay on a set of 34 steroids. [42] In a more recent study, Kolle et al. (2010) compared outcomes of a yeast androgen screen assay (YAS) with literature data from the Hershberger assay and showed that the YAS assay has a very high degree of concordance (95%) with androgenic effects in vivo. [7] Although several in vitro assays are developed for screening progestagenic activities, i.e., progesterone receptor (PR) binding assays, [45] yeast progesterone reporter gene screens [46] and mammalian cell-based reporter gene assays, [47-50] data on the in vivo endometrium differentiation assay (McPhail assay), an animal model for testing progestagenic activities in immature rabbits, are quite limited. Nevertheless, it has been shown that comparison between the U2OS PR-CALUX assay and the in vivo McPhail assay revealed a good correlation with $R^2 = 0.85$ based on a set of 50 compounds. On the one hand, these data imply that in vitro assays measuring direct effects mediated by AR and PR could cover a major part of the androgenic and progestagenic activities, respectively, observed in vivo. On the other

hand, such a hypothesis may not apply to the thyroid system, as most of the thyroid effects are not mediated via the thyroid receptor, but through interference with thyroid hormone triiodothyronine (T3) and/or thyroxine (T4) synthesis and by disturbing the transport of T3 and T4 in the bloodstream. This was demonstrated in a study by Jomaa et al. (2013), showing that among 11 thyroid-active compounds, known to affect pituitary and/or thyroid weights in vivo, only two compounds tested had an effect in the in vitro rat thyroid cell proliferation assay (TSH-screen). [51] Therefore, a broad range of in vitro assays that cover various modes of action of thyroid-active compounds are needed, for example, a thyroid peroxidase assay, or even more complex (vertebrate) "in vitro" models like zebrafish.

The selected in vitro assays, covering different endocrine disruption endpoints, can also contribute to establish testing programs and strategies to predict potential toxicity, and to develop a cost-effective approach to prioritize the toxicity testing of chemicals. For example, these in vitro assays can be implemented in the U.S. Environmental Protection Agency (EPA) ToxCast program, which is currently evaluating over 1,000 chemicals in more than 600 in vitro assays. However, as strengthened above, to ultimately replace the short-term in vivo screening assays for specific endocrine disruption effects, the selected in vitro bioassays have to be combined with other types of in vitro assays, including at least the H295R steroidogenesis assay as an in vitro model for steroid metabolism, as well as for in vitro (cellular) models for digestion, absorption, and bioavailability of the compounds under investigation.

In conclusion, the present thesis provides proof-of-principle that combining in vitro assays measuring different steps in the estrogen receptor signaling pathway enables accurate prediction of the estrogenic effects in vivo. By including the androgen reporter gene assays as well as the H295R steroidogenesis assay, the extended testing panel even goes beyond estrogenicity testing, as it can detect possible (anti)androgenic effects and effects on steroidogenesis that are not covered by the in vivo uterotrophic assay. The integrated in vitro testing strategy presented in this thesis may therefore allow easy high-throughput screening and prioritization of chemicals, thereby contributing to refinement, reduction and to some extent even replacement of current animal testing for estrogenic effects.

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9 Summary

SUMMARY

Testing chemicals for their endocrine-disrupting potential, including interference with estrogen receptor signaling, is an important aspect to assess the safety of currently used and newly developed chemicals. The standard test for disruption of normal estrogen function is the in vivo uterotrophic assay in immature or ovariectomised rodents with uterus weight as a crucial read-out parameter. Due to the high costs, ethical objections and labour intensiveness of the in vivo uterotrophic assay, the development of an in vitro test battery for in vivo estrogenicity has high priority. The aim of the present thesis was to develop an integrated testing strategy (ITS), based on existing and newly developed in vitro assays for estrogenicity testing, allowing easy high-throughput screening and prioritization of chemicals. An ITS preferentially based on in vitro assays would be a crucial step towards refinement, reduction, and ultimately replacement of current animal testing for estrogenic and other endocrine disrupting effects.

To reach this aim, several presently available and newly developed in vitro bioassays were selected and evaluated for optimal representation of the estrogenic effects occurring in the uterus/endometrium in vivo. In **chapter 2** of the thesis the estrogenic potency of 12 compounds was studied in four different estrogen-dependent cell proliferation assays in order to determine to what extent these in vitro tests provide alternatives for the in vivo uterotrophic assay. The cell lines used for proliferation assays were cell lines derived from three different female estrogen-sensitive tissues, i.e. breast (MCF-7/BOS and T47D), endometrial (ECC-1) and ovarian (BG-1) cells. These cell lines were characterised by investigating their relative estrogen receptor (ER) α and β levels, as the ERα/ERβ ratio is a known dominant factor determining the estrogen-dependent proliferative responses in estrogen sensitive cell lines. Results showed that all four cell lines clearly express the ERα type and a very low, yet detectable, amount of ERβ at both the mRNA and protein level, with the T47D cell line expressing the highest level of the ERβ type. Subsequently, a set of selected (estrogenic) reference compounds representing different modes of action and potency were used to investigate the proliferative response of the four cell lines. It was shown that the responses of all four cell lines towards the model compounds revealed a reasonable to good correlation with their in vivo uterotrophic effect, with the correlation being highest for the MCF-7/BOS cell line ($R^2 = 0.85$). The main differences between the in vivo uterotrophic assay and the in vitro proliferation assays were observed for tamoxifen and testosterone. The proliferative response of the MCF-7/BOS cells to testosterone was shown to be partially caused by its conversion to estradiol by aromatase or via androstenedione to estrone. It was concluded that of the four cell lines tested, the best assay to include in an ITS for replacement of the in vivo uterotrophic assay is the proliferation assay with the human MCF-7/BOS breast cancer cell line.

