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The assessment of estrogenic or anti-estrogenic activity of chemicals by the human stably transfected estrogen sensitive MELN cell line: Results of test performance and transferability

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ABSTRACT

The need for development and validation of *in vitro* hormone receptor transactivation assays as important alternative tools to study interactions with sex hormone receptors is outlined by international organisations, as such assays should be included in the OECD conceptual framework for the testing and assessment of endocrine active chemicals. Therefore as part of the European Union (EU)-sponsored 6th framework project ReProTect, the validation study with MELN cells, MCF-7 cells (ER+, estrogen receptor positive) which were stably transfected with the estrogen responsive gene ERE-βGlob-Luc-SVNeo was set up. Standard operating procedures including a prescreen assay for unknown chemicals, an ER-agonist assay and an ER-antagonist assay were developed at the Flemish Institute for Technological Research, Belgium, and successfully transferred to Bayer Schering Pharma AG, Germany. Test results were obtained for 16 chemicals, and it was demonstrated that the MELN assay is transferable, robust and reproducible which allowed to rank chemical compounds according to their strong to weak affinity for the estrogen-α receptor, or identify negative chemicals within the test range up to 10^{-5} M. Besides the screening for agonism, we demonstrated the suitability of MELN cells to test for antagonistic activity, which is of added value compared to current validated assays. As the MELN assay successfully passed the first modules of the ECVAM validation procedure, it now should be considered for further steps including the definition of a prediction model and application domain to get it accepted as an alternative screening assay, contributing to the 3R's with a reduction of animal experiments.

1. Introduction

Concern has been raised that certain environmental chemicals, natural or man-made cause adverse effects in both human and wildlife through their interaction with the endocrine system. This relates mainly to possible effects on the environmental or human health through (anti)estrogenic, (anti)androgenic or (anti)thyroid activity of chemicals [1–3]. Taking into account the likelihood of broad exposure of man and wildlife organisms, the need to develop a sound testing strategy with robust test methods has been highlighted in the past 10–15 years [4–6]. The related OECD activity, which is part of the OECD Test Guidelines Programme, is managed by the Task Force on Endocrine Disrupters Testing and Assessment (EDTA) and they provided the OECD conceptual framework [7]. This framework identifies assays of increasing complexity and detail to gather information on a chemical. It includes (a) structural activity relationships and *in vitro* assays that could identify the chemical based on intrinsic characteristics (e.g. estrogen receptor binding), (b) short-term *in vivo* (screening) assays (e.g. uterotrophic assay) and (c) definitive long-term assays involving exposure at different developmental stages of animals and evaluation of multi-generation effects [7].

With regard to the potential need to screen thousands of chemicals for endocrine activity, the use of animal studies is problematic in terms of costs, speed and ethical considerations. Both the recommendations to the US Environmental Protection Agency (EPA) of the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) [8] and the OECD conceptual framework for the testing and assessment of endocrine disrupting chemicals [7] addressed *in vitro* hormone receptor binding and

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transactivation assays as important fast screening tools to study interactions with sex hormone receptors. EPA asked the Interagency Coordination Committee on the Validation of Alternative Methods (ICCVAM) to evaluate the scientific validity of *in vitro* estrogen receptor (ER)- or androgen receptor (AR)-based assays. However, in a comprehensive review by ICCVAM on new, revised, and alternative toxicological test methods for screening endocrine disrupting activity and application for regulatory use [9] there appeared no adequately validated *in vitro* ER- or AR-based assays, and recommendations were made with regard to the four different types of assays and the minimal performance standards to be incorporated in an assay protocol. Recently results from a Japanese validation study of a stably transfected transactivation assay (STTA), using the hERα-HeLa-9903 cell line were made available by the Japanese Chemical Evaluation and Research Institute (CERI) through the OECD website [10]. The MVLN assay, that uses MCF-7 cells stably transfected with the vitellogenin-luciferase reporter plasmid [11], was among those considered by ICCVAM as one of the assays to be validated together with other binding and transactivation assays, the latter being of added value just because of their capability to discriminate both receptor agonists and antagonists. Therefore in a joint effort and through partial funding by the European Union (EU)-sponsored 6th framework project ReProTect, the Flemish Institute for Technological Research (VITO) did initiate in 2004 prevalidation studies with the MVLN cells. However, lack of reproducibility and persistent mycoplasm infection triggered discussions with INSERM, the inventor of MVLN cells, and INSERM recommended to use another reporter cell line, the MELN cells, MCF-7 cells which were stably transfected with the estrogen responsive gene ERE-βGlob-Luc-SVNeo [12]. With approval of the European Center for the Validation of Alternative Methods (ECVAM), the scientific coordinator of ReProTect, the prevalidation study by VITO was continued but oriented to the development of a standard protocol with the MELN cells. A test protocol for both agonist and antagonist activity was optimized for a limited panel of chemicals, an assay for simultaneous cytotoxicity assessment was included and results on intralaboratory variability were obtained [13]. Next, a generic test plan was developed by ReProTect partners in collaboration with ECVAM to proceed with the 2nd phase of prevalidation for two ER- and two AR-transactivation assays. The VITO standard protocols of the MELN assay were transferred to Bayer Schering Pharma AG (BSP), partner within ReProTect, and interlaboratory variability of the assay was evaluated for a common panel of test chemicals, which were defined by ECVAM within the task force for endocrine disrupting chemicals. The test plan for transactivation assays consisted of a prescreen to be run for 4 chemicals in order to identify the mode of action (agonist/antagonist), the concentration range of activity (up to maximum 10^{-5} M) and the cytotoxicity range. Next to the prescreen, a protocol was run for either the agonist assay or the antagonist assay for the ECVAM list of chemicals, which were classified according to known activity. These tests were set up in at least 3 independent runs in both labs in a concentration range defined by the leading lab. Raw data files and calculation files (template format) were made available to an independent statistician for data analysis. Results on the interlaboratory transferability, test performance and reproducibility of the MELN assay are reported and discussed.

2. Materials and methods

2.1. Test chemicals

Both laboratories used compounds from the same supplier and lot, as listed in Table 1. The stock solutions were prepared in DMSO, and aliquoted in glass vials for storage at −18 °C. For each experimental run, a new aliquot of stock solution was used and fresh preparations of dilutions of test chemicals according to instructions in the standard operating procedures (SOPs) were made.

2.2. Cell culture of MELN cells

MCF-7 cells, adherent human breast cancer cells which express the endogenous estrogen receptor α and β, the latter being of secondary importance [14,15], were stably transfected with the estrogen responsive gene ERE-βGlob-Luc-SVNeo. Those transfected cells are called MELN cells [12]. MELN cells were made available to VITO and BSP by Dr. P. Balaguer (INSERM, Montpellier, France) after signature of a material transfer agreement (MTA).

The cells were regularly checked for mycoplasm infection in order to guarantee experimental work with mycoplasm-free cells and comply with guidelines for good cell culture practice (GCCP). MELN cells were maintained in growth medium, which consisted of 90.5% DMEM: F12 (1:1) with GlutaMAX, 1% P/S (Gibco, Invitrogen, Merelbeke, Belgium), 1% G418 sulfate (100 mg/ml) (InvivoGen, Cayla-InvivoGen, Toulouse, France), 7.5% FCS (Harlan, IMP, Brussels, Belgium at VITO or PAA laboratories, Pasching, Austria at BSP) and 0.125% NaOH (1 M). The cell line was maintained in an incubator at 37 °C with a relative humidity of 95% and a CO_2 concentration of 5%. The cells were subcultured once a week, with in between medium refreshment. The cells can be used from passage number 4 up to passage number 25 in these MELN procedures for ER transactivation.

2.3. Pretreatment to hormone-depleted medium and exposure to test chemicals

The standard set up for compound exposure of MELN cells and measurements of ER- transactivation does include a pretreatment of cells in order to reduce the background signal caused by endogenous activity in the presence of FCS. Approximately 1 week before seeding (day 1, at Monday or Tuesday), the cells were subcultured in sterile cell culture flasks (75 cm^2). To test 3 compounds in the prescreen, or 6 compounds in the agonist or antagonist assay (3 × 96-well plates), 1 culture flask with cells which have reached approximately 95% confluence is required. In order to adapt the cells to charcoal/dextran stripped fetal calf serum, CS-FCS, the cells in the flasks which have grown for 4 days were rinsed with PBS and the growth medium was replaced with fresh test medium, consisting of OPTIMEM I without

Table 1
Information on identification and source of selected test chemicals and solvent used in MELN assays.

