Assessment of (Anti-)estrogenic compounds using a Stably Transfected Luciferase Reporter Gene Assay in the Human T47-D Breast Cancer Cell Line

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Introduction

An ever-growing list of environmental, industrial, natural and pharmaceutical chemicals have been identified as potentially estrogenic. Many of these chemicals have structures that deviate considerably from the natural steroid hormone 17β-estradiol (E2), but can evoke effects via a mechanism of action comparable to estrogens. Recombinant receptor and reporter gene assays based on stably transfected cell lines can provide a specific, responsive and biologically relevant means to assess substances for both anti-estrogenic and estrogenic effects. This type of assay is based on the receptor-mediated mechanism of action of estrogens and reporter gene expression is a culmination of molecular cascade of events involved in receptor transactivation. An estrogen receptor (ER)-mediated Chemical Activated Luciferase gene eXpression (ER-CALUX) assay for the assessment of (anti-)estrogenic substances was developed. T47D human breast adenocarcinoma cells expressing endogenous estrogen receptor were stably transfected with a newly constructed estrogen-responsive luciferase reporter gene, pEREtataLuc. Stable transfection of pEREtata-Luc in T47D cells resulted in a highly sensitive, responsive clone which was further characterized in dose-response studies with E2 as well as a number of (pseudo)estrogenic compounds. Compared to a recombinant yeast assay for estrogens, the ER-CALUX was more sensitive (EC50= 6 pM in ER-CALUX vs 100 pM in yeast) and responsive (induction = 75 fold for ER-CALUX vs 25 fold for yeast). Polar extracts of sediments were tested in the ER-CALUX assay to provide an indication of estrogenic activity in complex mixtures. The ER-CALUX assay can form a valuable contribution to a battery *of in vitro* and *in vivo* assays used to identify and determine the (anti-)estrogenicity of compounds.

Materials and Methods

Cell culture

The T47D human breast adenocarcinoma cell line was kindly provided by Dr. R L. Sutherland (Garvin Institute of Medical Research, Sydney, Australia). The T47D cells were cultured in a 1:1 mixture of Dulbeccos's modified Eagle's medium and Ham's F12 medium supplemented with 7.5% fetal calf serum (FCS). T47D cells were cultured at 37°C, 7.5% (v/v) CO_2. During ER-CALUX luciferase induction assays, T47D cells were maintained in medium without phenol red supplemented with 5% dextran-coated charcoal treated FCS (DCC-FCS). DCC-FCS was prepared by heat inactivation (30 minutes at 56°C) of FCS followed by two 45-minute DCC treatments at 45°C (2).

Stable transfection pEREtata-Luc

Transfection was carried out according to the calcium phosphate precipitation method (3), using 18 μg pEREtata-Luc and 2 μg pGK-Hyg. The DNA construct pEREtata-Luc consists of three estrogen response elements upstream of a TATA box regulating expression of an enhanced luciferase reporter gene construct. The plasmid pGK-Hyg confers hygromycin antibiotic resistance to stably transfected cells. Clones were grown in medium supplemented with 100 μg/ml hygromycin for about 2 weeks. Individual clones were isolated and tested for luciferase induction as described below.

ER-CALUX assay procedure

T47D.Luc cells stably transfected with pEREtata-Luc were plated in black 96 well viewplates (Packard, the Netherlands) at a density of 5000 cells in 0.1 ml DF without phenol red + 5% DCC-FBS per well. Following 24 hours incubation, medium was renewed and the cells incubated for another 24 hours. The medium was then removed and the cells were dosed in triplicate by addition of the dosing medium containing the chemical or extract to be tested dissolved in ethanol or DMSO (max. 0.2%). Control wells, solvent control wells and E2 calibration points (6 pM and 30 pM) were included in triplicate on each plate. (Pseudo-)estrogenic substances were tested as described elsewhere (4). Following 24 hours treatment, 50 μl luciferin solution (Luclite, Packard, the Netherlands) was added to the medium above cells. The plates were shaken gently for 10 min. at room temperature. Luciferase activity was assayed in the same plate in a scintillation counter (Hewlett-Packard) for 0.1 minute per well.

Recombinant yeast estrogen screen

Yeast cells stably expressing the human ER and ERE-lacZ expression plasmid were kindly provided by Glaxo Group Research Ltd, United Kingdom. Yeast culture and β-galactosidase measurement was carried out according to the assay procedure of Routledge and Sumpter (5).

Sediment extraction

Sediment was sampled from 12 marine locations in the Netherlands. Dried sediment samples of 5 g were extracted with hexane:acetone (1:1) for 2 hours in a Soxtec apparatus. After sulphur removal the extract was transferred to hexane resulting in a precipitate of acetone soluble component. This precipitate (representing the polar fraction) was redissolved in acetone, filtered over a 1 g Na2SO4 column, evaporated and taken up in 50 μl DMSO.