In addition to proliferation assays, reporter gene assays based on either mammalian or yeast cells are also widely used for estrogenicity testing. In **chapter 3** it was investigated which of these in vitro assays most accurately predict the in vivo uterotrophic effect and possible discrepancies between the in vitro assays and the in vivo uterotrophic assay were identified. To this end a larger set of 23 estrogenic reference compounds, most of which

were selected from the ICCVAM list of compounds defined for validation of in vitro tests for estrogenicity and representing diverse chemical classes, was tested in a series of in vitro reporter gene assays and in the MCF-7/BOS proliferation assay selected in a previous study (chapter 2). The reporter gene assays included two CALUX assays based on mammalian cell lines, i.e. the T47D ER-CALUX and U2OS ERα-CALUX, and a yeast based estrogen bioassay. Outcomes of these assays were compared with published results for ER binding assays and the OECD validated BG1Luc ER transcriptional activation (TA) assay, and then altogether compared with the outcomes of the in vivo uterotrophic assay in order to identify the in vitro assay(s) providing the best prediction of the in vivo uterotrophic response. All in vitro assays revealed a reasonable to good correlation (R^2 = 0.62-0.87) with the in vivo uterotrophic assay. The best correlation was obtained with the yeast estrogen bioassay (R^2 = 0.87), expressing a green fluorescent protein (yEGFP) in response to estrogens. The correlation with MCF-7/BOS cell proliferation assay in this study with 23 compounds was the same as obtained before in the study with the smaller set of 12 reference compounds (R^2 = 0.85). However, compared to the reporter gene and ER binding assays (1-2 days) the MCF7/BOS takes too long (6 days) and is therefore regarded as less suited for the high-throughput screening of compounds for prediction of in vivo estrogenicity. The combination of the yeast estrogen bioassay with the U2OS ERα-CALUX assay seemed the most promising for an ITS for in vitro estrogenicity testing. The main outliers identified when correlating data from the different in vitro assays and the in vivo uterotrophic assay are 4-hydroxytamoxifen, testosterone and to a lesser extent apigenin, tamoxifen and kepone. Based on the modes of action possibly underlying these discrepancies it becomes evident that to further improve the ITS and ultimately replace animal testing for estrogenic effects, the selected bioassays have to be combined with other types of in vitro assays, including in vitro models for bioavailability as a result of adsorption, distribution, metabolism and excretion (ADME characteristics) of the compounds under investigation.

In an effort to also develop new, especially omics-based, in vitro assays for estrogenicity, **chapter 4** describes a newly developed low-density DNA microchip for the detection of estrogenic compounds and their relative potencies. The low-density DNA microchip in array tube format contains probes for 11 different estrogen-responsive genes (marker genes) selected from a comprehensive gene expression study of estrogen-treated MCF-7/BOS cells and was used to test a set of 12 selected reference compounds. The results showed that the seven most informative marker genes on the microchip resulted in fingerprints that correctly predicted the estrogenic activity of the model compounds, except that of the negative control testosterone. Two marker genes (i.e., myeloid leukemia factor 1-interacting protein and ubiquitin-conjugating enzyme E2C) were even capable of correctly predicting the estrogenic potency of all five ER agonists tested and correlated well with the potencies as determined in the MCF-7/BOS proliferation assay and the in vivo uterotrophic assay. In addition, it was demonstrated that the estrogenic responses of testosterone, both in the array tube assay and in the proliferation assay, were partially due to the conversion of testosterone into estradiol by aromatase, but also due to formation of other estrogenic metabolites, the presence and estrogenic potency of which were confirmed by GC-MS/

MS analysis and a yeast-based reporter gene assay, respectively. The low-density DNA microchip-based analysis of marker gene expression fingerprints takes 3 days and is faster than the MCF-7/BOS cell proliferation assay which takes 6 days, but it is nevertheless quite laborious and requires highly skilled technicians; therefore, it is not ideal in its current format for the large-scale testing of chemicals as required by initiatives like REACH.