Name—abbreviation	Cas no.	Supplier	Supplier code	Batch identity	Purity (%)
17β-Estradiol—E2	50-28-2	Sigma	E-1024	026K18061	98.8
Dibutylphthalate	84-74-2	Aldrich	15,243-9	S34804	99.7
Diethylstilbestrol	56-53-1	Sigma	D-4628	106H0643	99.6
n-Butylparaben	94-26-8	Aldrich	54680	1210159	100
Corticosterone	50-22-6	Sigma	C-2505	035K1673	100
17α-Ethynylestradiol	57-63-6	Sigma	E-4876	045K1440	99
Equol	531-95-3	APIN chem	04392 e	23730	98.2
Genistein	446-72-0	Sigma	G-6649	104K1198	98.9
Hexestrol	84-16-2	Sigma	H-7753	112K0687	100
ICI 182.780	129453-61-8	Tocris	1047	18A/62566	>99
Nonylphenol	84852-15-3	Fluka	74430	1092230	96.5
Norethynodrel	68-23-5	Sigma	N-7253	088F0192	>99
4-OH-tamoxifen—4OH-tam	68047-06-3	Sigma	H-7904	076K4109	99
o,p′-DDT	789-02-6	Supelco	49018	LB39706	97.7
Raloxifene-HCl	82640-04-8	Sigma	R-1402	036K1054	99.5
Tamoxifen	10540-29-1	Sigma	T-5648	046K1569	99
Dimethylsulfoxide (DMSO)-VITO	67-68-5	Labscan	H34J11X	3552/6	99.5
Dimethylsulfoxide (DMSO)-BSP	67-68-5	Sigma	D-5879	51K0004	≥99.5

phenol red (Invitrogen) and 5% CS-FCS. At Vito, CS-FCS of a commercial source was used (Hyclone, Perbio, Belgium), while at BSP FCS was stripped in the laboratory. Therefore a batch of 500 ml FCS was stripped two times for 30 min with 2×5 g of a mixture of 0.5 g dextran T70 (Amersham Biosciences, Uppsala, Sweden) and 4.5 g activated charcoal (100 mesh particle size, Sigma Aldrich, Taufkirchen, Germany). The step of medium replacement was repeated twice, prior to seeding the cells in 96-well plates at day 8 (respectively Monday or Tuesday) for any of the MELN assays.

Following adaptation to hormone-depleted conditions cells were microscopically evaluated for infection and normal growth, before they were harvested for subsequent plating at a cell number of 8×10^5 cells/ml in black 96-well plates with flat clear bottom (Costar). Details are given by Berckmans et al. [13].

2.4. Prescreen assay

The prescreen procedure has been developed to allow testing when no information is available on the agonistic or antagonistic nature of the compound, neither on its overall toxicity to the cells. Therefore exposure of the test chemicals in this prescreen is set up in several ways. (1) A dilution series of the compound in test medium, which is supplemented with the solvent (e.g. DMSO). An increase of the luminescent signal in a concentration dependent way in comparison to the solvent control, does point to agonist activity. (2) A dilution series of the compound in test medium is supplemented with the EC_{50} of the reference agonist, 17β-estradiol (E2). When the luminescent signal increases in comparison with the solvent control (=EC_{50} of E2 in DMSO), the compound is an agonist. When the luminescent signal decreases in comparison to EC_{50} of E2, the compound is an antagonist (except see 3). (3) For some compounds the decrease of the luminescent signal can be non-specific, which either can be caused by cell death (to be evaluated by a cytotoxicity test, see below) or it can be due to other effects, e.g. inhibition of protein synthesis or mRNA transcription. To evaluate the latter, a dilution series of the compound is evaluated in test medium, supplemented with 1000× EC_{50} of E2. If the antagonistic effect is due to competition at the receptor, then exposing with an excess of reference agonist should shift the curve approximately 3 log units (1000×) to the right. When the decrease of the luminescent signal is non-specific, the curve is expected to shift equally or less than 1 log unit to the right.

Each of the test chemicals in the prescreen was evaluated at 5 concentrations in the range 1×10^{-5} M down to 1×10^{-13} M (1:100 dilution factor) and corresponding solvent control (SC, DMSO), with 3 replicate wells for each test concentration at these 3 different series of test conditions, to be set up at the same test plate. The final DMSO concentration in each of the test conditions in the prescreen was 0.2%. The 4 chemicals selected for evaluation in the prescreen, 17β-estradiol, norethynodrel, raloxifene-HCl and 4-OH-tamoxifen were set up at both laboratories in only 1 experimental run.

2.5. Agonist assay

Within each series of experiments, a complete concentration response was set up for the reference estrogen, 17β-estradiol on one plate. The upper part of this plate was filled from left to right with the solvent control (0.1% DMSO), eight concentrations of 17β-estradiol, E2 (3.3×10^{-13}, 1.0×10^{-12}, 3.3×10^{-12}, 1.0×10^{-11}, 3.3×10^{-11}, 1.0×10^{-10}, 3.3×10^{-10}, and 1.0×10^{-9} M) and positive plate control 1.0×10^{-9} M E2. The lower part of this plate was used to investigate a test compound and was filled from left to right with the positive plate control 1.0×10^{-9} M E2, the solvent control (0.1% DMSO) and 8 concentrations of the test compound. Other test plates were filled using a similar scheme consisting of 8 concentrations of a test chemical in the upper and lower part, respectively. Next to the reference agonist, the concentration range and dilution factors for test chemicals were set up as recommended by the leading lab. The chemicals evaluated in the agonist assay were dibutylphthalate (7.8×10^{-8} to 1.0×10^{-5} M, 1:2), diethylstilbestrol (6.1×10^{-13} to 1.0×10^{-8} M, 1:4), n-butylparaben (7.8×10^{-8} to 1.0×10^{-5} M, 1:2), corticosterone (1.3×10^{-10} to 1.0×10^{-5} M, 1:5), o,p′-DDT (4.6×10^{-9} to 1.0×10^{-5} M, 1:3), equol (1.3×10^{-10} to 1.0×10^{-5} M, 1:5), genistein (6.1×10^{-10} to 1.0×10^{-5} M, 1:4), hexestrol (3.1×10^{-13} to 5.0×10^{-9} M, 1:4), nonylphenol (2.3×10^{-9} to 5.0×10^{-6} M, 1:3) and 17α-ethynylestradiol (3.1×10^{-13} to 5.0×10^{-9} M, 1:4). Norethynodrel was tested at concentrations which were chosen by the individual laboratories based on the outcome of the prescreen assay. Each test chemical was evaluated at least three times by independent experiments.

2.6. Antagonist assay

Within each series of experiments, one plate was set up with a complete concentration series of the reference anti-estrogen, 4-OH-tamoxifen combined with the EC_{50} concentration of E2. According to the SOP, the EC_{50} concentration of E2 should be the mean value of at least 3 former agonist experiments, and was set by VITO at 3.7×10^{-11} M. This same concentration was used in the set up at BSP. The upper part of the plate was filled from left to right with the solvent control, 8 concentrations of 4OH-tam (1.3×10^{-11}, 6.4×10^{-11}, 3.2×10^{-10}, 1.6×10^{-9}, 8.0×10^{-9}, 4.0×10^{-8}, 2.0×10^{-7}, 1.0×10^{-6} M) and the positive plate control 1.0×10^{-6} M 4OH-tam. In all these conditions, the test medium in the well was supplemented with test medium containing E2, in order to reach a nominal E2 concentration in the well of 3.7×10^{-11} M corresponding to the EC_{50} concentration of E2. The solvent concentration in all the test conditions was 0.2% DMSO. The lower part of this plate was used to investigate a test compound and was filled from left to right with the positive plate control 1.0×10^{-6} M 4OH-tam with EC_{50} E2, the solvent control (0.2% DMSO with EC_{50} E2) and 8 concentrations of the test compound with EC_{50} E2. Other test plates were filled using a similar scheme with 8 concentrations of a test chemical in the upper and lower part, respectively. Next to the reference antagonist, the concentration range and dilution factors for test chemicals were set up as recommended by the leading lab. The chemicals evaluated in the antagonist assay were dibutylphthalate (7.8×10^{-8} to 1.0×10^{-5} M, 1:2), corticosterone (1.3×10^{-10} to 1.0×10^{-5} M, 1:5), o,p′-DDT (4.6×10^{-9} to 1.0×10^{-5} M, 1:3), genistein (1.3×10^{-10} to 1.0×10^{-5} M, 1:5), ICI 182.780 (1.3×10^{-12} to 1.0×10^{-7} M, 1:5), nonylphenol (3.1×10^{-10} to 5.0×10^{-6} M, 1:4) and tamoxifen (3.1×10^{-10} to 5.0×10^{-6} M, 1:4). Raloxifene-HCl and norethynodrel were evaluated at a concentration range derived from the prescreen assay, specific for each of the laboratories. For each test chemical, at least 3 independent experiments were run.

2.7. Cytotoxicity assessment

At the end of the exposure period, plates were removed from the incubator and inspected for infection and visual signs of cell death prior to further analysis for cytotoxicity and luminescence.

The CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega cat nr G7890 or G7891) is a sensitive fluorometric method, which was optimised to detect the release of lactate dehydrogenase (LDH) from damaged cells in MELN assays, next to luciferase measurements on the same test plates. Details on the procedure are given by Berckmans et al. [13]. Fluorescence was measured either at an excitation/emission wavelength of 544 nm/590 nm (Fluoroskan, Labsystems at VITO) or 540 nm/580 nm (Tecan Ultra fluorimeter, Tecan, Crailsheim, Germany, at BSP). After subtracting the background fluorescence signal (=no cell control), mean values and standard deviation of replicate wells were calculated. LDH activity in the media from treated cells was related to LDH activity in media from corresponding SC cells, set at 100%.

2.8. Determination of luciferase activity in cells

At the end of the exposure period, and after visual observations of the cell cultures and removal of 100 μl of test medium to quantify cytotoxicity, the remaining test medium was removed by flipping the plates and the luciferase assay was performed as described in former work [13]. Measurements of luminescence were made using either the Luminoskan (Labsystems) equipped with an automatic dispenser at VITO or the Tecan Ultra luminometer (Tecan, Crailsheim, Germany) at BSP. The luciferase assay reagent (Promega, E1482) was applied at 50 μl in each well, and immediately after addition, luminescence was measured for 5 s. Data were expressed as relative light units (RLU).

