<LIFE98 ENV/B/000260>

Detection and elimination of human exposure to environmental hormone disrupting substances.

Yeast assay protocol

<February 2003>

Ghent University Partners:	Funded by:
Laboratory for Andrology	European Commission: Life98 fund
Laboratory of Microbial Ecology	Ghent University: different projects
Laboratory of Hormonology	
Laboratory of Toxicology	
Hydraulics Laboratory	

Experimental procedure Yeast assay.

A Chemicals

17β-estradiol (E_2; >98% pure), estrone (>99% pure), 17α-ethynylestradiol (EE2; >98% pure), 4-hydroxytamoxifen (OHT; >98% pure), tamoxifen (>99% pure), coumestrol (>98% pure), daidzein (>98% pure), diosgenin (~95% pure), enterolacton (~95% pure), genistein (>98% pure), benzyl butyl phthalate (BBP; 98% pure), 4,4'-biphenol (97% pure), biphenyl (99.5% pure), 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (4,4'-DDD; >99% pure), 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (4,4'-DDE; 99% pure), 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (4,4'-DDT; >99% pure), 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethanol (dicofol; 97.2% pure),1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane or methoxychlor (MeOCl; 97% pure), diphenylmethane (99% pure), 4,4'-isopropylidenediphenol or Bisphenol A (BPA; >99% pure), γ-hexachlorocyclohexane (lindane; 97% pure), 1-naphthyl N-methylcarbamate (carbaryl; 99.7% pure), 4-nonylphenol (technical grade), 4-n-octylphenol (99% pure), permethrin (>96% pure) and 4-phenylphenol (99% pure) were purchased from Sigma-Aldrich N.V. (Bornem, Belgium). Endosulfanαβ (99.5% pure) was from Chem Service (West Chester, USA). 8-Prenylnaringenin and naringenin (>99% pure) were a gift from Prof. Dr. D. De Keukeleire (Ghent University, Belgium). Mestranol, testosterone and dihydrotestosterone (DHT) were kindly provided by Prof. Dr. J.M. Kaufman (Ghent University, Belgium). ICI 182.780 was a gift from Dr. D. D'Hulster (AstraZeneca, Ukkel, Belgium). The compounds were dissolved in HPLC-grade absolute ethanol (Merck-Eurolab, Leuven, Belgium) or dimethylsulfoxide (DMSO; 99.5%, Sigma-Aldrich) and were kept at 4°C in amber vials.

B Recombinant Yeast screen

A yeast assay to screen the ability of compounds to interact with the human estrogen receptor alfa was originally developed at GLAXO (Glaxo Group Research Ltd., Middlesex UK) and was kindly provided by Professor J. Sumpter (Brunel University, UK). Details of the yeast estrogenicity assay (YES) (including details of the medium components) have been previously described ({Routledge & Sumpter 1996 ID: 2003}). In brief, yeast cells, stably transfected with the genetic code for the human estrogen receptor alfa together with *LacZ* expression plasmids (coding for secretory β-galactosidase enzyme) under control of estrogen responsive elements (ERE), were incubated in medium, containing test compounds and the chromogenic β-galactosidase substrate chlorophenol red-β-D-galactopyranoside (CPRG). Ligands that bind to the estrogen receptor in a way that enables receptor dimerization and interaction of the ligand/receptor dimer with the estrogen responsive elements and subsequently with the general transcription machinery, induce β-galactosidase expression which is quantified through the conversion of the yellow CPRG into chlorophenol red.

C Yeast stock production

To produce the 10x yeast stock, four 50 mL cultures were grown (growth medium: see further) to an optical density of 1.0 at 640 nm and transferred to a sterile 50 mL centrifuge tubes. The cultures were

centrifuged at 4°C for 10 min at approximately 2000x*g*. The supernatant was decanted and the separate cell batches were resuspended in 5 mL fresh minimal medium with 15% glycerol. The yeast strain was stored at -80°C in 0.5 mL aliquots in 2 mL sterile cryogenic ampoules (Simport, Quebec, Canada).