To further develop an in vitro ITS for refinement, reduction and ultimately replacement of in vivo tests for estrogenicity testing, **chapter 5** describes a 155-plex high-throughput in vitro coregulator binding assay enabling the study of ligand-modulated interaction of coregulators with ERα. This assay uses a 96-well PamChip plate with a peptide microarray mounted in each well representing 155 different coregulator-nuclear receptor binding motifs. The relative estrogenic potencies of the tested compounds were determined based on ligand-induced ERα binding to 57 (out of 155) coregulator peptides on the PamChip peptide array that displayed a clear E2 dose-dependent response (goodness of fit of a logistic dose-response model of 0.90 or higher). The estrogenic potencies thus obtained were compared to the relative estrogenic potencies as determined in the in vivo uterotrophic assay. It was shown that the estrogenic potencies predicted for 18 selected compounds based on induction of ERα-coregulator binding correlated well with their in vivo potencies in the uterotrophic assay, with coefficient of determination (R^2) values for 30 coactivators even being higher than or equal to 0.85. Moreover, this coregulator binding assay was able to distinguish ER agonists from ER antagonists, as selective estrogen receptor modulators (SERMs), such as tamoxifen, showed distinct profiles for coregulator activation from pure ER agonists, such as dienestrol. It was concluded that combination of this coregulator binding assay with other types of in vitro assays, e.g., reporter gene assays and the H295R steroidogenesis assay, will provide an in vitro test panel suitable for screening and prioritization of chemicals, thereby contributing to reduce and ultimately replace animal testing for estrogenic effects.

With a view to practical application of the coregulator binding assay described in chapter 5, for example in a regulatory toxicology setting, **chapter 6** presents the results on the technical characteristics of the assay, such as the reproducibility and robustness. A set of 14 model compounds recommended by the Office of Prevention, Pesticides and Toxic Substances (OPPTS) for testing laboratory proficiency in estrogen receptor transactivation assays were tested in the coregulator binding assay. With a median coefficient of variation of 5.0 % and excellent correlation (R^2 = 0.993) between duplicate measurements, the reproducibility of the ERα-coregulator binding assay was better than the reproducibility of other commonly used in vitro ER functional assays. In addition, the coregulator binding assay is correctly predicting the estrogenicity for 13 out of 14 compounds tested. When the potency of the ER-agonists to induce ERα-coregulator binding was compared to their ER binding affinity, their ranking was similar, and the correlation between the respective EC_{50} values was excellent (R^2 = 0.96), as was the correlation with their potency in a transactivation assay (R^2 = 0.94). Moreover, when the ERα-coregulator binding profiles were hierarchically clustered using Euclidian cluster distance, the structurally related compounds were found to cluster together, e.g., the steroid test compounds having an aromatic A-ring were separated from those with a cyclohexene A-ring. The results obtained in chapter 5 and 6 showed that

the coregulator binding assay is capable to distinguish ERα agonists from ERα antagonists and is even reflecting structural similarity of ERα agonists, indicating its potential to achieve identification and classification of ERα endocrine disruptors with high fidelity.

Further research, as described in **Chapter 7**, focused on the evaluation and extension of the previously established ITS for in vitro estrogenicity. Ten estrogenic compounds with modes of action in part different from ER binding were tested in the previously defined ITS, i.e., in a yeast estrogen reporter gene assay, a U2OS ERα-CALUX reporter gene assay and a cell-free coregulator binding assay. Results show that the two reporter gene assays in the ITS accurately predict the estrogenicity of the model compounds and revealed 100% concordance with the in vivo uterotrophic assay. Moreover, the coregulator binding profiles provided new mechanistic insights into estrogen receptor signaling of the model compounds tested. Moreover, by adding androgen reporter gene assays as well as the H295R steroidogenesis assay to the ITS, several model compounds also showed potent antiandrogenic properties and effects on steroidogenesis that might potentiate the estrogenic effects in vivo. By covering these additional mechanisms of estrogen-related endocrine disruption, this extended ITS will go beyond in vivo estrogenicity testing by the uterotrophic assay, thereby contributing to refinement, reduction and to some extent even a replacement of current animal testing for estrogenic effects.

Chapter 8 presents a discussion on the selection of the in vitro assays for the ITS based on the in vitro - in vivo correlation approach, taking also into account other aspects such as costs, fastness, simplicity, reproducibility and adaptability for high-throughput applications. Altogether, the results of the present thesis reveal that an ITS consisting of the U2OS ERα-CALUX, yeast estrogen bioassay and ERα coregulator binding assay enables an accurate prediction of the estrogenic effects in vivo and provides mechanistic insights. By including the H295R steroidogenesis assay as well as an androgen reporter gene assay, the extended ITS will even go beyond in vivo estrogenicity testing as performed by the uterotrophic assay. The extended ITS presented in this thesis allows easy high-throughput screening and prioritization of chemicals, and was shown to be able to contribute to the refinement, reduction and to some extent even a replacement of current animal testing for estrogenic effects.

