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

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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.

1. 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

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[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 signalling 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].

2. Materials and methods

2.1. Chemicals

Estradiol (E2), dienestrol, zearalenone, corticosterone, tamoxifen, 4-hydroxytamoxifen, EGF and β-mercaptoethanol were obtained from Sigma–Aldrich Chemie BV (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-5 h-inden-5-ylidene)bisphenol from Acros Organics (Fisher Emergo BV, 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).

2.2. 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.

2.3. 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 24 h. After 24 h, 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 5000 cells/well and allowed to attach for 24 h. After 24 h, 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 5000 cells/well, respectively and both cell lines were exposed for 5 days.

2.4. LDH cytotoxicity test

At the end of the proliferation assay (described in Section 2.3), lactate dehydrogenase (LDH) released by damaged cells into the cell

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′

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.

2.5. 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.

2.6. 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.

2.7. 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, 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 50 V for 30 min followed by 100 V for 30 min using anode buffer (0.2 M 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 BV, Amsterdam Zuidoost, The Netherlands) at 100 V 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).

2.8. 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 24 h 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.

2.9. 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

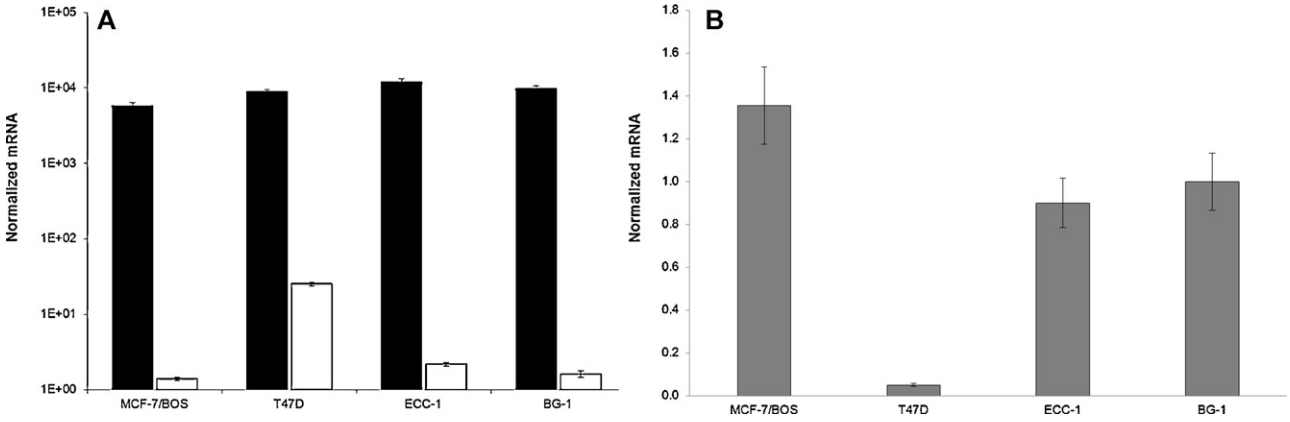

Fig. 1. Relative estrogen receptor and aromatase mRNA expression measured in MCF-7/BOS, T47D, ECC-1 and BG-1 cells. (A) ERα (black column) and ERβ (white column) expression are normalised to HP-1 mRNA, (B) aromatase (grey column) expression is normalised to GAPDH mRNA. Average fold change and standard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

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].

3. Results

3.1. 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.

3.1.1. ERα, ERβ and aromatase mRNA levels

Total RNA was isolated from each of the four cell lines 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. Fig. 1 shows the ERα, ERβ and aromatase mRNA levels as determined in the four human cell lines. Fig. 1A shows that, compared to the MCF-7/BOS cell line, the T47D, ECC-1 and BG-1 cell lines have about 1.5–2.0 times higher ERα mRNA amounts and that the T47D cell line expresses a relative high amount of the ERβ mRNA. Fig. 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.

3.1.2. 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. Fig. 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. Fig. 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

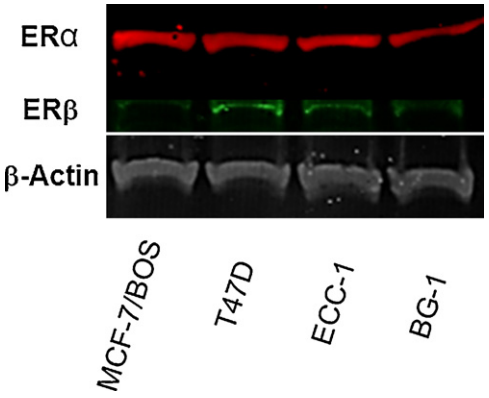

Fig. 2. ERα and ERβ protein expression measured in MCF-7/BOS, T47D, ECC-1 and BG-1 cells. Upper part shows a Western blot of a total protein extract after treatment with ER antibodies, and the lower part the same blot after treatment with β-actin antibodies to show that equal amounts of protein had been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

