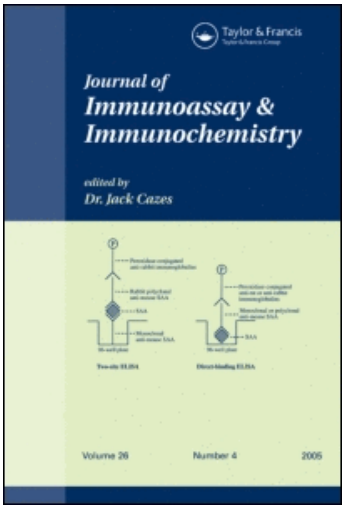

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Development of a Bio-Assay for Estrogens using Estrogen Receptor Alpha Gene Expression by MCF7 Cells as Biomarker

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Abstract: Estrogenic endocrine disruptors (EDCs) have been identified in soil, food, air, and water, and may produce adverse health effects in both humans and wildlife. Various *in vitro* assays, including the E-screen that measures estrogen dependent proliferation of the MCF-7 human breast cancer cell line, have been developed and implemented to screen for environmental estrogenic EDCs. This study describes a new amendment to the well known E-screen. A direct ELISA to quantify ERα protein levels on MCF-7 cells cultured in a high through put 96-well format were validated as a biomarker for estrogenicity. The ELISA shows that there is an inverse correlation between ERα levels and 17β-estradiol (E2) concentration ($R^2 = 1$). The detection range of the assay is between 1 and 1000 nM for E2. Results obtained with the ERα ELISA showed a good inverse correlation with total cellular LDH levels that is conventionally used to quantify MCF-7 cell proliferation. This ELISA was employed to assess environmental water extracts for estrogenicity.

Keywords: Bio-assay, Estrogen receptor alpha gene expression, Estrogens, MCF7

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INTRODUCTION

There is an increased concern regarding the potential adverse effects of pollutants found in the environment and also food on human and wildlife health. In the environment, these compounds occur as complex mixtures containing different congeners and isomers of both natural and anthropogenic chemicals. Some of these compounds exert their adverse effects by disrupting the natural hormone balance of the animal.[1]

These chemicals are collectively named endocrine disrupting chemicals (EDCs). Most of the known adverse effects of these EDCs have been attributed to environmental estrogens (xenoestrogens).[2–10] Some of the reported diverse range of effects/defects that estrogenic EDCs may have on human and animal health include reduced fertility, congenital malformations of the reproductive tract and increased incidence of cancer in estrogen responsive tissues.[11,12]

The molecular structure of exogenous natural and synthetic estrogens may be similar or extremely different from endogenous natural estrogens.[13–15] Despite the vast structural diversity among environmental estrogens, estrogenic compounds can be characterized by their ability to bind to and activate the estrogen receptor (ER). Ingestion of xenoestrogens results in either initiation (agonist) or inhibition (antagonist) of estrogenic responses.[16,17] Upon binding of an estrogenic compound to the ligand binding domain of the ER (located predominantly in the nucleus of cells), the associated heat shock protein complex, which masks the DNA binding domain dissociates. The ligand occupied receptor undergoes conformational changes that allow the ER-hormone complex to bind as a homo- or heterodimer[18] to specific sites on the DNA called the estrogen response element (ERE).[19,20] Once bound to the DNA, this ER-hormone complex modulates the transcription of the specific target genes.[21,22] ER complexes bound to an ERE may recruit additional transcription factors, leading to increased or decreased gene transcription and synthesis of proteins.[23,24] Other modes of actions of estrogenic EDCs include the binding of these chemicals to numerous other nuclear receptors and/or signal transduction pathways resulting in the modulation of steroidogenesis and catabolism of active steroid hormones.[25] To date, at least two subtypes of ERs have been identified and described namely ERα [NR3A1] and ERβ [NR3A2].[26] Another subtype, ERγ, has been identified in fish.[27] ERα has been well characterized, whereas ERβ was later discovered in the rat,[28] mouse,[29] and human.[30]