A

Samenvatting

Acknowledgements

Curriculum Vitae

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SAMENVATTING

Het testen van chemicaliën op hun hormoonverstorende werking is een belangrijk aspect bij de beoordeling van de veiligheid van reeds in gebruik zijnde en nieuw ontwikkelde chemicaliën. De verstoring van de signaaltransductie via de oestrogeenreceptor is hiervan een belangrijk onderdeel. De standaardtest voor het meten van verstoring van de normale hormoonfunctie van oestrogenen is de zogenaamde uterotrofe assay, een in vivo test die gebruik maakt van muizen of ratten, hetzij van juveniele dieren of van dieren waarvan de eierstokken zijn verwijderd, met uterusgewicht als de voornaamste meetwaarde. Vanwege de ernstige nadelen van de in vivo uterotrofe assay, zoals de hoge kosten, de ethische bezwaren en het arbeidsintensieve karakter, heeft de ontwikkeling van een in vitro testbatterij voor in vivo oestrogeniteit hoge prioriteit. Het doel van dit proefschrift was een integrale teststrategie (ITS) te ontwikkelen, gebaseerd op bestaande en nieuw-ontwikkelde in vitro testen voor oestrogene werking, die het onderzoeken van grote aantallen monsters ("high-throughput screening") en prioritering van chemicaliën mogelijk maakt en vereenvoudigt. Een ITS, bij voorkeur gebaseerd op in vitro testen, zou een cruciale stap zijn op de weg naar verfijning, vermindering en de uiteindelijk vervanging (3Vs) van de huidige dierproeven voor oestrogene en andere hormoonverstorende effecten.

Om dit doel te bereiken, werden meerdere reeds beschikbare- en nieuw ontwikkelde in vitro bioassays geselecteerd en werd geëvalueerd welke testen de oestrogene effecten op de uterus/het endometrium in vivo het beste weergeven. In **hoofdstuk 2** van het proefschrift werd de oestrogene potentie van 12 stoffen bestudeerd in vier verschillende celproliferatietesten, testen gebaseerd op humane cellijnen die prolifereren in afhankelijkheid van oestrogenen, om te bepalen in welke mate deze in vitro testen een alternatief bieden voor de in vivo uterotrofe assay. De gebruikte cellijnen voor de proliferatietesten waren afkomstig uit drie verschillende vrouwelijke oestrogeengevoelige weefsels, t.w. borst- (MCF-7/BOS en T47D), endometrium- (ECC-1) en ovariumcellen (BG-1). Deze cellijnen werden gekarakteriseerd door hun relatieve oestrogeenreceptor (ER) α- en β-niveaus te bepalen, omdat de ERα/ERβ-ratio een dominante factor is die bepalend is voor de proliferatierespons. De resultaten toonden aan dat alle vier de cellijnen duidelijk ERα en een zeer lage, zij het detecteerbare, hoeveelheid ERβ tot expressie brengen (zowel op mRNA- als eiwitniveau). De T47D cellijn vertoonde nog de hoogste expressie van ERβ, maar ook daar was ERα dominant. Vervolgens werd voor een aantal geselecteerde referentiestoffen met verschillende werkingsmechanismen en oestrogene potentie de proliferatieve respons van de vier cellijnen onderzocht. Het bleek dat de respons van alle vier cellijnen op de modelstoffen een redelijk goede correlatie vertoonde met hun in vivo uterotrofe effect, waarbij de correlatie het hoogst was voor de MCF-7/BOS-cellijn (R^2 = 0.85). De belangrijkste verschillen tussen de in vivo uterotrofe assay en in vivo celproliferatie werden waargenomen voor tamoxifen en testosteron. De onverwachte proliferatieve respons van de MCF-7/BOS cellen na blootstelling aan testosteron bleek deels veroorzaakt te worden door de omzetting van testosteron in estradiol door aromatase of via androsteendion naar estron. Geconcludeerd werd dat van de vier geteste cellijnen, de proliferatietest met de humane MCF-7/BOS borstkankercellijn de beste is om mee te nemen in een ITS ter vervanging van de in vivo uterotrofe assay.

Behalve proliferatietesten worden ook reportergen-assays, gebaseerd op hetzij zoogdiercellen, hetzij gistcellen, veelvuldig voor oestrogeniteitsonderzoek gebruikt. In **hoofdstuk 3** werd onderzocht welke van deze in vitro assays het meest accuraat het uterotrofe effect in vivo voorspelt en mogelijke discrepanties tussen de in vitro assays en de in vivo uterotrofe assay werden geïdentificeerd. Hiervoor werd een grotere groep van 23 oestrogene referentiestoffen getest in een serie in vitro reportergen-assays en in de al eerder geselecteerde MCF-7/BOS proliferatie-assay (hoofdstuk 2). De meeste van deze stoffen waren geselecteerd uit de ICCVAM-lijst van stoffen die opgesteld is voor de validatie van in vitro oestrogeniteitstesten, en die verschillende chemische structuurklassen vertegenwoordigt. De reportergen-assays behelsden twee CALUX-assays gebaseerd op humane borstkankercellijnen, te weten de T47D ER-CALUX en U2OS ERα-CALUX, en een op gist gebaseerde bioassay voor oestrogenen. De resultaten van deze assays werden vergeleken met gepubliceerde resultaten van ER-receptorbindingstesten en de door de OECD-gevalideerde BG1Luc ER-transcriptie-activatie (TA) test, en vervolgens allemaal vergeleken met de uitkomsten van de in vivo uterotrofe assay om zo de in vitro assay(s) te identificeren met het beste voorspellend vermogen voor de in vivo uterotrofe respons. Alle in vitro assays lieten een redelijk goede tot goede correlatie zien (R^2 = 0.62-0.87) met de in vivo uterotrofe assay. De bioassay gebaseerd op gist, die een groen fluorescerend eiwit (yEGFP) tot expressie brengt bij blootstelling aan oestrogenen, gaf de beste correlatie te zien (R^2 = 0.87). De correlatie met de MCF-7/BOS celproliferatietest in deze studie met 23 stoffen was hetzelfde als eerder verkregen in de studie met de kleinere set van 12 referentiestoffen (R^2 = 0.85). Vergeleken met de reportergen- en ER receptorbinding-assays (1-2 dagen) neemt de MCF7/BOS celproliferatietest te veel tijd in beslag (6 dagen) en wordt daarom beschouwd als minder geschikt voor het snel screenen van grote aantallen stoffen. De combinatie van de gistbioassay voor oestrogenen met de U2OS ERα-CALUX assay leek het meest veelbelovend in het kader van een ITS voor het testen van oestrogene activiteit in vitro. De belangrijkste afwijkende uitkomsten, na correleren van de data van de verschillende in vitro assays met de in vivo uterotrofe assay, werden gevonden voor 4-hydroxytamoxifen, testosteron en - in mindere mate – apigenine, tamoxifen en chloordecon (kepone). Gebaseerd op de werkingsmechanismen die mogelijk ten grondslag liggen aan deze afwijkende uitkomsten, wordt duidelijk, dat om de ITS verder te verbeteren en uiteindelijk een volledige vervanging van dierproeven voor het testen van oestrogene effecten te bereiken, de geselecteerde bioassays moeten worden gecombineerd met andere typen in vitro assays, inclusief in vitro modellen voor biobeschikbaarheid als gevolg van absorptie, distributie, metabolisme en excretie (ADME eigenschappen) van de onderzochte stoffen.