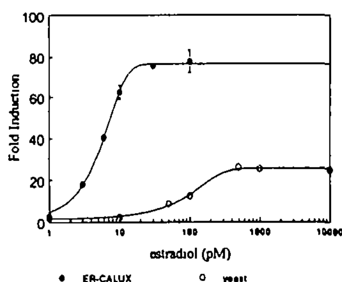

Figure 1: Reporter gene induction by estradiol in the ER-CALUX assay with T47D.Luc cells and recombinant yeast assay.

Results and Discussion

Response to estradiol

Exposure of T47D.Luc cells to E2 for 24 hours resulted in a detection limit of 0.5 pM and an EC50 of 5.5 pM was calculated (Figure 1). Maximum induction at 30 pM E2 relative to control was about 75 fold. No clear reduction in luciferase activity over prolonged periods of cell culture was found, demonstrating stable integration of the luciferase gene. To our knowledge, in comparison to other recombinant receptor and/or reporter gene assays using stably or transiently transfected mammalian cells, the ER-CALUX assay with T47D.Luc cells is the most sensitive and responsive stably transfected estrogen-responsive cell line in existence (4). Comparison of theE2 standard lines in the ER-CALUX and recombinant yeast assay demonstrated that the ER-CALUX is more sensitive (detection limit YES = 10 pM) and has an EC50 about 20 times lower (EC50 YES = 100 pM) (Figure 1). The maximum fold induction of 25 relative to solvent control was found in in the recombinant yeast assay.

Table 1: Potency of (pseudo-)estrogens relative to the estradiol following 24 hour treatment in the ER-CALUX assay with T47D.Luc cells. Potency calculated at concentration at which estrogenic effect is detected (LOEC) and 50% of maximum luciferase activity is reached (EC50).

	LOEC (nM)	Relative potency (LOEC)	EC50 (nM)	Relative potency (EC50)	Max. effect conc. (nM)	% effect rel. to E2
Estradiol	0.0005		0.006	1	0.030	100
o,p'-DDT	100	5 E-6	390	1.5 E-5	1000	154 (7.7)
methoxychlor	1000	5 E-7	n.c.*		10000	142 (4.2)
dieldrin	10000	5 E-8	n.c.		10000	55 (2.6)
endosulfan	1000	5 E-7	n.c.		10000	92 (6.8)
chlordane	1000	5 E-7	n.c.		10000	74 (2.0)
lindane	1000	5 E-7	n.c.		10000	58 (6.4)
kepone	1000	5 E-6	n.c.		10000	89 (1.4)
4-nonylphenol	100	5 E-6	260	2.3 E-5	1000	111 (13.0)
Bisphenol A	100	5 E-6	770	7.8 E-6	10000	153 (2.3)
genistein	10	5 E-5	15	4 E-4	100	125 (13.0)
Diethylstilbesterol	0.0005	1	0.0025	2.4	0.10	110 (2.0)

* n.c.: not calculated; no saturation curve achieved

Response to (mixtures of) estrogenic compounds
(Pseudo-)estrogenicity of various substances was demonstrated in the ER-CALUX assay, though luciferase induction by pesticides and industrial chemicals was elicited only at high concentration(> 100 nM) (Table 1). In testing environmental mixtures of chemicals, polar acetone extracts demonstrated higher estrogenicity than nonpolar hexane extracts (6). Polar extractsof sediments taken from the port of Rotterdam and the Dutch coast showed elevated estrogenic activity (Table 2).

Table 2: Estradiol equivalents (EEQ) following 24 hour treatment of T47D.Luc cells with polar extracts of sediments collected from marine locations in the Netherlands

Location	EEQ[1]	Location	EEQ
Port of Rotterdam:		**Port of Amsterdam:**	
Nieuwewaterweg Benelux Tunnel	38.4 (9.5)	IJmuiden Harbour (inner)	7.3 (0.5)
Nieuwewaterweg Splitsingdam	22.0 (4.5)	North Sea Canal km 10	5.4 (1.5)
Dutch Coast:		North Sea Canal km 18	7.1 (2.9)
Loswal North km 53	6.4 (1.5)	North Sea Canal km 29	6.0 (1.2)
Loswal North km 12	4.5 (0.6)	**"Reference" areas:**	
Noordwijk km 2	15.0 (5.7)	Lake IJssel	7.5 (0.6)
IJmuiden Harbour (outer)	27.1 (10.1)	Eastern Scheldt	4.9 (0.8)

[1]pmol/g sediment, average ± std

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