2.9. Data analysis and statistical evaluation

Only for test conditions which showed no cytotoxicity to the cells, luciferase activity was calculated. A test condition was judged cytotoxic when % LDH leakage > 110%, $p < 0.01$ (Student's t-test, comparison to SC), and the response curve for cytotoxicity showed concentration dependency.

For the analysis of data from the prescreen assay, mean RLU values and a 95% confidence interval were calculated for replicate wells. The mean value for the RLU response of the test chemical in the presence of 1000× EC_{50} of E2 was fixed at 100%, and all other measurements of that chemical were calculated relative to this value and summarized in excel sheets as mean values with 95% confidence interval. A software package GraphPad Prism (version 2.01 and version 5.0) was used to fit sigmoid concentration response curves with a variable slope, if possible. Therefore relative RLU values for luciferase activity were plotted as a function of the $\log_{10}$ transformation of the concentration of test compounds. Next to data on cytotoxicity, these graphs could be used to identify the mode of action and the concentration range of receptor activity.

With respect to the evaluation of chemicals in the agonist and antagonist assays, all raw data were transferred to the statistician (MW) and treated according to standard procedures. Prior to fitting a dose–response model and estimation of EC_{50} (agonist mode) or IC_{50} (antagonist mode), the data were normalised as the RLU response of treated wells was divided by the mean of the corresponding solvent control. The four-parametric log-logistic function

$$f(x) = \theta_1 + \frac{\theta_2 - \theta_1}{1 + \exp(\theta_3(\log(x) - \theta_4))}$$

was fitted to the transformed data using the `drm` function of `R`-package `drc`. EC_{50}/IC_{50} and confidence intervals were calculated using the `ED` function of the same package [16,17]. Parameter θ_4 is the log of the EC_{50}/IC_{50}, i.e. the log of the concentra-

tion with a response half-way between upper response limit θ_2 and lower response limit θ_1. Parameter θ_3 is the slope parameter. Parameters θ_1 und θ_2 were constrained to be non-negative.

The relative agonistic/antagonistic activity (RAA) of each compound was calculated as the ratio of the EC_{50}/IC_{50}-value of each compound and the EC_{50}/IC_{50}-value of the positive control compound (E2 or 4OH-tam). This RAA value does allow to rank chemicals according to their potency for estrogen receptor activation or suppression.

2.10. Test performance criteria

The prescreen assay was designed as a preliminary test, and except for the exclusion of wells with infection or cytotoxicity for luminescence analysis, thus far no specific quality criteria were defined. In some cases, EC_{50} or IC_{50}-values could be calculated in the prescreen, but it was mainly a qualitative evaluation of the concentration response curves, obtained by GraphPad curve fitting to decide about the nature of the chemical (agonism, true antagonism or cytotoxicity).

When screening for agonism, test performance criteria were defined for the reference agonist (E2) only. This included that (1) a sigmoid concentration response should be observed, (2) the EC_{50} of E2 should be in the range 1×10^{-11} M to 10×10^{-11} M and (3) the E2 induction factor (average RLU at 1×10^{-9} M of E2/average RLU of SC) should be > 4.0. The first 2 criteria are evaluated on the dilution series of E2, which is planned in each experimental run. The third criterion is evaluated plate by plate, using the average values of internal plate controls (SC and 1×10^{-9} M of E2).

Test performance criteria defined for 4OH-tam, reference chemical in the antagonist assay were (1) a sigmoid response curve with clear antagonist response (=suppression of EC_{50} E2 signal) and (2) an inhibition factor (average RLU at SC + EC_{50} E2/average RLU at 10^{-6} M 4OH-tam + EC_{50} E2) which should be >10.0. The latter criterion is evaluated plate by plate using the average values of internal plate controls.

3. Results

3.1. Prescreen

In order to evaluate the applicability of the prescreen approach, and simulate testing of chemicals with no background information on estrogenic properties, 4 chemicals of the ECVAM list were selected: 17β-estradiol, norethynodrel, 4-OH-tamoxifen and raloxifene-HCl. The prescreen therefore included testing for agonism (compound only), for antagonism (compound in the presence of EC_{50} of E2) and for non-specific inhibition to be assessed by the response to an excess of estrogenic stimulus (compound in presence of 1000× the EC_{50} of E2). The 3 test conditions for 4 test chemicals in the prescreen, run at VITO and at BSP, did show similar responses. These results are summarised in graphs (a–h) for each chemical and each laboratory in Fig. 1 for the purpose of qualitative assessment of the nature of each compound. Both at VITO and BSP, luciferase activity was induced at concentrations of $\geq 10^{-11}$ M for E2 (Fig. 1a and b), and at concentrations of $\geq 10^{-9}$ M for norethynodrel (Fig. 1c and d), both in the absence and presence of EC_{50} of E2 until a plateau was reached. It further appeared that these chemicals, exposed in combination with 1000× the EC_{50} of E2 were not able to further modify the high luciferase activity at 1000× the EC_{50} of E2 (3.7×10^{-8} M). These features clearly indicated ER agonism. In only one test condition, for the test plate with 17β-estradiol run at BSP, cytotoxicity was measured at the highest test concentration (10^{-5} M) in the presence of the EC_{50} of E2. Therefore this point for luciferase activity was omitted on the curve (Fig. 1b). Results for raloxifene-HCl and 4OH-tam in the prescreen demonstrated the absence of luciferase induction, pointing to the lack of agonism activity (Fig. 1e–h). On the other hand, a clear concentration dependent suppression of luciferase activity was observed, both in the absence and in the presence of the EC_{50} concentration of the reference agonist. The suppressive effects were also confirmed in the presence of 1000× the EC_{50} of E2, while the curves shifted approximately 3 log units to the right. This points to true antagonism by both chemicals, while no indication for unspecific effects was apparent. For none of these chemicals, neither at VITO nor at BSP, cytotoxicity was noticed at any of the test conditions. Thus, raloxifene-HCl and 4OH-tam were further tested in the antagonism assay.

3.2. Testing for agonist activity

Next to 17β-estradiol as reference estrogen, the other chemicals dibutylphthalate, diethylstilbestrol, n-butylparaben, corticosterone, o,p′-DDT, equol, genistein, hexestrol, nonylphenol and 17α-ethynylestradiol were tested by both laboratories using the same test concentrations as defined in the test plan by the leading lab. Only for norethynodrel each lab used test conditions which were slightly different, as these were derived from results of the prescreen and according to instructions in the test procedure. At VITO, norethynodrel was evaluated in the range 4.8×10^{-12} to 1.0×10^{-5} M, with a dilution factor 1:8. At BSP, norethynodrel was tested at the following concentrations 1.0×10^{-11}, 1.0×10^{-10}, 1.0×10^{-9}, 3.3×10^{-9}, 1.0×10^{-8}, 3.3×10^{-8}, 1.0×10^{-7}, and 1.0×10^{-6} M. Comparison of results at both laboratories showed similar responses: except for corticosterone and dibutylphthalate, all the chemicals did induce maximum luciferase activity higher than 300% compared to solvent control (3-fold induction) and EC_{50}-values could be calculated. Based on the current data set of test chemicals, an induction of at least 3-fold compared to the SC is considered as positive for agonist activity. A few examples of concentration response curves generated at VITO and BSP from independent experimental runs, and representative for MELN agonist assays are shown in Fig. 2a and b. Table 2 lists the calculated EC_{50}-values with upper and lower 95% confidence intervals (CI) of individual experiments at each lab, as well as the mean EC_{50}, with standard deviation (SD) and intralaboratory coefficient of variation (CV) per chemical obtained at VITO or at BSP. For tests at VITO, the mean CV value on calculated EC_{50} for all chemicals was 32.1% (range 5.7–69.1%), while the mean CV value at BSP was 56.8% (range 20.1–157%). This higher mean value was mainly caused by an exceptional high CV for DES (157%), which could be attributed to a deviation in one experimental run with diethylstilbestrol, likely due to an error with preparation of test concentrations. Despite some intralaboratory variability, a very good comparability of the EC_{50}-values for each of the chemicals between labs was seen, and this is also illustrated in Fig. 3a. These estimates of EC_{50}-values at both labs resulted in similar ranking for the majority of chemicals with same results if ranking was based on absolute EC_{50} or on relative agonist activity (RAA), calculated as the ratio EC_{50} TC (test chemical)/EC_{50} E2 (reference agonist). Ranking from highest to lowest estrogenic potency gave the same at VITO and BSP for 17α-ethynylestradiol > 17β-estradiol > hexestrol > diethylstilbestrol > norethynodrel > nonylphenol > n-butylparaben followed by corticosterone and dibutylphthalate which appeared negative up to the highest tested concentration 10^{-5} M. For 3 test chemicals with moderate to low estrogenic activity (to be ranked between nonylphenol and n-butylparaben) the ranking at VITO (genistein > equol > o,p′-DDT) was different from BSP (equol > o,p′-DDT > genistein). However, as the EC_{50}-values for these 3 chemicals were in a very narrow range from 0.99×10^{-6} to 3.35×10^{-6} M, these differences should have no major impact on the ranking of chemicals using the MELN assay as a screening test in a tiered approach. For all test compounds, the CytoTox-ONE™ assay for LDH leakage was applied, and only at VITO in 1 out of 3–4 experimental runs per chemical, cytotoxicity was seen at only the highest test concentration 10^{-5} M for equol, genistein and o,p′-DDT (Table 2). Test performance criteria were met for all MELN agonist assays which were run at BSP. At VITO in majority of test conditions, the performance criteria were met except that results of only 1 test plate (total 25 plates tested in 8 experimental runs) were discarded due the induction factor for the reference agonist