In een poging om ook nieuwe, in het bijzonder op omics gebaseerde in vitro assays voor oestrogeniteit te ontwikkelen en te testen, wordt in **hoofdstuk 4** een nieuw ontwikkelde DNA microchip met lage probe-dichtheid beschreven voor de detectie van oestrogene stoffen en hun relatieve potenties. Deze "low-density" DNA-microchip in "array tube" format bevat probes voor 11 verschillende oestrogeen-responsieve genen (marker genen) die geselecteerd werden op basis van een genoomwijde gen-expressiestudie van met oestrogeen behandelde MCF-7/BOS-cellen. Deze microchip werd gebruikt om een groep

van 12 geselecteerde referentiestoffen te testen. De resultaten toonden aan dat de zeven meest informatieve genen op de microchip "vingerafdrukken" opleverden die de oestrogene activiteit van de modelstof correct voorspelden, met uitzondering van de negatieve controle testosteron. Twee marker genen (te weten myeloid leukemia factor 1-interacting protein en ubiquitin-conjugating enzym E2C) waren zelfs in staat de oestrogene potentie te voorspellen van alle vijf de geteste ER-agonisten, en correleerden dus goed met de potenties zoals gemeten in de MCF-7/BOS celproliferatietest en de in vivo uterotrofe assay. Daarnaast werd aangetoond dat de oestrogene respons van testosteron, zowel in de array tube assay als in de proliferatietest, gedeeltelijk veroorzaakt werd door de omzetting van testosteron in estradiol door aromatase, maar ook door de vorming van andere oestrogene metabolieten. De aanwezigheid en oestrogene potentie van die metabolieten werden bevestigd door respectievelijk een GC-MS/MS analyse en een op gist gebaseerde reportergen-assay. De analyse van deze "vingerafdrukken" gebaseerd op markergenexpressie gemeten door middel van de "low-density" DNA microchip duurt 3 dagen en is weliswaar sneller dan de MCF-7/BOS celproliferatietest (6 dagen), maar is nogal arbeidsintensief, vereist een hoog technisch niveau en duurt langer dan de in vitro reportergen-assays en bindingsassays (hoofdstuk 3). Daarom is deze test in de huidige vorm niet ideaal voor het op grote schaal testen van chemicaliën zoals in het kader van REACH gewenst is.

Om een in vitro ITS te ontwikkelen voor verdere verfijning, reductie en uiteindelijk een volledige vervanging van in vivo proeven voor oestrogeniteitsonderzoek, beschrijft **hoofdstuk 5** een in vitro coregulator-bindingstest, geschikt voor grote aantallen monsters (high-throughput), die de bestudering mogelijk maakt van door ligandbinding gemoduleerde interactie van ERα met 155 verschillende coregulatoren. Deze assay gebruikt een 96-well PamChip-plaat met in elke well een microarray met peptiden welke 155 verschillende bindingsmotieven representeren voor binding van coregulatoren aan kernreceptoren. De relatieve oestrogene potenties van de onderzochte stoffen werden bepaald op basis van de binding, geïnduceerd door de desbetreffende ligand, tussen ERα en 57 (van de 155) coregulatorpeptiden op de PamChip peptide-array die een duidelijke, van de E2 dosis afhankelijke respons lieten zien (een goodness-of-fit van een logistisch dosis-responsmodel van 0.90 of hoger). De aldus gevonden oestrogene potenties werden vergeleken met de relatieve oestrogene potenties zoals bepaald in de in vivo uterotrofe assay. Daaruit bleek dat de oestrogene potenties die waren voorspeld voor 18 geselecteerde stoffen, goed correleerden met hun in vivo potenties in de uterotrofe assay, waarbij de correlatiecoëfficiënten (R^2) voor 30 co-activatoren zelfs groter of gelijk waren aan 0.85. Bovendien was deze coregulator-bindingstest in staat ER agonisten te onderscheiden van ER antagonisten, aangezien selectieve modulatoren van de oestrogeenreceptor (SERMs), zoals tamoxifen, profielen vertoonden voor coregulator-activatie welke duidelijk verschilden van die van pure ER-agonisten, zoals dieenestrol. De conclusie was dat de combinatie van deze coregulator-bindingstest met andere typen in vitro testen, bijvoorbeeld reportergen-assays en de H295R steroidogenese assay, een in vitro test panel kunnen opleveren dat geschikt is voor het screenen en prioriteren van stoffen, en zo bijdragen aan het verminderen en uiteindelijk vervangen van dierproeven voor het onderzoeken van oestrogene effecten.