The development of *in vitro* models for screening environmental samples for endocrine disrupting characteristics as a first screen has drawn continued interest over the past years.[31] Many approaches have been used to identify EDCs using a variety of biological screening assays. There are three main categories of *in vitro* bio-assays available to assess

estrogenic or androgenic activity of single compounds or complex mixtures, namely, competitive receptor binding assays, cell proliferation assays and reporter gene assays, and direct immunoassay detection of single active compounds.[32,33] As an alternative, *in vitro* assays could be based on the quantitation of estrogen-induced changes in the expression levels of endogenous genes and proteins. Examples of estrogen-induced changes in the expression levels of endogenous genes and proteins as biomarker for EDCs include the induction of vitellogenin (VTG) by liver cultures of oviparous animals.[34]

The MCF-7 cell line is a human breast cancer cell line that has been derived from a patient with metastatic breast adenocarcinoma at the Michigan Cancer Foundation.[35] The MCF-7 cell line has been extensively used to study the molecular interactions of estrogens and anti-estrogens with the ER.[36] Cellular bio-assays such as the MCF-7 breast cancer cell proliferation assay (E-Screen), which measure the potency of a sample to induce cell proliferation, has been used as a screening assay for estrogenicity.[16,37] However, the quantification of ERα protein levels using a direct ELISA on cultured MCF-7 cells in a high throughput 96-well format has not been validated as a biomarker for estrogenicity before. The aim of this study was to development a direct ELISA to quantify ERα levels of cultured MCF-7 cells as a biomarker for estrogenicity.

EXPERIMENTAL

Reagents

RPMI-1640 with L-glutamine and phenol red as well as RPMI-1640 modified without L-glutamine and phenol red were supplied by Sigma Aldrich. Glutamax (L-glutamine), fetal bovine serum (FBS), antibiotic-antimycotic solution, phosphate buffered saline (PBS) tablets, serum replacement solution, trypsin in ethylenediaminetetraacetic acid (trypsin-EDTA) solution and 17β-estradiol (E2) were also supplied from Sigma Aldrich. Estradiol was prepared as stock solution (1 mg/ml) in Dimethyl sulfoxide (DMSO). MCF-7 maintenance medium was prepared by adding 10 ml FBS and 1 ml antibiotic-antimycotic solution to 100 ml RPMI-1640 medium containing phenol red and L-glutamine. MCF-7 serum deprived medium were prepared by adding 1 ml antibiotic-antimycotic solution, 1 ml glutamax and 2 ml serum replacement solution to 100 ml RPMI-1640 without L-glutamine and phenol red. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a SDS-PAGE gel preparation kit supplied from Sigma Aldrich. Nitrocellulose membranes were supplied from AEC-Amersham

International. All other protein blotting detection reagents, solvents and biochemicals were supplied by Roche International. The standard protein molecular mass markers were from Sigma Chemical Company.

Solid Phase Extraction of Hydrophobic Molecules from Water Samples

Water samples were collected from a pristine site and also from a sewage treatment plant outlet. The pristine site is a river site near the origin of the river and not impacted by human activity. Sewage water is from a sewage treatment plant servicing a population of approximately 40,000 inhabitants. The hydrophobic content of environmental water samples were extracted on C_{18} SPE columns (Analtech) using a method previously described by Swart and Pool.[38] In brief: C_{18} columns were pre-washed with 4 mL of solvent mixture (40% hexane, 45% methanol and 15%, 2-propanol), followed by another wash with 4 mL of ethanol. The column was then washed with one column volume of reverse osmosis water after which the water sample was applied onto the column. The column was then air-dried. The bound hydrophobic substances were eluted with solvent mixture. The eluate was dried under air and then reconstituted to 1/1,000 of the original sample volume with dimethyl sulfoxide (DMSO). The samples were stored at $-20^{\circ}C$ until further use.

MCF-7 Cell Culture

MCF-7 cells were maintained and harvested as previously described by Soto et al.[16,37] MCF-7 cells were suspended in maintenance medium to a concentration of 5×10^5 cells/mL. The cell suspension were then dispensed at 200 µL per well in a 96-well flat bottom tissue culture plate (Greiner, AEC-Amersham). Cells were allowed to adhere to the wells for a minimum of 5 hours before the medium was decanted. The wells were then rinsed twice with PBS pre-heated to 37°C. This was followed by the addition of 200 µL estrogen deprived medium per well. Cells were cultured for 48 hours in order to deplete estrogen levels. Medium was then replaced with 200 µL per well estrogen deprived medium containing 1% v/v of the 17β-estradiol standards, environmental water extracts or dimethyl sulfoxide (DMSO) controls. The cells were cultured for another 48 hours before analysis.