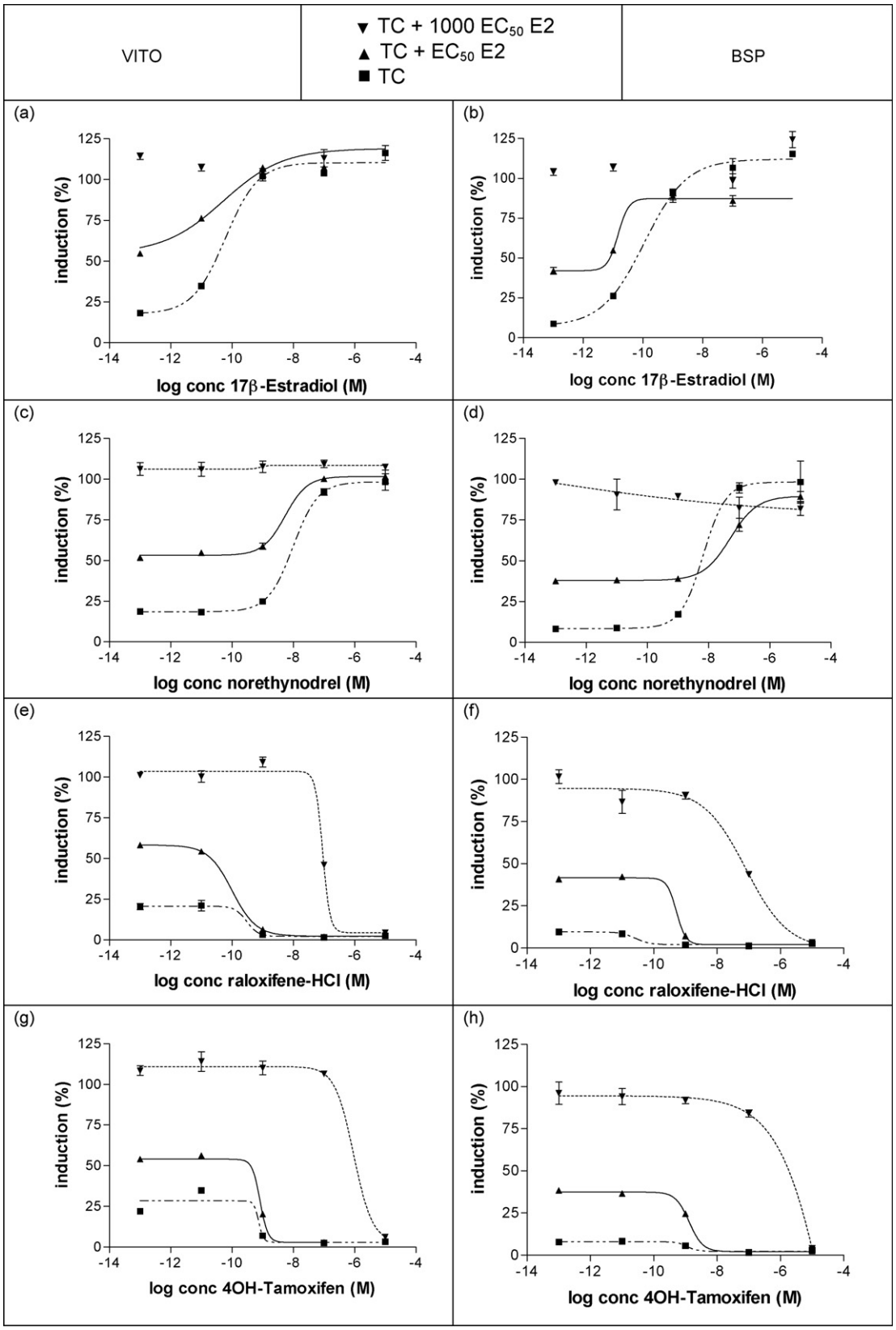

Fig. 1. Graphs of prescreen results including 3 test set ups for 4 different test chemicals (TC): 17β-estradiol (a and b), norethynodrel (c and d), raloxifene-HCl (e and f) and 4-OH-tamoxifen (g and h), run at both laboratories VITO (left panel) and BSP (right panel). The curves represent mean values with SD ($n = 3$) and if possible, fitting was made by GraphPad software.

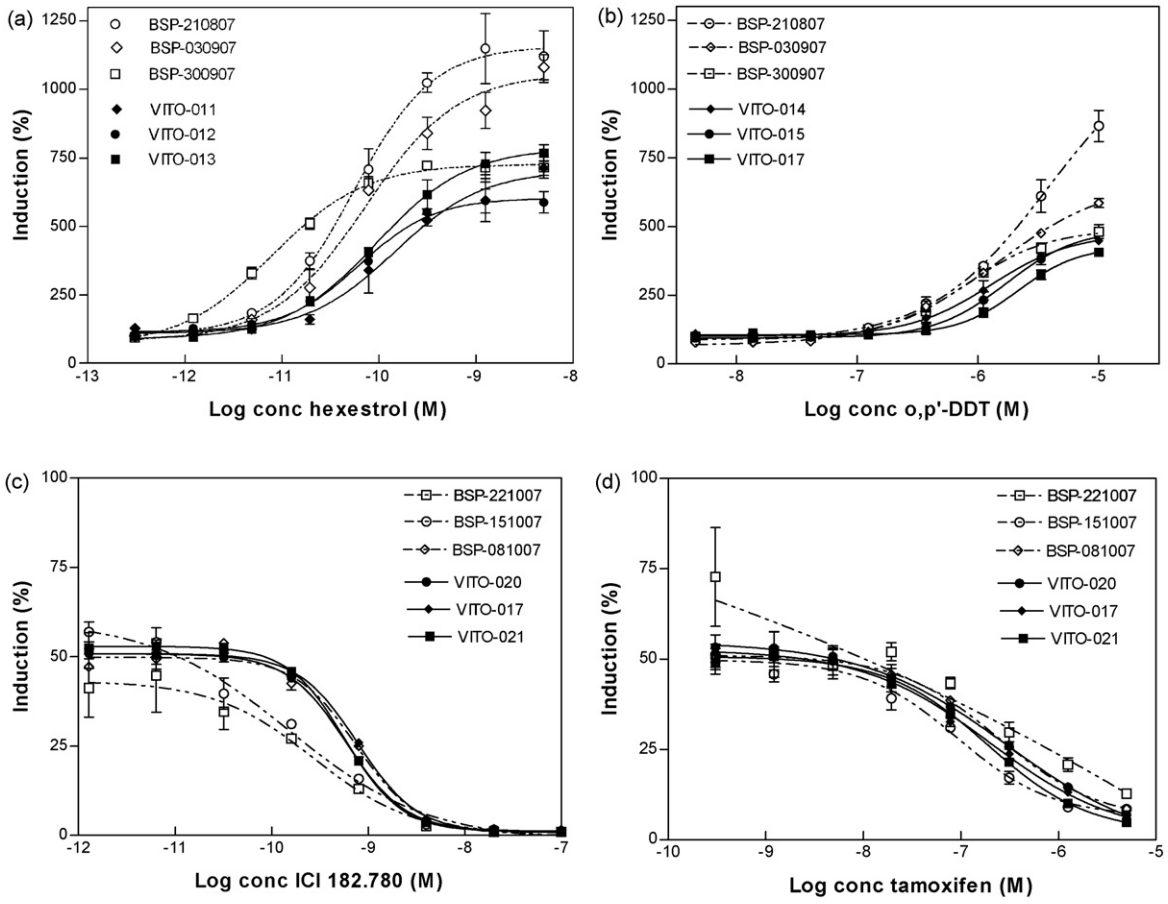

Fig. 2. Examples of agonist assays with hexestrol (a) and o,p′-DDT (b) and antagonist assays with ICI 182.780 (c) and tamoxifen (d), for at least 3 independent experiments run at VITO or at BSP, respectively. The curves represent mean values with SD ($n = 3$) and fitting was made by GraphPad software.

which was not met: average RLU at 1×10^{-9} M of E2/average RLU of SC = 3.96, thus <4.0.