Met het oog op de praktische toepassing van de coregulator-bindingstest, zoals beschreven in hoofdstuk 5, bijvoorbeeld in de toxicologische regelgeving, laat **hoofdstuk 6** de resultaten zien van de technische karakterisering van de assay, zoals de reproduceerbaarheid en de robuustheid. Een groep van 14 modelstoffen, aanbevolen door het Office of Prevention, Pesticides and Toxic Substances (OPPTS) voor het onderzoeken van de bekwaamheid van onderzoekslaboratoria in het uitvoeren van transactivatie-assays, werden getest in de coregulator-bindingstest. Met een mediaan van 5% voor de variatiecoëfficiënt en een excellente correlatie (R^2 = 0.933) tussen duplometingen was de reproduceerbaarheid van de ERα-coregulator-bindingstest beter dan de reproduceerbaarheid van andere, standaard in vitro functionele ER-testen. Bovendien voorspelt de coregulator-bindingstest de oestrogeniteit van 13 van de 14 onderzochte stoffen op correcte wijze. Bij vergelijking van de potentie van deze ER-agonisten om ERα-coregulatorbinding te induceren met hun ER bindingsaffiniteit, bleek hun rangorde overeenkomstig te zijn, en de correlatie tussen de respectievelijke EC_{50}-waarden was uitstekend (R^2 = 0.96), evenals de correlatie met hun potentie in een transactivatie-assay (R^2 = 0.94). Bovendien bleken de structureel verwante stoffen samen te clusteren wanneer de ERα-coregulator bindingsprofielen hiërarchisch werden geclusterd op basis van Euclidische afstand, bijvoorbeeld de geteste steroïden met een aromatische A-ring werden gescheiden van de teststoffen met een cyclohexeenring. De resultaten die in hoofdstuk 5 en 6 werden verkregen, tonen aan dat de coregulator-bindingstest in staat is onderscheid te maken tussen ERα-agonisten en ERα-antagonisten en zelfs structurele gelijkenis weergeeft van ERα-agonisten, hetgeen zijn vermogen aantoont om een betrouwbare identificatie en classificatie van endocriene verstoorders (endocrine disruptors) van ERα tot stand te brengen.

Verder onderzoek, zoals beschreven in **hoofdstuk 7**, richtte zich op de evaluatie van de eerder ontwikkelde ITS voor in vitro oestrogeniteit. Tien oestrogene stoffen, deels met werkingsmechanismen verschillend van ER binding, werden getest in de eerder gedefinieerde ITS, te weten de gist reportergen-assay voor oestrogenen, de U2OS ERα-CALUX reportergen-assay en de celvrije coregulator-bindingstest (dedicated peptide microarray). De resultaten tonen aan dat de twee reportergen-assays in de ITS nauwkeurig de oestrogeniteit voorspelden van de modelstoffen met 100% overeenkomst met de in vivo uterotrofe assay. Daarenboven geven de coregulator-bindingsprofielen nieuwe mechanistische inzichten in de signaaltransductie via de oestrogeenreceptor geïnduceerd door de geteste modelstof. Door zowel reportergen-assays voor androgenen als de H295R steroidogenese assay aan de ITS toe te voegen, kwamen bij verschillende modelstoffen tevens potente antiandrogene eigenschappen en effecten op de steroidogenese aan het licht, welke zouden kunnen leiden tot potentiering van de oestrogene effecten in vivo . Door deze additionele mechanismen van aan oestrogenen gerelateerde endocriene verstoring mee te nemen, gaat deze uitgebreide ITS verder dan in vivo oestrogeniteitsonderzoek door middel van de uterotrofe assay.