SDS PAGE and Western Blotting of ERα

MCF-7 cell lysates were prepared according to a method described by Abcam International (www.abcam.com/technical). Lysis buffer contains

150 mM NaCl, 1% v/v Triton X-100, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS and 50 mM trishydroxymethylaminomethane (Tris) pH 8. A 10X concentrated stock of lysis buffer was prepared. Cells were harvested as described by Soto et al.[16,37] and then suspended in PBS to yield 1×10^7 cells/mL. Concentrated lysis buffer was added to the cell suspension (100 μL lysis buffer for every 900 μL cell suspension). The suspension was gently agitated for 30 min at 4°C after which it was centrifuged for 10 min 10,000 × g to remove cell rests. Supernatants were removed and aliquots of the supernatant were frozen at −80°C until further use. Protein content of cell lysates were determined according to the method of Bradford (39) using bovine serum albumin (BSA) as a standard protein (Sigma). SDS-PAGE gels and buffers were prepared according to the kit manufacturer's instructions. MCF-7 protein preparations (5 μg) were resolved in 10% w/v polyacrylamide gels. Polyacrylamide gels were run at 120 V in a Hoefer Mighty Small II slab electrophoresis unit.

Separated polypeptides were transblotted onto nitrocellulose in Towbin buffer (25 mM Tris-HCl, 192 mM glycine and 20% (v/v) methanol) at 15 Volts for 1 hour. Following transfer, the blots were stained in Ponceau-S (0.2% w/v Ponceau-S, 3% w/v trichloroacetic acid). The blots were then thoroughly rinsed in saline and blocked for 1 hour at room temperature in blocking solution containing 3% w/v low fat milk powder in saline. All subsequent steps in the procedure were carried out at room temperature. Blots were incubated overnight in saline containing 0.3% w/v low fat milk powder, 0.01% v/v tween and 1/500 anti-ERα. The following day unbound antibody was removed by washing the nitrocellulose membranes four times for five minutes each in wash solution (saline and 0.01% v/v Tween). The nitrocellulose was then incubated for 1 hour in saline containing 1/2,500 v/v horseradish peroxidase conjugated anti-mouse immunoglobulin and 0.1% w/v serum albumin. The nitrocellulose was washed as before and finally it was stained using BM Blue precipitating peroxidase substrate. After staining the nitrocellulose, it was washed with distilled water, dried and stored in an aluminum foil envelope.

ERα ELISA

Following the culture of MCF-7 cells, the medium was carefully removed from the wells and cells fixated to the tissue culture plate as previously described by Maggiolinie et al.[40] In short, 200 μL of 2% v/v paraformaldehyde in PBS were dispensed in all the wells and the plate was then incubated for 45 minutes at room temperature. The paraformaldehyde solution was then replaced with 200 μL of 3% v/v H_2O_2 in methanol

and incubated for another 45 minutes. After fixation, wells were washed twice with PBS. Protein adsorption sites in the well were then blocked with 3% w/v low fat milk powder in PBS for 1 hour at room temperature with gently shaking. Anti-ERα (Santa Cruz Biotechnology, INC.) was diluted 1/200 in saline containing 0.3% w/v milk powder and dispensed at 50 μL per well. The plate was incubated for 2 hours at room temperature. The wells were then washed 4 times with 200 μL PBS. Horseradish peroxidase conjugated anti-mouse immunoglobulin (AEC-Amersham International) was diluted 1/2,500 with PBS containing 1% w/v human serum albumin and 0.01% v/v Tween and dispensed at 50 μL per well. Plates were incubated for another hour and the same wash procedure was followed. BM Blue soluble peroxidase substrate was heated to 37°C and dispensed at 50 μL per well. Plates were incubated at room temperature for 30 minutes followed by the addition of 50 μL per well of stop solution (0.5 M H_2SO_4). The optical density was lastly determined at 450 nm. All ELISA readings were corrected for background. Background controls are wells that receive all procedures except that the anti-ERα step is replaced by an incubation step with 0.3% (w/v) milk powder in saline. Results were expressed as ERα OD/mg cell protein which was calculated using the formula: ERα OD/mg cell protein = (OD experimental well – OD background control)/cellular protein (mg). In order to compare the results of multiple plates with each other, ELISA results of the experimental wells being analyzed were expressed as a percentage of the ERα OD/mg protein obtained for the 0.1 nM E2 exposure control used on all 96 well plates.