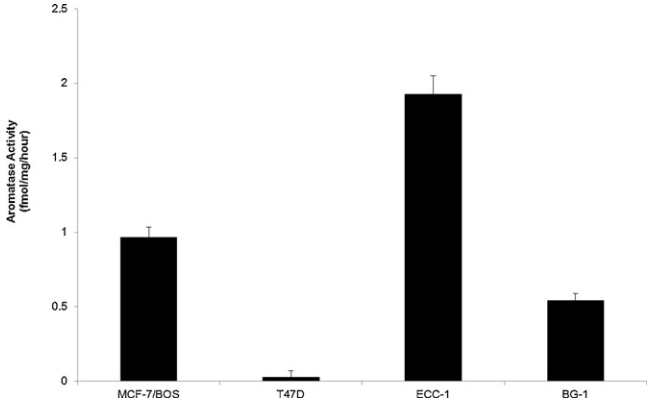

Fig. 3. Aromatase enzyme activity measured in MCF-7/BOS, T47D, ECC-1 and BG-1 cells. Aromatase activity was measured as described in Section 2.8. Results are presented as the mean aromatase enzyme activity ± SD ($n = 3$) expressed as fmol estrogen formed/mg protein/h.

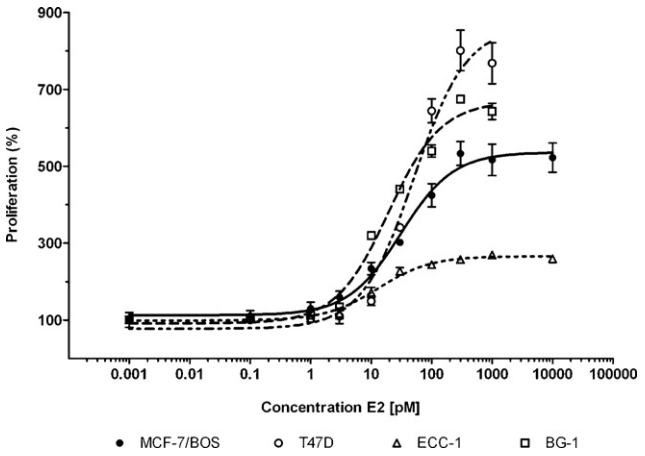

Fig. 4. Proliferative response of 17β-estradiol obtained in the four estrogen-sensitive human cell lines. Results are representative of at least five independent experiments and are presented as the mean proliferative response ±SD ($n = 3$) expressed as a percentage of the solvent (DMSO) control.

aromatase activity in the ECC-1 cell line being about 2-fold higher than what would be expected based on the mRNA level.

3.2. Cell proliferation assays

Fig. 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. Fig. 5a–d 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 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. Fig. 6 shows that letrozole partially inhibited the proliferative response induced by testosterone.

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.

Compounds	Uterotrophic assay	MCF-7/BOS		T47D		ECC-1		BG-1	
	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. [26]

[e] Obtained from Fang et al. [27].

[f] EGF is able to mimic the uterotrophic effects of estrogen in the rodent [28].