Hoofdstuk 8 geeft een discussie betreffende de selectie van in vitro testen voor de ITS, gebaseerd op de in vitro – in vivo correlatie, en ook rekening houdend met andere aspecten zoals kosten, snelheid, eenvoud, reproduceerbaarheid en toepasbaarheid bij grote aantallen monsters ("high-throughput" toepassingen). Concluderend laten de resultaten van dit

proefschrift zien dat een ITS die bestaat uit de U2OS ERα-CALUX, de gist bioassay voor oestrogenen en de ERα coregulator-bindingstest, een nauwkeurige voorspelling mogelijk maakt van de oestrogene effecten in vivo en mechanistisch inzicht verschaft. Door zowel de H295R steroidogenesetest als een reportergen-assay voor androgenen toe te voegen, stijgt de ITS zelfs uit boven in vivo oestrogeniteitsonderzoek, zoals uitgevoerd in de uterotrofe assay. De uitgebreide ITS, zoals gepresenteerd in dit proefschrift, maakt "high-throughput" screening en prioritering van chemicaliën op eenvoudige wijze mogelijk en blijkt ook in staat te zijn bij te dragen aan de verfijning, reductie en tot op zekere hoogte zelfs vervanging van het huidige proefdieronderzoek naar oestrogene effecten.

ACKNOWLEDGEMENTS

First and foremost, I extend my sincere gratitude to my thesis supervisors. I'm greatly indebted to Ivonne for her guidance, vision, constructive feedback and encouragement throughout my work towards this thesis. I am extremely **G**rateful (with a capital and bold g) to Toine, as his passion, expertise, and inspiration have been indispensable to my growth as a scientist over these past years. I would like to sincerely thank Jac. His good advice, support and willingness to discuss the minutiae of the different obstacles I encountered have been invaluable.

I gratefully thank Ad, Laura and Jeroen for their valuable contributions to the project. I am thankful to René and Rinie for the enjoyable collaboration on the PamGene projects and lending me their expertise and intuition to my scientific and technical problems. Thanks also to Diana for her excellent technical support with the PamChip experiments.

Furthermore, many thanks to staff and (former) PhD students at Toxicology (Irene, Gre, Hans, Bert, Tinka, Gerrit, Ans, Marelle, Reiko, Wasma, Jochem, Justine, Barae, Erryana, Samantha, Myrthe, Karsten, Arif, Alexandros, Suzanne, Rungnapa, Elise, Jaime, Henrique, Ana, Walter, Alicia, Merel, Linda) and colleagues at RIKILT (Evelien, Geert, Hans, Peter. H, Peter. S, Henri, Richard, Gerlof, Nathanna, Agata, Meike, Eva, Oscar, Angeline, Arjen, Ron, Lonneke, Robert, Gerrit, Jenneke) whose friendship and support have made Wageningen not only a workplace but also a second home town for me. I would especially like to thank my female office mates: Astrid, Liza, Ainhoa, and Jia for the companionship. They made the office an enjoyable place and full of "estrogens". A big thank you goes to my paranymphs Nynke and Jonathan, for their valuable support and help. Thanks to my students Marieke and Dimitrios for their hard work and dedication.

I cannot forget friends who went through tough times together and celebrated each accomplishment: Yinghua, Yin, Jue, Feifei, Xinxin, Lijin, Zhaoying, Huaifeng, Huiyan, Kun, Jing, Xifeng, Aichun, Junyou, Xu, Wei, Ke, Shiyang, Xiao and all my soccer teammates.

Above all, I would like to thank my parents, for their love, trust and support.

Finally, my other half Hequn, whose love, encouragement and great patience at all times allowed me to complete this important chapter in my life. She already has my heart, so I will just give her a heartfelt "thanks."

Thank you all for your encouragement and support during this long but fulfilling journey!

CURRICULUM VITAE

Si Wang was born on June 12th,1983 in Chengde, China. After his secondary education in Chengde in 2001, he started his undergraduate study in Food Technology at China Agricultural University. In 2003, he moved to the Netherlands to continue his study at Wageningen University. After receiving his BSc degree in 2006, he continued with a master study in Food Safety and specialised in toxicology at Wageningen University. During his master, Si conducted a thesis research at RIKILT – Institute of Food Safety, and afterwards he spent a 6 month internship at Kraft Foods RD&Q Munich, Germany. After completing his master in 2008, he was appointed as a junior researcher at RIKILT on a project involving the implementation of the precision-cut liver slice techniques. From March 2009 until March 2013, he worked as a PhD student on the project presented in this thesis, which was a collaboration between the Division of Toxicology and RIKILT, Wageningen UR. During his PhD study, he followed several postgraduate courses in toxicology which enabled him to register as a European Toxicologist.

LIST OF PUBLICATIONS

Wang, S., Rijk, J. C. W., Poortman, J. H., Kuijk, S., Peijnenburg, A. A. C. M., and Bovee, T. F. H. (2010) Bovine liver slices combined with an androgen transcriptional activation assay: an in-vitro model to study the metabolism and bioactivity of steroids. *Analytical and Bioanalytical Chemistry*, *397*, 631-641.

Wang, S., Aarts, J. M. M. J. G., Evers, N. M., Peijnenburg, A. A. C. M., Rietjens, I. M. C. M., and Bovee, T. F. H. (2012) Proliferation assays for estrogenicity testing with high predictive value for the in vivo uterotrophic effect. *The Journal of Steroid Biochemistry and Molecular Biology*, *128*, 98-106.