3.3. Testing for antagonist activity

The antagonist assay was set up with 4OH-tam as the reference antagonist, and to evaluate its mode of action, the EC_{50} level of E2 was added in the test medium at all conditions. Other test chemicals, dibutylphthalate, corticosterone, o,p′-DDT, genistein, ICI 182.780, nonylphenol and tamoxifen were evaluated in the concentration range as recommended by the leading lab and listed in the test plan. Norethynodrel, though negative for antagonism in the prescreen both at VITO and BSP, was nevertheless evaluated in the antagonist assay as it was on the ECVAM list also marked as a chemical with potential antagonist activity. At VITO, norethynodrel was evaluated in the range 4.77×10^{-12} M to 1.0×10^{-5} M, with a dilution factor 1:8. At BSP, norethynodrel was tested at 1.0×10^{-11}, 1.0×10^{-10}, 1.0×10^{-9}, 3.3×10^{-9}, 1.0×10^{-8}, 3.3×10^{-8}, 1.0×10^{-7}, and 1.0×10^{-6} M. Raloxifene-HCl was clearly positive for antagonism activity in the prescreen and each of the laboratories defined the test conditions for the MELN antagonist assay. At BSP, the test was set up at 1.0×10^{-12}, 1.0×10^{-11}, 3.3×10^{-11}, 1.0×10^{-10}, 3.3×10^{-10} 1.0×10^{-9}, 1.0×10^{-8}, and 1.0×10^{-7} M, while at VITO a quite similar concentration series with raloxifene-HCl was set up from 1.28×10^{-12} to 1.0×10^{-7} M, with a dilution factor 1:5. Only 3 of 9 test chemicals demonstrated clear antagonist mode of action by a concentration dependent suppression of the EC_{50} E2 signal, similar to the reference antagonist 4OH-tam. Thus chemicals were considered positive for antagonist activity if the minimum luciferase activity was at least less than 40% compared to the solvent control with EC_{50} E2 (=50%). In Fig. 2c and d, a few graphs with data from individual experiments run at VITO and BSP are given as representative for the antagonist activity of chemicals as a function of concentrations. There were 4 chemicals, being norethynodrel, genistein, nonylphenol and o,p′-DDT which showed a concentration dependent increase of luciferase activity compared to solvent control condition at both laboratories, and thus agonistic properties were confirmed. Finally, dibutylphthalate and corticosterone showed only slight differences of RLU signals compared to solvent control and a clear concentration response relationship was not seen. These chemicals can be classified as negative for antagonism activity, at least within the tested range up to 10^{-5} M. Table 3 gives a summary of calculated IC_{50}-values with upper and lower 95% confidence intervals (CI) of individual experiments at each lab, as well as the mean IC_{50}, with standard deviation (SD) and intralaboratory coefficient of variation (CV) per chemical obtained at VITO or at BSP. Reproducibility within the labs, based on a limited number of experimental runs was good with a mean CV value of 18.5% at VITO and 45% at BSP. Interlaboratory comparison of mean IC_{50} between both laboratories gave almost similar values per chemical, as is illustrated in Fig. 3b, and this was also confirmed by the same ranking according to antagonistic potency. Strong antagonist activity was seen for raloxifene-HCl, followed by ICI 182.780 and 4OH-tam, while tamoxifen showed moderate potency at both laboratories. All test compounds were studied in parallel for potential cytotoxicity. For experiments run at VITO, occasionally a cytotoxic effect was present at the highest test concentration (10^{-5} M), but only in 2 of 3 experimental runs with either o,p′-DDT or dibutylphthalate (Table 3). For none of these conditions, neither for the other chemicals cytotoxicity was

Table 2
Comparative data from agonist assays run at VITO and BSP for each of selected test chemicals.

Compound	Experiment identification	EC_{50} (M)	Lower CI EC_{50} (M)	Upper CI EC_{50} (M)	Mean EC_{50} (M)	SD (M)	CV (%)	*n*
17β-Estradiol	e2.VITO.011-01.1	5.72E−11	4.69E−11	6.98E−11				
	e2.VITO.012-01.1	3.57E−11	3.06E−11	4.17E−11				
	e2.VITO.013-01.1	4.22E−11	3.93E−11	4.54E−11				
	e2.VITO.014-01.1	3.64E−11	3.28E−11	4.04E−11	4.23E−11	7.90E−12	18.7	8
	e2.VITO.015-01.1	4.40E−11	4.03E−11	4.80E−11				
	e2.VITO.016-01.1	3.65E−11	3.32E−11	4.00E−11				
	e2.VITO.017-01.1	5.04E−11	4.57E−11	5.55E−11				
	e2.VITO.022-04.1	3.64E−11	3.14E−11	4.21E−11				
	e2_03.09.07_Bayer_1_1	1.02E−11	5.06E−12	2.06E−11				
	e2_04.09.07_Bayer_1_1	2.29E−11	1.96E−11	2.67E−11				
	e2_21.08.07_Bayer_1_1	2.26E−11	1.86E−11	2.74E−11	2.36E−11	9.61E−12	40.7	5
	e2_28.08.07_Bayer_1_1	3.72E−11	3.08E−11	4.50E−11				
	e2_30.09.07_Bayer_1_1	2.53E−11	1.63E−11	3.92E−11				
17αEthynylestradiol	ee2.VITO.011-02.2	3.12E−11	2.69E−11	3.62E−11				
	ee2.VITO.012-02.2	1.62E−11	1.39E−11	1.89E−11	2.39E−11	7.50E−12	31.4	3
	ee2.VITO.013-02.2	2.43E−11	2.13E−11	2.79E−11				
	ee2_04.09.07_Bayer_3_2	1.26E−11	9.30E−12	1.70E−11				
	ee2_28.08.07_Bayer_3_2	1.74E−11	1.26E−11	2.42E−11	1.64E−11	3.36E−12	20.6	3
	ee2_30.09.07_Bayer_3_2	1.90E−11	1.36E−11	2.66E−11				
Diethylstilbesterol	des.VITO.011-02.1	1.71E−10	1.27E−10	2.29E−10				
	des.VITO.012-02.1	9.75E−11	7.52E−11	1.26E−10	1.66E−10	6.70E−11	40.3	3
	des.VITO.013-02.1	2.31E−10	1.89E−10	2.84E−10				
	des_04.09.07_Bayer_2_1	2.46E−12	1.08E−12	5.57E−12				
	des_28.08.07_Bayer_2_1	4.49E−10	3.88E−10	5.19E−10	1.60E−10	2.51E−10	157.0	3
	des_30.09.07_Bayer_2_1	2.83E−11	2.00E−11	4.01E−11				
Hexestrol	hex.VITO.011-03.1	1.45E−10	9.06E−11	2.31E−10				
	hex.VITO.012-03.1	6.13E−11	4.95E−11	7.59E−11	1.00E−10	4.19E−11	41.8	3
	hex.VITO.013-03.1	9.49E−11	7.90E−11	1.14E−10				
	hex_03.09.07_Bayer_1_2	5.30E−11	1.91E−11	1.47E−10				
	hex_21.08.07_Bayer_1_2	5.54E−11	4.33E−11	7.10E−11	3.91E−11	2.62E−11	67.1	3
	hex_30.09.07_Bayer_6_2	8.83E−12	7.22E−12	1.08E−11				
Equol	equ.VITO.011-03.2	9.76E−07	7.81E−07	1.22E−06				
	equ.VITO.012-03.2	2.17E−06	5.39E−07	8.72E−06	1.08E−06	7.49E−07	69.1	4
	equ.VITO.013-03.2	6.79E−07	5.77E−07	8.00E−07				
	equ.VITO.022-04.2[b]	5.12E−07	4.30E−07	6.09E−07				
	equ_04.09.07_Bayer_4_1	8.10E−07	5.44E−07	1.21E−06				
	equ_28.08.07_Bayer_4_1	1.19E−06	9.35E−07	1.51E−06	1.10E−06	2.55E−07	23.2	3
	equ_30.09.07_Bayer_4_1	1.30E−06	5.59E−07	3.00E−06				
Genistein	gen.VITO.012-04.1	1.01E−06	4.75E−07	2.14E−06				
	gen.VITO.013-04.1	7.10E−07	4.24E−07	1.19E−06	9.90E−07	3.87E−07	39.1	4
	gen.VITO.022-05.1	1.53E−06	9.52E−07	2.47E−06				
	gen.VITO.022-05.2[b]	7.08E−07	4.52E−07	1.11E−06				
	gen_04.09.07_Bayer_4_2	2.77E−06	1.68E−06	4.56E−06				
	gen_28.08.07_Bayer_4_2	1.16E−06	6.80E−07	1.97E−06	3.35E−06	2.53E−06	75.7	3
	gen_30.09.07_Bayer_4_2	6.12E−06	2.12E−06	1.77E−05[a]				
n-Butylparaben	dib.VITO.014-01.2	4.72E−06	4.32E−06	5.16E−06				
	dib.VITO.015-01.2	5.01E−06	4.41E−06	5.70E−06	5.41E−06	9.54E−07	17.6	3
	dib.VITO.016-01.2	6.50E−06	5.11E−06	8.26E−06				
	dib_04.09.07_Bayer_2_2	5.29E−06	4.89E−06	5.72E−06				
	dib_28.08.07_Bayer_2_2	7.54E−06	3.63E−06	1.57E−05[a]	6.13E−06	1.23E−06	20.1	3
	dib_30.09.07_Bayer_2_2	5.56E−06	4.79E−06	6.45E−06				
Norethynodrel	nor.VITO.011-01.2	8.01E−09	6.85E−09	9.38E−09				
	nor.VITO.012-01.2	7.33E−09	5.49E−09	9.77E−09	7.52E−09	4.32E−10	5.74	3
	nor.VITO.013-01.2	7.22E−09	6.43E−09	8.10E−09				
	nor_03.09.07_Bayer_2_2	7.00E−09	5.68E−09	8.61E−09				
	nor_21.08.07_Bayer_2_2	4.50E−09	3.61E−09	5.62E−09	5.28E−09	1.48E−09	28.1	3
	nor_30.09.07_Bayer_5_2	4.36E−09	3.21E−09	5.93E−09				
Nonylphenol	non.VITO.014-02.1	5.98E−07	4.84E−07	7.39E−07				
	non.VITO.015-02.1	1.01E−06	8.42E−07	1.22E−06	7.43E−07	2.34E−07	31.6	3

Table 2 (*Continued.*)