D Standard assay procedure

All the ingredients, unless stated elsewhere, were purchased from Sigma-Aldrich NV, (Bornem, Belgium) and were research grade biochemicals suitable for cell culture. Minimal medium (pH 7.1) was prepared by adding 13,61 g KH_2PO_4 (Merck, Overijse, België), 1,98 g $(NH_4)_2SO_4$ (Merck, Overijse, België), 4,2 g KOH (Merck, Overijse, België), 0,2 g $MgSO_4$ (UCB), 1 ml $Fe_2(SO_4)_3$ solution (40 mg/ 50 ml H_2O), 50 mg L-leucine, 67 mg L-histidine-HCl, 50 mg adenine, 20 mg L-arginine-HCL, 20 mg methionine, 30 mg L-thyrosine, 30 mg L-isoleucine, 30 mg L-lysine-HCl, 25 mg fenylalanine, 115 mg L-glutamic acid-Sodium, 150 mg L-valine, and 375 mg L-serine to 1000 mL of purity double distilled water. Aliquots of 45 mL were dispensed into 100 mL flasks, sterilized at 121°C for 15 min, and stored at room temperature. The vitamin solution was prepared by adding 8 mg thiamine, 8 mg pyridoxine, 8 mg pantothenic acid, 40 mg inositol, and 20 ml biotin oplossing (2 mg/100 ml H_2O) to 180 mL of purity double distilled water. The solution was filter sterilized through a 0.2µm pore size Pall Gelman Acrodisc filter and 10 mL aliquots were stored at 4°C in sterile glass tubes. A 20% w/v solution of D-(+) glucose was sterilized in 20 mL aliquots at 121°C for 15 min and stored at room temperature. A stock solution of 4 mg/mL L-aspartic acid was sterilized in 20 mL aliquots at 121°C for 10 min and stored at room temperature. A stock solution of 24 mg/mL L-threonine was sterilized in 5 mL aliquots at 121 °C for 15 min and stored at 4°C. A 20 mM copper (II) sulfate solution was prepared and filter sterilized through a 0.2µm pore size Pall Gelman Acrodisc filter and 5 mL aliquots were stored at room temperature in sterile glass tubes. A 10 mg/mL solution of chlorophenol red β-D-galactopyranoside (CPRG) was prepared and filter sterilized through a 0.2µm pore size Pall Gelman Acrodisc filter and 5 mL aliquots were stored at 4°C in sterile glass tubes. Growth medium was prepared by adding 5mL glucose solution, 1.25 mL L-aspartic acid solution, 0.5 mL vitamin solution, 0.4 mL L-threonine solution and 125µL copper(II) sulfate solution to 45 mL minimal medium in a sterile conical flask. At day zero, 5mL of growth medium was inoculated with 12.5µl of the 10x yeast stock and incubated at 28°C for 24 hours on an orbital shaker (150 rpm) or until an absorbance of 1.0 at 620nm was reached. The following day, assay medium was prepared by adding 0.5mL of the 24 yeast culture and 0.4mL of a CPRG solution (10mg/mL) to 50mL of growth medium (yeast concentration approximately $5x10^5$ cells/mL). Yeast assays were carried out in a type II Biohazard laminar air flow cabinet. Chemicals (2 or 1 g/L in ethanol or DMSO) were serially diluted in absolute ethanol or DMSO and 10µl (ethanol) or 5µl (DMSO) aliquots of each concentration were then transferred in duplicate to separate 96-well optically flat bottom microtiter plates. The ethanol was allowed to evaporate to dryness on the assay plate and 200µl of assay medium were added. In the DMSO assay 195µl of assay medium were added yielding a final DMSO concentration of 2.5%. This concentration did not inhibit yeast growth. Each plate contained two solvent blank rows and one row with a standard curve of 17β-estradiol (1E-8M-4.9E-12M for ethanol or half these values for DMSO). The plates were sealed with parafilm and incubated in a naturally ventilated heating cabinet at 32°C for up to ten days (DMSO assays) or longer (ethanol assays). After two days of incubation, all wells were homogenized using a multichannel pipet, and further incubated for the appropriate time. The enzymatic reaction was followed at an absorbance of 540nm. Yeast growth was checked using a second reading at 620nm.

E Prolonged incubation

In several experiments the yeast assays were run for more than the usual three days. For this, after absorbance measurement, plates were resealed with parafilm foil and re-incubated at 32°C. Microscopal evaluation at the end of the experiments did not reveal detectable amounts of microbial contamination in the wells.