Total Cellular Protein Level Determination

Following the last 48 hour culture period of the MCF-7 cells, cultures were washed twice with PBS. Each well then received 100 μL of a 1 M NaOH solution. The plates were allowed to gently shake (300 rpm) at room temperature for 30 minutes. A multi-pipet was used to gently suspend all particulates by slowly pipetting well contents up and down. Total protein concentration of the cell hydrolysates were determined according to the method of Bradford,[39] using bovine serum albumin (BSA) as a standard protein (Sigma).

Total Cellular Lactate Dehydrogenase (LDH) Determination

Total LDH activity was determined as an indicator of total cell number or proliferation. LDH was measured using the instructions of a cytotoxicity detection kit supplied from Roche Applied Science. In short: Cells

were grown in a in a 96-well format as described in the previous section. In order to obtain total LDH levels, culture medium was removed from the exposed cells after the last 48 hour incubation period. Estrogen deprived medium containing 2% (v/v) Triton X-100 was then added at 200 μL/well. The plate was incubated on a shaker for 10 min at 300 rpm, after which the cell lysates were assayed for LDH. LDH reaction mixture was prepared according to the manufacturer's instructions immediately before use. To determine the LDH activity, 100 μL of LDH reaction mixture was added to 100 μL of 1:10 cell lysate preparation in an optically clear 96-well flat bottom plate. The absorbance was immediately measured at 492 nm to obtain a background reading. The plate was then incubated in the dark for 30 min at room temperature, where after the absorbance was again measured. Absorbance readings were corrected by subtracting the background reading for the specific sample.

Statistical Analysis

Differences in response of MCF-7 cells between controls groups and treatments were analyzed using analysis of variance (ANOVA). Tuckey's HSD test was used for all pair wise multiple comparisons.

RESULTS

Anti ERα Specificity

In order to use the anti-ERα antibody in an ELISA system, the antibody specificity was first confirmed by protein blotting (Fig. 1). The Coomassie stain of cells cultured in maintenance medium (lane 2) and estrogen deprived medium (lane 3) shows that equal amounts (10 μg) of protein were loaded onto lanes of the SDS-Page gel. In lanes 4 and 5 it can be observed that the anti-ERα antibody recognized a single peptide with an apparent molecular weight of around 55 kDa. It can also be observed that the ERα protein levels are higher in cells cultured in estrogen deprived medium (lane 5) compared to cells cultured in medium containing FBS and phenol red (lane 4).

ERα ELISA Validation

The optimum anti-ERα titer was determined using a direct ELISA as discussed in the Experimental section. In order to obtain the optimum

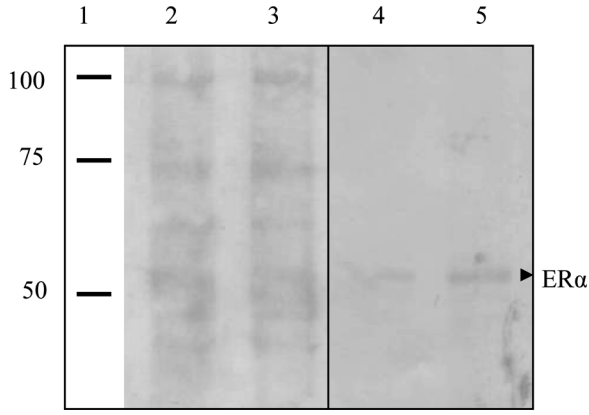

Figure 1. SDS-Page and protein blotting of MCF-7 whole cell lysates. Commercially available anti-ERα antiserum was checked for specificity. MCF-7 cells were cultured in maintenance- and estrogen deprived medium where after their whole cell lysates were subjected to SDS-Page and protein blotting. Lanes 1, 2 and 3 are a SDS-Page gel stained with Coomassie, whereas lanes 4 and 5 are protein blot with anti-ERα. Lane 1, Molecular weight marker; lane 2, maintenance medium whole cell lysate; lane 3, estrogen deprive whole cell lysate; lane 4, maintenance medium whole cell lysate; lane 5, estrogen deprive whole cell lysate.