Compound	Experiment identification	EC_{50} (M)	Lower CI EC_{50} (M)	Upper CI EC_{50} (M)	Mean EC_{50} (M)	SD (M)	CV (%)	*n*
	non.VITO.017-01.2	6.17E−07	5.21E−07	7.30E−07				
	non_03.09.07_Bayer_2_1	2.89E−07	1.28E−07	6.55E−07				
	non_21.08.07_Bayer_2_1	1.27E−06	9.53E−07	1.69E−06	8.49E−07	5.04E−07	59.4	3
	non_30.09.07_Bayer_5_1	9.91E−07	6.76E−07	1.45E−06				
o,p′-DDT	opd.VITO.014-02.2	1.28E−06	1.03E−06	1.59E−06				
	opd.VITO.015-02.2[b]	1.65E−06	9.00E−07	3.04E−06	1.69E−06	4.35E−07	25.7	3
	opd.VITO.017-02.1	2.15E−06	1.93E−06	2.39E−06				
	opd_03.09.07_Bayer_3_1	1.40E−06	1.08E−06	1.82E−06				
	opd_21.08.07_Bayer_3_1	3.63E−06	2.06E−06	6.39E−06	1.95E−06	1.49E−06	76.4	3
	opd_30.09.07_Bayer_6_1	8.06E−07	6.64E−07	9.79E−07				
Dibutylphthalate	dbp.VITO.014-03.2	Negative: no response for agonism						
	dbp.VITO.015-03.2	Negative: no response for agonism			3			
	dbp.VITO.016-03.2	Negative: no response for agonism						
	dbp_04.09.07_Bayer_1_2	Negative: no response for agonism						
	dbp_28.08.07_Bayer_1_2	Negative: no response for agonism			3			
	dbp_30.09.07_Bayer_1_2	Negative: no response for agonism						
Corticosterone	cor.VITO.014-03.1	Negative: no response for agonism						
	cor.VITO.015-03.1	Negative: no response for agonism			3			
	cor.VITO.016-03.1	Negative: no response for agonism						
	cor_04.09.07_Bayer_3_1	Negative: no response for agonism						
	cor_28.08.07_Bayer_3_1	Negative: no response for agonism			3			
	cor_30.09.07_Bayer_3_1	Negative: no response for agonism						

[a] Upper level of EC_{50} confidence interval is out of dose range.
[b] Cytotoxicity at highest test concentration 10^{-5} M.

observed in experiments run at BSP. Test performance criteria for antagonist assays including a sigmoid response curve with clear antagonist suppression of EC_{50} E2 signal by the reference antagonist in each experimental run and an inhibition factor (average RLU at SC + EC_{50} E2/average RLU at 10^{-6} M 4OH-tam EC_{50} E2) > 10.0 on each test plate were met by both laboratories.

4. Discussion

The need for validated *in vitro* assays, such as the hormone sensitive transactivation assays, has been well recognised by international organisations involved in the establishment of testing guidelines. The development of test protocols with MELN cells providing a means to distinguish chemicals with agonist or antagonist properties for the estrogen receptor, and the validation exercise within the framework of ReProTect does anticipate to these testing needs. The different steps of the validation exercise were in agreement with the modular approach as outlined by Hartung et al. [18] and 4 building blocks test definition, within-laboratory variability, test transferability and between-laboratory variability have been considered so far.

The stably transfected MCF-7 cells with the estrogen responsive gene ERE-βGlob-Luc-SVNeo were developed at INSERM, France [12], and named MELN cells. VITO, the leading lab did evaluate the cells for a selected panel of chemicals and demonstrated their ER-responsiveness. Technical issues were improved for cost-efficient screening, establishing a reproducible assay compliant with test performance criteria, and accordingly standard operating procedures were developed for either evaluation of agonist properties or antagonist properties. Next to evaluation of the interaction of chemicals with the hormone receptor, it is crucial to identify potential interference of 'unknown' chemicals with cellular processes, including the reporter gene activation system and consequently an objective method to assess cytotoxicity should be included. However, a number of studies with ER-reporter assays used for screening an extended set of chemicals did even not mention any procedural step to account for potential cytotoxic effects [19–21]. A few other studies evaluated cytotoxicity by setting up an additional plate with exposed cells, next to the test plate for measurements of reporter gene activation. This approach was included in the screening with stably transfected T47D breast cancer cells using the 'Cytolite' luminescent assay for cell viability [22], with MVLN cells using the neutral red assay [23], as well as in the recently adopted OECD test guideline 455 with the stably transfected hERα-HeLa-9903 which did not define the cytotoxicity test to be used [24]. As part of protocol optimisation for the MELN assay, we searched for a cost-efficient approach to evaluate cytotoxicity and thus selected a fluorometric assay assessing lactate dehydrogenase (LDH) leakage in the cell culture medium, and compared its sensitivity to detect cytotoxicity for a panel of chemicals with the neutral red assay [13]. We demonstrated that the CytoTox-ONE™ kit, which seemed more sensitive than measurements of LDH leakage based on a colorimetric method, is a good alternative to test cytotoxicity with a major advantage that estrogenicity can simultaneously be measured for luciferase activity using the same test plates with MELN cells [13].

The likelihood of occurrence of cytotoxicity is highly dependent on the choice of chemicals, e.g. industrial chemicals versus pharmaceutical drugs such as synthetic hormones, and the highest concentration to be tested. Starting with the prescreen assay in order to deal with chemicals or drugs of unknown estrogen-like activity or toxicity, it was agreed to select 10^{-5} M as the highest test concentration, in agreement with OECD recommendations at that time (personal communication by M. Jacobs). In exceptional cases our test results showed cytotoxicity for a few chemicals, o,p′-DDT, equol, dibutylphthalate and genistein only at 10^{-5} M (Tables 2 and 3). On the other hand, it appeared that this highest test concentration of 10^{-5} M might not be high enough to test estrogenic activity of moderate to weak compounds, as this appeared from incomplete concentration response curves (lack of plateau value) for a few chemicals (equol, genistein and possibly dibutylphthalate). Provided that an adequate cytotoxicity test is included, and provided that solubility of chemicals is accurately evaluated, it is recommended that the highest test concentration might be increased up to 10^{-4} or 10^{-3} M. In that case, the use of

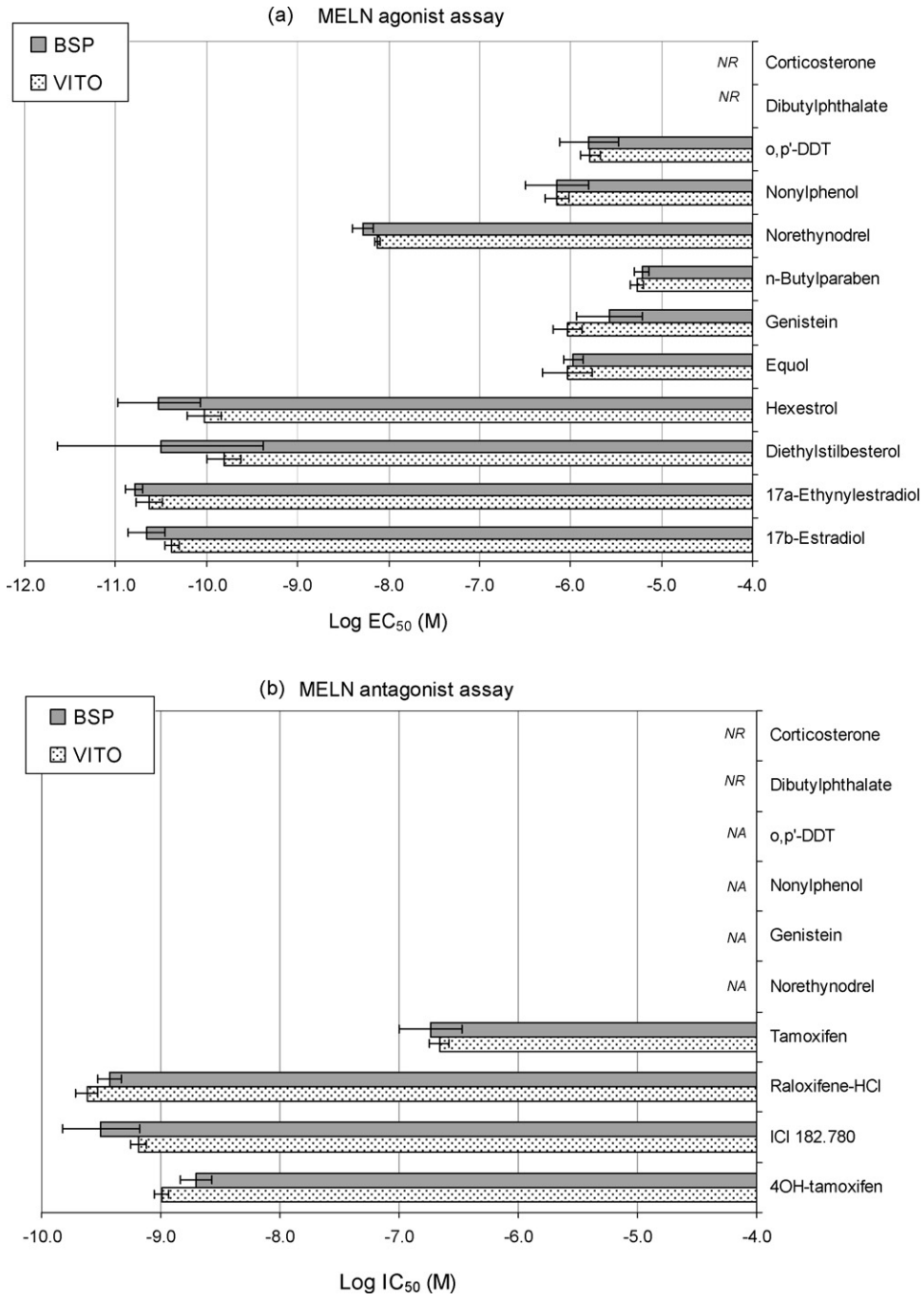

Fig. 3. Comparison of log EC_{50} (a) or log IC_{50} (b) obtained in agonist or antagonist assays, respectively, for selected test chemicals, run at VITO or at BSP. The bars represent mean values with SD (n = 3–8). NR: no response, NA: no antagonist response.

a cost-efficient cytotoxicity assay complementary to the luciferase assay is of major importance. The OECD guideline 445, an agonist assay with hERα-HeLa-9903 cells does suggest testing up to 10^{-3} M, and recommends the screening for cytotoxicity within the range of solubility of the test chemicals [24].