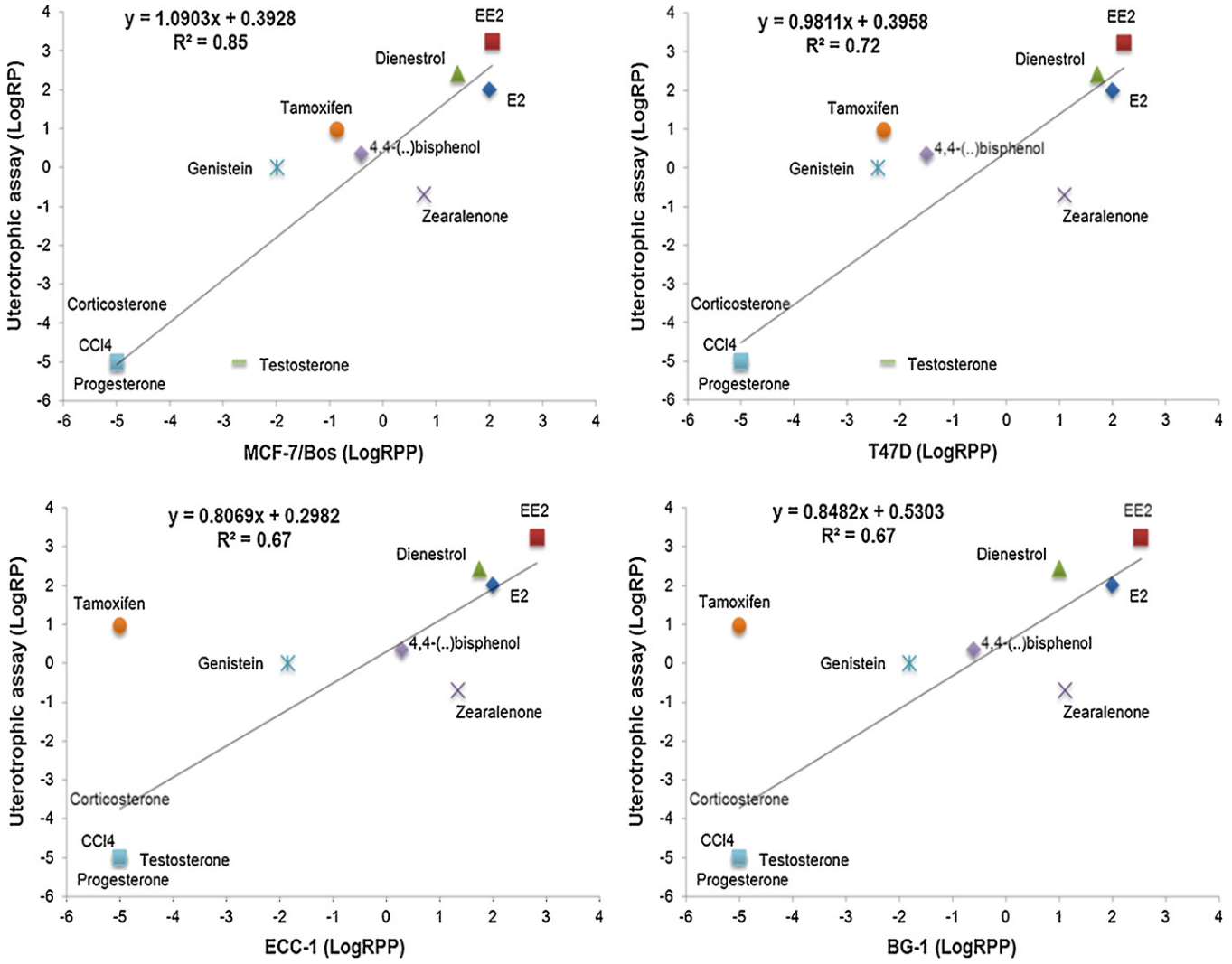

Fig. 5. Comparison of the *in vivo* uterotrophic assay with the proliferation assays using four different estrogen-responsive human cell lines. With tamoxifen excluded, the ECC-1 and BG-1 cell lines show a $R^2 = 0.92$, $y = 0.97x + 0.06$ and $R^2 = 0.91$, $y = 1.02x + 0.34$, respectively.

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 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

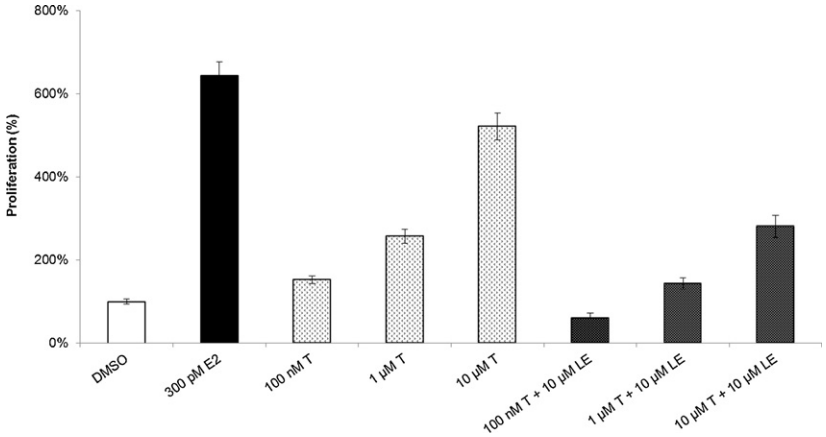

Fig. 6. Effect of the aromatase inhibitor letrozole (LE) on testosterone induced cell proliferation in MCF-7/BOS cells. Results are representative of at least three independent experiments and are presented as the mean proliferative response ±SD ($n = 3$) expressed as a percentage of the solvent (DMSO)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

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 uterotrophic effect[a]	NE[b]	NE	NE	NE
EGF + E2	Enhanced uterotrophic effect	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.

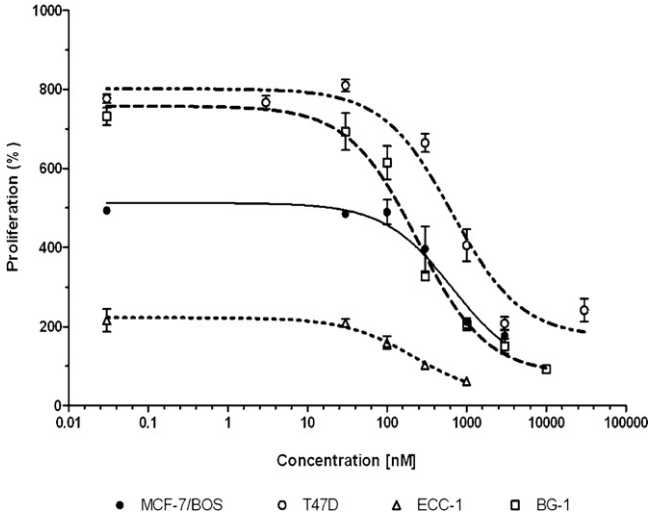

Fig. 7. Antagonistic effect of tamoxifen measured in MCF-7/BOS, T47D, ECC-1 and BG-1 cells. Cells were treated with 300 pM 17β-estradiol in combination with different concentrations of tamoxifen. Results are expressed as the mean proliferative response ±SD ($n = 3$) presented as a percentage of the solvent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

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 compounds were studied as well. Fig. 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.

4. 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 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, Fig. 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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