antiserum dilution, anti-ERα was added in a two fold dilution series between dilutions ranging from 1/100 to 1/3,200. An antiserum dilution of 1/200 gave an OD response midway in the linear region of the titration graph (results not shown). This antiserum dilution factor was chosen to perform all direct ELISAs on the MCF-7 cells using ERα as the primary antibody. Figure 2 displays the results obtained (expressed as OD/mg protein) when the ELISA was performed with No. 1° antibody (background OD/mg protein), 1/200 diluted anti-ERα antiserum (assay OD/mg protein) as well as the differences in OD/mg protein observed between the two antibody controls (corrected OD/mg protein). This ELISA was performed on MCF-7 cells exposed to estrogen deprived medium complemented with DMSO vehicle control (0 nM E2), 0.1 and 10 nM E2. No significant differences in the background OD/mg protein (ELISA performed with No. 1° antibody) can be observed between the three different treatments (0 nM E2, 0.1 E2 and 10 nM E2). When this ELISA is performed with 1/200 anti-ERα antiserum, cells exposed to 10 nM E2 displays significantly lower OD/mg protein compared to cells exposed to 0.1 and 0 nM E2. There was no statistical difference in the OD/mg protein for 0 nM and 0.1 nM E2 exposures.

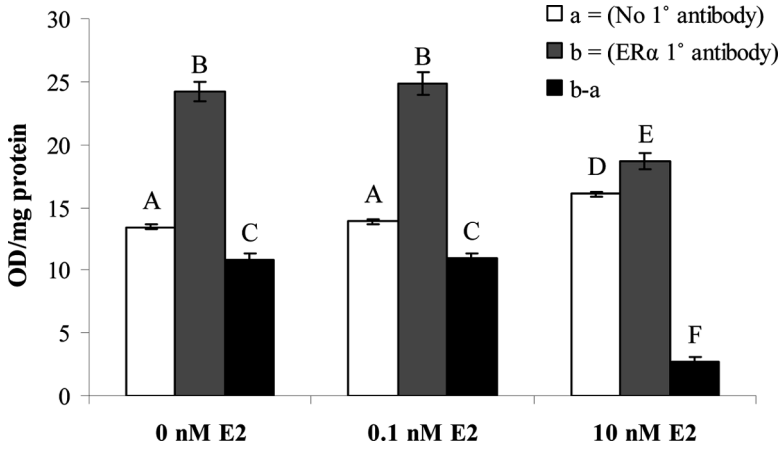

Figure 2. Anti-ERα ELISA validation. The ERα antibody was employed in a direct ELISA on the cultured and then fixated MCF-7 cells. MCF-7 cells were exposed to the DMSO vehicle control (0 nM E2), 0.1 nM E2 and 10 nM E2. White bars represent the OD/mg protein obtained when the ELISA was performed with no 1° antibody (background OD/mg protein), grey bars with the ERα antiserum (1/200 diluted) as the 1° antibody (assay OD/mg protein) and black bars are the corrected OD/mg protein obtained using the formula described in the methods section. Each data bar represents three replicates. Vertical error bars represent standard deviations of the mean. Letters above the data bars of each treatment represent significant differences ($P < 0.001$).

Effect of E2 Concentration on ERα Levels

MCF-7 cells were cultured in estrogen deprived medium supplemented with various concentrations of E2. The same volume of the vehicle control (DMSO) was used with all E2 concentrations so that the E2 concentration was the only variant. ERα-ELISA was done as described in the methods. The ERα OD/mg protein shows a good inverse correlation ($R^2 = 0.97$) with the log of the E2 concentration to which the MCF-7 cells were exposed to (Fig. 3). The lowest concentration of E2 that result in a significant reduction in ERα OD/mg protein compared with the control (0 nM E2) was 1 nM E2. The range of the assay was between 1-1000 nM E2.

Correlation of ERα and Total LDH Levels

The total LDH levels obtained for MCF-7 cells exposed to the dilution series of E2 between 1 and 1,000 nM E2 were correlated with the ERα