With respect to transferability of MELN protocols, it is demonstrated through comparative figures for the prescreen (Fig. 1), from comparative tables (Tables 2 and 3) and figures (Figs. 2 and 3) for agonist and antagonist assays that similar conclusions can be derived from experiments run either at VITO or at BSP. The prescreen did allow to find the appropriate working range and the similar mode of action of the chemicals at both labs to continue further testing. The agonist assays or antagonist assays did, similarly at both labs, allow to distinguish chemicals with a strong activity from those with a moderate to weak activity or no activity within the tested range (Fig. 3). The minor differences in EC_{50} ranking for agonist activity of chemicals between both labs, while no differences for IC_{50} ranking of antagonist chemicals, are considered to be negligible, provided that the MELN assay in a tiered strategy is used for screening purposes to classify chemicals as strong, moderate, weak or negative covering the tested range from 10^{-13} to 10^{-5} M. Indeed based on mean EC_{50}-values for chemicals with agonist activity (n = 10), differences between VITO and BSP were very low and ranged from a factor 0.3–2.6, while this variation was even smaller for IC_{50}-values in antagonist assay with a factor from 0.5 to 1.7 (n = 4).

Table 3
Comparative data from antagonist assays run at VITO and BSP for selected test chemicals.

Compound	Experiment identification	IC_{50} (M)	Lower CI IC_{50} (M)	Upper CI IC_{50} (M)	Mean IC_{50} (M)	SD (M)	CV (%)	*n*
4-OH-tamoxifen	4oh.VITO.017-03.1	1.09E−09	9.11E−10	1.29E−09				
	4oh.VITO.020-01.1	1.20E−09	1.04E−09	1.38E−09				
	4oh.VITO.021-01.1	9.54E−10	8.35E−10	1.09E−09	1.03E−09	1.44E−10	14.0	4
	4oh.VITO.022-01.1	8.76E−10	7.65E−10	1.00E−09				
	4oh_08.10.07_Bayer_1_1	2.17E−09	1.81E−09	2.60E−09				
	4oh_09.10.07_Bayer_1_1	2.59E−09	2.21E−09	3.03E−09				
	4oh_15.10.07_Bayer_1_1	1.45E−09	8.35E−10	2.51E−09	2.06E−09	5.57E−10	27.1	6
	4oh_16.10.07_Bayer_1_1	2.31E−09	1.84E−09	2.91E−09				
	4oh_22.10.07_Bayer_1_1	1.29E−09	7.70E−10	2.16E−09				
	4oh_23.10.07_Bayer_1_1	2.54E−09	1.54E−09	4.18E−09				
ICI 182.780	ici.VITO.017-03.2	7.85E−10	6.75E−10	9.14E−10				
	ici.VITO.020-01.2	5.98E−10	5.57E−10	6.42E−10	6.58E−10	1.10E−10	16.8	3
	ici.VITO.021-01.2	5.91E−10	5.40E−10	6.47E−10				
	ici_08.10.07_Bayer_1_2	7.42E−10	6.19E−10	8.89E−10				
	ici_15.10.07_Bayer_1_2	2.14E−10	1.28E−10	3.56E−10	3.84E−10	3.10E−10	80.6	3
	ici_22.10.07_Bayer_1_2	1.97E−10	9.82E−11	3.96E−10				
Raloxifene-HCl	ral.VITO.017-05.1	2.82E−10	2.38E−10	3.34E−10				
	ral.VITO.020-03.1	3.00E−10	2.78E−10	3.23E−10	2.44E−10	5.43E−11	22.2	4
	ral.VITO.021-03.1	2.01E−10	1.73E−10	2.34E−10				
	ral.VITO.022-02.2	1.95E−10	1.57E−10	2.41E−10				
	ral_08.10.07_Bayer_2_2	4.80E−10	4.14E−10	5.58E−10				
	ral_15.10.07_Bayer_2_2	3.08E−10	2.48E−10	3.82E−10	3.78E−10	9.04E−11	23.9	3
	ral_22.10.07_Bayer_2_2	3.47E−10	2.83E−10	4.26E−10				
Tamoxifen	tam.VITO.017-04.1	1.98E−07	1.27E−07	3.08E−07				
	tam.VITO.020-02.1	2.72E−07	1.81E−07	4.11E−07	2.19E−07	4.58E−08	20.9	3
	tam.VITO.021-02.1	1.88E−07	1.46E−07	2.43E−07				
	tam_08.10.07_Bayer_3_1	2.44E−07	1.54E−07	3.89E−07				
	tam_15.10.07_Bayer_3_1	9.24E−08	5.97E−08	1.43E−07	2.05E−07	9.89E−08	48.3	3
	tam_22.10.07_Bayer_3_1	2.78E−07	3.53E−08	2.19E−06				
Norethynodrel	nor.VITO.017-04.2	Compound is not an antagonist, response indicates agonism						
	nor.VITO.020-02.2	Compound is not an antagonist, response indicates agonism						
	nor.VITO.021-02.2	Compound is not an antagonist, response indicates agonism						4
	nor.VITO.022-02.1	Compound is not an antagonist, response indicates agonism						
	nor_09.10.07_Bayer_3_1	Compound is not an antagonist, response indicates agonism						
	nor_16.10.07_Bayer_3_1	Compound is not an antagonist, response indicates agonism						3
	nor_23.10.07_Bayer_3_1	Compound is not an antagonist, response indicates agonism						
Genistein	gen.VITO.012-04.1	Compound is not an antagonist, response indicates agonism						
	gen.VITO.013-04.1	Compound is not an antagonist, response indicates agonism						
	gen.VITO.022-05.1	Compound is not an antagonist, response indicates agonism						4
	gen.VITO.022-05.2	Compound is not an antagonist, response indicates agonism						
	gen_08.10.07_Bayer_3_2	Compound is not an antagonist, response indicates agonism						
	gen_15.10.07_Bayer_3_2	Compound is not an antagonist, response indicates agonism						3
	gen_22.10.07_Bayer_3_2	Compound is not an antagonist, response indicates agonism						
Nonylphenol	non.VITO.017-06.1	Compound is not an antagonist, response indicates agonism						
	non.VITO.020-04.1	Compound is not an antagonist, response indicates agonism						3
	non.VITO.021-04.1	Compound is not an antagonist, response indicates agonism						
	non_09.10.07_Bayer_2_2	Compound is not an antagonist, response indicates agonism						
	non_16.10.07_Bayer_2_2	Compound is not an antagonist, response indicates agonism						3
	non_23.10.07_Bayer_2_2	Compound is not an antagonist, response indicates agonism						
o,p′-DDT	opd.VITO.017-06.2[a]	Compound is not an antagonist, response indicates agonism						
	opd.VITO.020-04.2[a]	Compound is not an antagonist, response indicates agonism						3
	opd.VITO.021-04.2	Compound is not an antagonist, response indicates agonism						
	opd_09.10.07_Bayer_2_1	Compound is not an antagonist, response indicates agonism						
	opd_16.10.07_Bayer_2_1	Compound is not an antagonist, response indicates agonism						3
	opd_23.10.07_Bayer_2_1	Compound is not an antagonist, response indicates agonism						
Dibutylphthalate	dbp.VITO.017-07.2	Negative: no response for antagonism, neither for agonism						
	dbp.VITO.020-05.2[a]	Negative: no response for antagonism, neither for agonism						3
	dbp.VITO.021-05.2[a]	Negative: no response for antagonism, neither for agonism						
	dbp_09.10.07_Bayer_1_2	Negative: no response for antagonism, neither for agonism						
	dbp_16.10.07_Bayer_1_2	Negative: no response for antagonism, neither for agonism						3
	dbp_23.10.07_Bayer_1_2	Negative: no response for antagonism, neither for agonism						

Table 3 (*Continued.*)

Compound	Experiment identification	IC_{50} (M)	Lower CI IC_{50} (M)	Upper CI IC_{50} (M)	Mean IC_{50} (M)	SD (M)	CV (%)	*n*
Corticosterone	cor.VITO.017-07.1	Negative: no response for antagonism, neither for agonism						
	cor.VITO.020-05.1	Negative: no response for antagonism, neither for agonism						3
	cor.VITO.021-05.1	Negative: no response for antagonism, neither for agonism						
	cor_08.10.07_Bayer_2_1	Negative: no response for antagonism, neither for agonism						
	cor_15.10.07_Bayer_2_1	Negative: no response for antagonism, neither for agonism						3
	cor_22.10.07_Bayer_2_1	Negative: no response for antagonism, neither for agonism						

[a] Cytotoxicity at highest test concentration 10^{-5} M.