F Inter-Laboratory validation test

Initially, major inter-laboratory differences were seen in background colour formation of the yeast assay. As a result of that, the maximal time before the plates completely turned pink varied between 4 to 10 days for the DMSO and ethanol protocol. Extensive measures were taken to reduce possible background contamination including thorough cleaning of glassware and laboratory surfaces, and separating rooms for assay performance, incubation and stock preparation. These measures significantly improved the assay, making it possible for all participating laboratories to keep the ethanol and DMSO tests for at least 10 days. Three laboratories enrolled in an inter-laboratory validation test for the yeast assay following the standard assay procedure. The 8 chemicals were weighed and dissolved in ethanol or DMSO in a concentration of 2g/L, except for genistein: 0.5 g/L. After coding, 1.5mL portions of identical batches were aliquoted in amber glass vials (1.5mL) with screw cap and tefflon lining. The panel of test compounds were increased with two vials containing 1.5mL of 200nm 17β-estradiol in ethanol or DMSO and two vials containing solvent blank. The 17β-estradiol solution was made after the other compounds were weighed and dissolved. All test panels were kept at 4°C until transferral to the different laboratories. All compounds were tested in duplicate and experiments were repeated at least twice. The identity and molecular weights of the compounds were revealed to the laboratories after the experiments had terminated.

G Calculations and parameters:

G.1 Parameters

Dose response curves for β-galactosidase activity were obtained using corrected absorbance units (CAU): CAU = $(Abs_{540})_{compound}$ –[$(Abs_{620})_{compound}$ –$(Abs_{620})_{blank}$]. The best fitting curve was calculated using SigmaPlot for Windows, version 4 (SPSS, Heverlee, Belgium) by a four parameter logistic regression as advocated by Moore *et al.* (1997), using the Marquardt-Levenberg algorithm according to the formula:

$$CAU = min + \frac{max - min}{1 + 10^{(\log EC_{50} - x) Hillslope}}$$

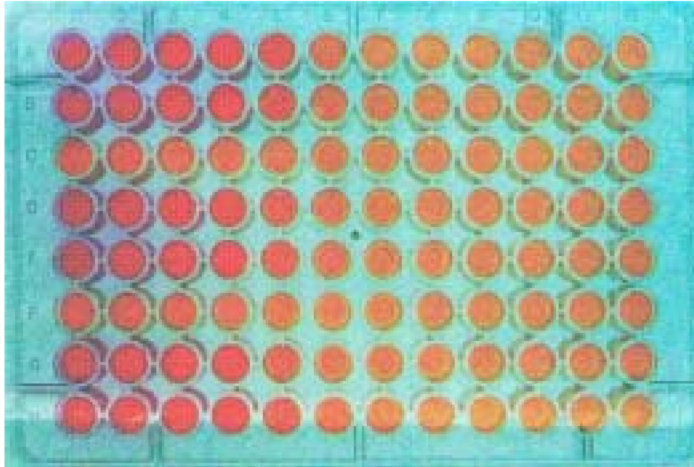
where min and max are the minimal (baseline) and maximal absorbance; Hillslope is the slope of the curve at the transition centre and EC_{50} is the concentration of test compound yielding half maximal effects. The relative potency (RP) of test compounds was determined as the ratio of the concentration of 17β-estradiol that caused the same CAU as the compound at its half maximal response, and the compound's EC_{50} x100. The relative induction efficiency (RIE) was the ratio between the (max-min) absorbance achieved with the test compound and that of E_2 x100. Because the maximal and background absorbance values changed during the incubation period, levelling of at an absorbance value of approximately 2.6 due to substrate limitation, the RIE went through a maximum. Unless stated differently, this value is reported here, as it represents the maximal additional induction effect of the compound above background.

The detection limit is defined as the concentration of E_2 corresponding to an effect equal to the mean of the corrected absorbances of the blank rows plus three times its standard deviation. In all experiments the blank rows showed colour formation dynamics, identical to the lower part of the estradiol curve. Finally a compound was considered to exhibit an estrogenic activity when in at least two separate experiments, it caused a dose dependent (two consecutive concentrations) colour formation above the detection limit.

G.2 Statistical analysis

All additional statistics were performed using SPSS 10.0 (SPSS Inc. Chicago Illinois, USA).

H Example of a test plate



I References

Routledge, E.J. and Sumpter, J.P. (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ.Toxicol.Chem.* **15**, 241-248.