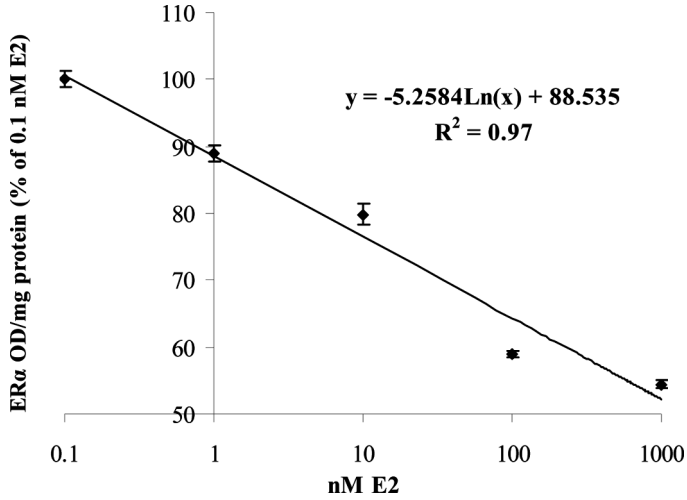

Figure 3. ERα-ELISA and E2 dose responsiveness. MCF-7 cells were cultured in estrogen deprived medium after which it was exposed to a dilution series of E2. ERα protein levels were determined and is expressed as the ERα OD/mg protein as a percentage of the 0.1 nM E2 exposure. Vertical error bars represents the standard deviation of the mean for ten replicates.

ELISA OD/mg protein (Fig. 4). There is an inverse correlation between the ERα ELISA and total LDH activity ($R^2 = 1$).

Analysis of Environmental Water Extracts for Estrogenicity

ERα levels of MCF-7 cells exposed to extracts of environmental samples were measured as biomarkers for estrogenicity of the sample (Fig. 5). Analysis of the ERα levels showed a highly significant ($P < 0.001$) decrease in ERα levels in the presence of 1,000 nM E2 standard ($55.01\% \pm 3.15$) compared with the 0.1 nM E2 standard ($100\% \pm 7.39$). There is no significant difference between the ERα levels induced by the 0.1 nM E2 standard and the pristine site. However, extracts of the sewage effluent resulted in significantly lower ERα levels compared to the 0.1 nM E2 standard ($P < 0.001$) and also the 1,000 nM E2 standard ($P < 0.001$).

DISCUSSION

Several characterized EDCs have been reported to act in an estrogen-like manner in living organisms.[37] When estradiol or estrogenic substances

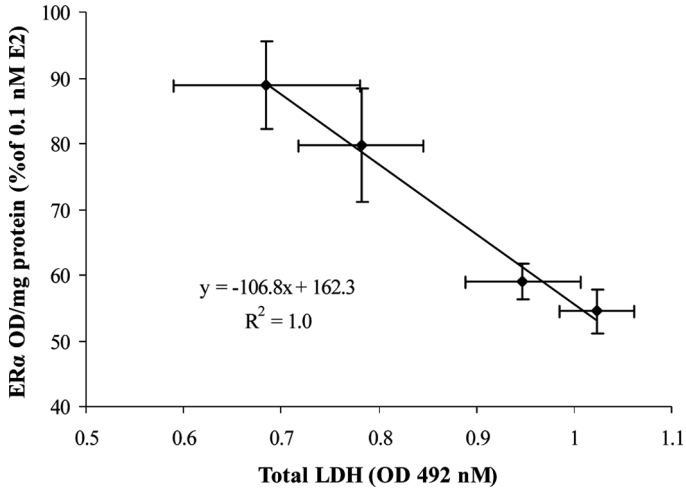

Figure 4. Correlation of ERα ELISA and total LDH activity. MCF-7 cells were exposed to a 10 fold concentration series between 1 and 1000 nM of E2. Total LDH activity is plotted against ERα OD/mg protein. Vertical and horizontal error bars represent the standard deviation of the mean for ten replicates.

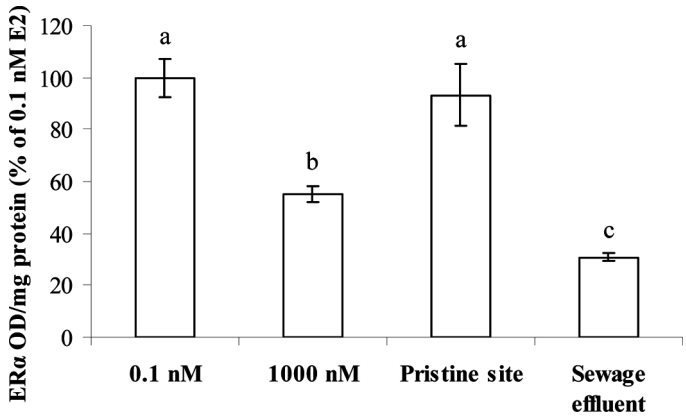

Figure 5. ERα protein levels of MCF-7 cells exposed to environmental water samples. Water samples collected from a pristine site and sewage effluent were subjected to the MCF-7 screen for estrogenicity and cytotoxicity. ERα protein levels were determined with the ERα-ELISA. Vertical error bars represents the standard error of the mean of six assays. Different letters indicate significant differences among treatments ($P < 0.001$).