Good agreement between both labs was also the consequence of overall compliance with test performance criteria. The reference chemicals showed adequate sigmoid response curves and for each experimental run the obtained EC_{50} for E2 in the agonist assay was within the range of 1.0×10^{-11} to 10.0×10^{-11} M, respectively a range of 3.57×10^{-11} to 5.72×10^{-11} M at VITO and 1.02×10^{-11} to 3.72×10^{-11} M at BSP. Based on these EC_{50}-values for E2, one might suggest a shift of the range from 0.8×10^{-11} to 8.0×10^{-11} M, though transfer to a third lab might be more appropriate prior to adopting the test performance criteria for a final test protocol. Another criterion was the induction factor of the E2 positive control at each plate, calculated as the ratio of average RLU at 1.0×10^{-9} M of E2 and average RLU of SC which should be >4.0. Except for 1 out of 25 test plates run at VITO, and for all 20 test plates run at BSP this criterion was always met. A higher variation of the E2 induction factor was seen at BSP, but it seemed to have no influence on the calculated EC_{50}-value, which remained within the prescribed range 1.0×10^{-11} to 10.0×10^{-11} M. To illustrate this, the results of both EC_{50} and induction factor for the concentration response curves of the reference agonist E2, have been summarised in a plot for each of the experiments run at VITO and at BSP (Fig. 4a). This figure also shows that based on EC_{50} for E2, the MELN assay run at BSP is slightly more sensitive, which could be due to culture conditions (e.g. type of serum). However this was not a systematic difference between both labs, as can be seen from comparison of EC_{50} for all chemicals (Fig. 3a), and thus indicates robustness of the MELN agonist assay. The test performance criteria in the antagonist assay were met in all conditions at both laboratories. The RLU signal obtained by E2 at the EC_{50} concentration was in a sigmoid concentration dependent manner suppressed by a concentration series of 4OH-tam, the reference antagonist set up in each experimental run. Furthermore, for the positive control on each test plate, the inhibition factor obtained by the ratio of average RLU at SC + EC_{50} E2 and average RLU at 10^{-6} M 4OH-tam EC_{50} E2 had to be higher than >10.0. In Fig. 4b, the inhibition factor and the corresponding IC_{50}-value for each of the response curves with 4OH-tam is given for both laboratories. Though this plot indicates that higher inhibition factors correspond to lower IC_{50}-values at both laboratories, it is unlikely to be of any significance as the variation of IC_{50} is within a very narrow range 0.88×10^{-9} to 2.59×10^{-9} M. On the other hand, this Fig. 4b does allow to identify a range for an IC_{50}-value of the reference agonist to be included as test performance criterion for the MELN SOP, e.g. 0.5×10^{-9} to 5.0×10^{-9} M. For these experiments with 4OH-tam again some systematic difference appeared with a lower range of IC_{50}-values at VITO compared to BSP, but this could not be generalised based on comparison of IC_{50}-values for the other tested chemicals. Data for the reference chemical 4OH-tam indicate that some background estrogenic activity may be present in the MELN assay, as appears from an inhibition factor in the range 12.7–24.4 (see Fig. 4b), while the induction factor for E2 in the agonist assay was in the range 4.2 up to 13.9 (see Fig. 4a). This background activity can have several reasons, such as residual estrogens (incomplete removal by charcoal treatment), and/or estrogen sulfate, being rather polar passing the treatment and which then can be cleaved by cells, or residual androgen levels converted by aromatase enzyme present in the cells. Though a slight shift of maximum a factor 2–3 for EC50/IC50-values can occur, it is within biological variability, and not significant in this screening assay to rank chemicals from strong to moderate or weak antagonist.

Finally, obtained results with MELN assay have been compared with available literature data for common chemicals tested. A comparison could be made for chemicals tested in an agonist mode with ERα-transactivation assays using stably transfected human cell lines [10,20,22,25], while published data for a comparable antagonist assay were scarce. Mean EC_{50}-values of BSP and VITO obtained by the MELN assay were compared to 3 other reporter assays for 6 chemicals (Table 4). The HELN-ERα assay is based on HeLa cells transfected first with the ERE-βGlobin-Luc-SVNeo plasmid and next with the pSG5-ERα-puro plasmid [25]. The hERα-HeLa-9903 assay also uses HeLa cells transfected with human ERα expression vector and a firefly luciferase reporter vector bearing five tandem repeats of estrogen respon-

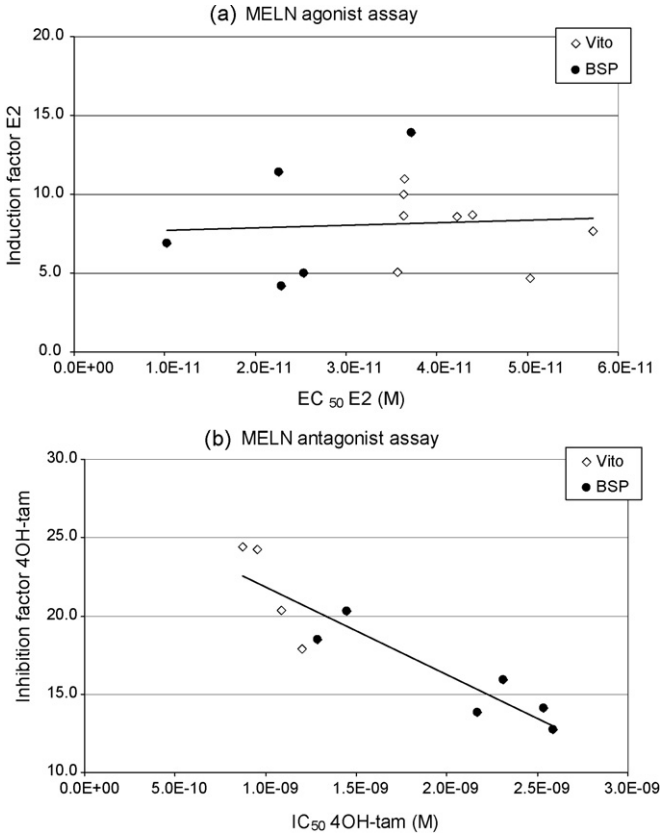

Fig. 4. (a) Comparison of EC_{50} and induction factor for the concentration response curves of the reference agonist, 17β-estradiol (E2), for each individual experiment run at VITO or BSP. (b) Comparison of IC_{50} and inhibition factor for the concentration response curves of the reference antagonist 4-OH-tamoxifen (4OH-tam), for each individual experiment run at VITO or at BSP.

Table 4
Comparison of EC_{50}-values obtained in MELN assay to literature data for other agonist assays. Mean ± SD are given, if available.

Chemical	MELN [this study][a]	HELN-ERα [25]	HeLa-hER-9903 [10]	ER-α Calux (U2-OS cells) [20]	ER-α Calux (T47D cells) [22]
17β-Estradiol	3.51E−11 ± 1.25E−11	1.7E−11 ± 0.3E−11	8.17E−12	6.0E−12	1.58E−11
Diethylstilbestrol	1.63E−10 ± 1.64E−10	n.a.	2.40E−11	n.a.	3.98E−11
17α-Ethynylestradiol	2.01E−11 ± 0.66E−11	8.0E−12 ± 3.0E−12	5.68E−12	n.a.	7.94E−12
Genistein	2.01E−6 ± 1.95E−6	3.8E−8 ± 1.6E−8	2.45E−8	n.a.	5.02E−8
Nonylphenol	7.96E−7 ± 3.57E−7	n.a.	4.91E−7	2.6E−7	n.a.
o,p′-DDT	1.82E−6 ± 0.99E−6	n.a.	n.a.	6.6E−7	n.a.

[a] Mean value calculated for VITO and BSP; n.a.: no data available.

sive element (ERE), driven by a mouse metallothionein promoter TATA element [10]. Further data on agonist activity of chemicals were obtained for the ERα-Calux assay, using T47D cells [22] or U2-OS cells [20], stably transfected with pEREtata-Luc. Except for genistein, the estimated EC_{50}-values with the MELN assay appeared comparable to the other assays with interassay differences for EC_{50} per chemical from a factor 2.7–6.8 and the EC_{50} in the MELN test was always the highest compared to the other assays. For genistein the EC_{50}-value in the MELN assay was almost 2 orders of magnitude higher (factor 82 compared to the most sensitive hERα-HeLa-9903 cells). This difference could not yet been explained but should be further investigated in the context of differences between cellular systems with respect to co-activation of estrogenic signalling by phyto-estrogens, and interference through antagonism of the aromatic hydrocarbon receptor (Ah-R) if present as hypothesized by Freyberger and Schmuck [23].

Our results obtained within two laboratories have demonstrated that the MELN assay is transferable, robust and reproducible which allowed to rank chemical compounds according to their strong to weak affinity for the estrogen-α receptor. We demonstrated the use of MELN cells to test for antagonism activity, which might be of added value compared to the current OECD guideline [24]. Moreover, the prescreen procedure with the purpose to define the mode of action with the corresponding working range, and exclude chemicals, or test concentrations with cytotoxicity or unspecific effects was shown to be applicable if chemicals with unknown properties have to be screened. The validity of this prescreen assay should be further confirmed in a study with coded chemicals. By this study we thus demonstrated that the MELN assay successfully passed the first modules of the ECVAM validation procedure. Further evaluation of the transferability to a 3rd laboratory is recommended prior to next validation steps including the definition of a prediction model and application domain to get it accepted as an alternative screening assay at level 2 of the OECD conceptual framework [7], contributing to the 3R's with a reduction of animal experiments.

Conflict of interest

The authors declare that there are no conflicts of interest.

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