Wang, S., Rijk, J. C. W., Pen, M. J., Aarts, J. M. M. J. G., Peijnenburg, A. A. C. M., Rietjens, I. M. C. M., and Bovee, T. F. H. (2013) A low-density DNA microchip for the detection of (anti-) estrogenic compounds and their relative potencies. *Analytical Biochemistry*, *435*, 83-92.

Wang, S., Houtman, R., Melchers, D., Aarts, J., Peijnenburg, A., Van Beuningen, R., Rietjens, I., and Bovee, T. (2013) A 155-plex high-throughput in vitro coregulator binding assay for (anti-)estrogenicity testing evaluated with 23 reference compounds. *ALTEX*, *30*, 145-157.

Wang, S., Aarts, J., de Haan, L., Argyriou, D., Peijnenburg, A., Rietjens, I., and Bovee, T. Towards an integrated in vitro strategy for estrogenicity testing. *Submitted for publication to Journal of Applied Toxicology*.

Aarts, J. M. M. J. G., Wang, S., Houtman, R., van Beuningen, R. M. G. J., Westerink, W. M. A., Van De Waart, B. J., Rietjens, I. M. C. M., and Bovee, T. F. H. (2013) Robust Array-Based Coregulator Binding Assay Predicting ERα-Agonist Potency and Generating Binding Profiles Reflecting Ligand Structure. *Chemical Research in Toxicology*, *26*, 336-346.

Rijk, J. C. W., Bovee, T. F. H., Wang, S., Poucke, C., Peteghem, C. v., and Nielen, M. W. F. (2009) Detection of anabolic steroids in dietary supplements: The added value of an androgen yeast bioassay in parallel with a liquid chromatography-tandem mass spectrometry screening method. *Analytica Chimica Acta*, *637*, 305-314.

Bovee, T. F. H., Hendriksen, P. J. M., Portier, L., Wang, S., Elliott, C. T., Egmond, H. P. v., Nielen, M. W. F., Peijnenburg, A. A. C. M., and Hoogenboom, L. A. P. (2011) Tailored Microarray Platform for the Detection of Marine Toxins. *Environmental Science and Technology*, *45*, 8965-8973.

Szalowska, E., Stoopen, G., Rijk, J. C. W., Wang, S., Hendriksen, P. J. M., Groot, M. J., Ossenkoppele, J., and Peijnenburg, A. A. C. M. (2013) Effect of oxygen concentration and selected protocol factors on viability and gene expression of mouse liver slices. *Toxicology in Vitro*, *27*, 1513-1524.

Evers, N. M., Klundert, T. M. C. v. d., Aesch, Y. M. v., Wang, S., Roos, W. K. d., Romano, A., Haan, L. H. J. d., Murk, A. J., A.G.H.Ederveen, Rietjens, I. M. C. M., and Groten, J. P. (2013) Human T47D breast cancer cells with tetracycline-dependent ERβ expression reflect ERα/ERβ ratios in rat and human breast tissue. *Toxicology in Vitro*, *Accepted for Publication*.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

Organ Toxicology, Postgraduate Education in Toxicology (PET), Nijmegen, 2010
Molecular Toxicology, PET, Amsterdam, 2010
Toxicogenomics, PET, Maastricht, 2010
Medical and Forensic Toxicology, PET, Utrecht, 2011
Lab. Animal science, PET, Utrecht, 2011
Cell Toxicology, PET, Leiden, 2012
Pathobiology, PET, Utrecht, 2012
Environmental Toxicology, MSc course, Wageningen, 2012

Meetings

NVT annual meeting, Zeist, 2011-2013 (1 oral and 2 poster presentations)
Netherlands Toxicogenomics Centre (NTC) annual meeting, Amsterdam, 2010-2012 (1 oral and 2 poster presentations)
51st annual meeting of the American Society of Toxicology, San Francisco, 2012 (poster presentation)
First Joint German-Dutch Meeting of the Societies of Toxicology, Düsseldorf, 2012 (poster presentation)
Joint BELTOX-INVITROM congress, Edegem, 2012 (oral presentation)
2nd Congress on Steroid Research, Chicago, 2013 (poster presentation)

General courses

VLAG PhD week, Venlo, 2010
Techniques for Writing and Presenting a Scientific Paper, Wageningen, 2011
Voice matters, Wageningen, 2012
Reviewing a Scientific Paper, Wageningen, 2012
Education-career development workshops at SOT meeting, San Francisco, 2012
Netherlands Toxicogenomics Centre training on valorization of research results, Amsterdam, 2012

Optional activities

Preparing PhD research proposal, 2009
Attending research in progress presentations at Division of Toxicology, 2009-2012
Attending business unit research presentations at Rikilt, 2009-2012
Organization and participation of Toxicology PhD trip to Switzerland and Italy, 2011

Approved by Graduate School VLAG

The studies described in this thesis were conducted within a collaborative project of Wageningen University and RIKILT - Institute of Food Safety. The research was financially supported by the Netherlands Genomics Initiative (Netherlands Toxicogenomics Centre, grant number 6162500134).

Financial support from Wageningen University and RIKILT - Institute of Food Safety for printing this thesis is gratefully acknowledged.

Layout and printing: Off Page, Amsterdam, The Netherlands.