enter a target cells, it rapidly complexes with the ER, translocates into the nucleus where it binds to chromatin and induces gene activation and an acceleration of biosynthetic processes.[41] Estrogenic EDCs such as phytoestrogens, mycoestrogens and xenoestrogens have the ability to interact with the ER which can then initiate (agonist) or inhibit (antagonist) estrogen-like actions.[16,17] Various *in vitro* assays have been developed, based on the quantitation of estrogen-induced changes in the expression levels of endogenous genes and proteins in estrogen responsive tissues.[42] The human breast cancer cell line, MCF-7, has been well characterized as an *in vitro* screen for estrogenicity (E-Screen).[16,37] The E-Screen assay is based on the dose-response relationship between the proliferation of the cells and the concentration of estrogen to which the cells are exposed.[37] Previous studies have reported that the molecular weight of ERα is in the region of 65 kDa.[43] The antibody used for this study reacted with a single peptide with an apparent molecular weight of ±60 kDa, similar to previous reports using this specific antibody.[44,45] More over, protein blot analysis showed that ERα levels were elevated in estrogen deprived medium and suppressed in maintenance medium (containing E2). Previous studies have shown that ERα expression is upregulated by estrogen deprivation.[46]

This antibody was used for the development of an ELISA to directly monitor ERα expression by MCF-7 cells cultured and subsequently fixed in 96 well trays. When MFC-7 cells were cultured in serum free medium high levels of ERα was expressed. The levels of ERα expression is down-regulated by the addition of estrogen to the culture medium. There is an inverse relationship between the ERα expression and log of the estrogen concentration ($R^2 = 0.97$). Previous studies using ERα expression by MCF-7 cells have employed ELISAs on cell homogenates of the cultured cells and obtained similar results.[46]

Results obtained using ERα protein levels as biomarker showed a good correlation ($R^2 = 1$) with total LDH as biomarker for estrogenicity when MCF-7 cells were exposed to the same E2 dilution series. Previous investigations have found that the detection limit of the E-Screen varies widely and reported values are 0.03 nM[16] and 0.001 nM.[47] Various factors may influence the sensitivity of MCF-7 for E2 including: differences between cell line clones; culture conditions; receptor level differences; differences in cell density and clone heterogeneity.[48]

The newly developed ELISA was used to monitor ERα levels as a biomarker to assess the estrogenicity of environmental water extracts. Water samples were obtained from a pristine site, previously described by Swart et al.[38] as well as sewage treatment work effluent from a local town. The level of ERα expression by MCF-7 cells cultured in the presence of hydrophic extracts of water collected from the pristine site was similar to the ERα expression by MCF-7 cells cultured in the presence

of the 0.1 nM E2 control ($P > 0.05$). This indicates that either the water from the pristine site does not contain estrogen or if this sample contains E2 or estrogenic compounds, it is well below the detection limit of the current assay. However as expected, the sewage treatment effluents resulted in significantly lower ERα levels compared to the E2 negative control ($P < 0.001$). More over, the sewage treatment effluent also resulted in significant lower ERα levels compared with the 1000 nM E2 standard ($P < 0.001$). These results therefore suggest that the pristine site shows no signs of estrogenicity, whereas the sewage effluent samples induced estrogenic responses greater than that observed for MCF-7 cells exposed to 1000 nM E2.

In this study, we extended the range of end-points of the classical E-Screen by using ERα expression as a biomarker for estrogenicity. We validated the use of a commercially available anti-ERα antibody and showed that this antibody can be successfully employed in a direct ELISA on MCF-7 cells fixed to the 96-well culture tray. This assay has a broad detection range between 1 and 1000 nM for E2. Previous studies have also investigated the use of ERα as a biomarker for estrogenicity using the MCF-7 cell line. Results obtained in our study corroborate the results obtained by Villabos et al.[46] However, the ELISA described by us is performed on cultured and fixated cells in a 96 well format and does not require protein preparation of cultured cells. The assay described by us is therefore less time consuming, easy to use, sensitive and can be used as a high throughput *in vitro* screen for environmental estrogens.

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