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SHORT COMMUNICATION

Oestrogenic activity of CPRG (chlorophenol red-β-D-galactopyranoside), a β-galactosidase substrate commonly used in recombinant yeast oestrogenic assays

ELS VANDERPERREN[1]*, WIM DEMARÉ[1], RONNY BLUST[2], KRIS COOREMAN[1] and PETER BOSSIER[1]

[1]Ministry of Small Enterprises, Traders and Agriculture, Agricultural Research Centre-Ghent, Department of Sea Fisheries, Ankerstraat 1, B-8400 Ostend, Belgium. e-mail: vdperren@mail.dma.be
[2]University of Antwerp, Department of Biology, Groenborgerlaan 171, B-2020 Antwerp, Belgium

The finding that a variety of chemicals display oestrogenic activity has resulted in the development of *in vitro* and *in vivo* assays to assess oestrogenic activity. One such assay, the yeast oestrogen assay (YES) makes use of recombinant yeast cells that harbour an oestrogen receptor expression cassette and a reporter construct, coding for β-galactosidase. The induction mechanism starts with the binding of oestrogenic compounds to the oestrogen receptor. This complex activates the production of β-galactosidase. The β-galactosidase activity is thus a measure of the oestrogenic activity of chemical compounds. In the YES assay, the β-galactosidase activity may be quantified with the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG). In the present study it is reported that CPRG or its β-galactosidase degradation product chlorophenol red act in the YES as an oestrogenic compound itself. The implications of this finding are described. It is especially argued that chlorophenol red production after prolonged incubation of the assay might be misinterpreted as an oestrogenic effect of the test compound.

Keywords: oestrogenic activity, yeast assay, chlorophenol red-β-D-galactopyranoside (CPRG).

Introduction

The topic of endocrine disrupters and that of environmental oestrogens in particular, including their effects on both humans and wildlife, has recently received considerable attention. Several studies (e.g. Gaido *et al*. 1997, Sonnenschein and Soto 1998, Tyler *et al*. 1998) demonstrated that a variety of environmental contaminants have the potential to mimic or partially mimic the natural female hormone oestrogen—17β-oestradiol (E_2)—despite the fact that their chemical structures do not resemble this prototypical natural oestrogen. Since the chemical structure of a substance alone does not allow its possible oestrogenic potency to be predicted, several *in vitro* and *in vivo* screening assays have been developed to assess the oestrogenic activities of substances (Gülden *et al*. 1997, Zacharewski 1997).

* Corresponding author: Els Vanderperren, Ministry of Small Enterprises, Traders and Agriculture, Agricultural Research Centre-Ghent, Department of Sea Fisheries, Ankerstraat 1, B-8400 Ostend, Belgium.

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Some *in vitro* assays use genetically modified yeast strains, which harbour an oestrogen receptor expression cassette and a reporter construct (Klein *et al*. 1994, Arnold *et al*. 1996, Routledge and Sumpter 1996). Upon binding of an oestrogenic compound to the oestrogen receptor, the complex interacts with the oestrogen responsive -cis-acting element (ERE) in the promoter of the reporter construction, resulting in the transcription of a reporter gene (e.g. *LacZ*). The recombinant yeast used by Routledge and Sumpter (1996, 1997) was developed to identify compounds that can interact with the human oestrogen receptor (hER). In their assay (further referred to as RSassay) the activity of the *LacZ* product (β-galactosidase) is measured by conversion of the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG) into chlorophenol red. The strain has been used to determine oestrogenic activities of representatives of a class of surfactants, namely alkylphenol polyethoxylates and some of their principal degradation products (Routledge and Sumpter 1996) and to determine structure–function relationship of alkylphenolic chemicals (Routledge and Sumpter 1997). In addition a variety of chemicals have been verified for their oestrogenic and anti-oestrogenic effects (Sohoni and Sumpter 1998).

Validation of the assay revealed that high blank values could be obtained, especially after prolonged incubation. A similar effect was seen when higher yeast cell densities were used in the incubation vials, compared with the cell densities used in the RS-assay. These were the first indications that CPRG or its β-galactosidase reaction product chlorophenol red might possess oestrogenic characteristics in the assay. The purpose of this paper is to present evidence for the latter hypothesis.

Material and methods

The recombinant yeast screen

The method for the recombinant yeast screen assay used in this paper is described in Routledge and Sumpter (1996, 1997). In brief, this assay makes use of a modified strain developed by Glaxo Wellcome. In order to construct this strain the cDNA sequence coding for the hER was integrated into the genome of the yeast *Saccharomyces cerevisiae*, under the control of a yeast promoter. The yeast also contains a plasmid carrying an ERE-LacZ construct controlling the oestrogen-induced expression of the reporter gene *LacZ* encoding the enzyme β-galactosidase. Thus, in the presence of an oestrogen the yeast synthesizes β-galactosidase, which splits the yellow chromogenic substrate CPRG, present in the assay-medium, into galactose and the chromophore chlorophenol red, yielding a dark red compound. The β-galactosidase activity is quantified spectrophotometrically at 540 nm (Routledge and Sumpter 1996).

Chemicals

All chemicals used in these experiments were pro analysis grade. The concentrations in stock solutions of the natural oestrogen 17β-oestradiol (E_2; ICN Biomedicals, Belgium), and the chromogenic substrates CPRG (Boehringer Mannheim, Germany), and *o*-nitrophenyl-β-D-galactopyranoside (ONPG; ICN Biomedicals, Belgium;) were, respectively, 2×10^{-7} M in absolute ethanol, 16.5 mm in double distilled water and 33.2 mM in double distilled water, They were filter-sterilized to 0.2 µm; Schleicher & Schuell, Belgium) and stored at 4°C. Chlorophenol red (CPR) (ICN Biomedicals, Belgium) was dissolved in absolute ethanol (stock solution: 23.6 mM) and phenolphthalein (Ferak, Berlin, Germany) was dissolved in double distilled water (stock solution: 31.4 mM) and filter sterilized to 0.2 µm. The chromogenic substrate resorufine-β-D-galactopyranoside (RFG; Sigma, Belgium) was directly solubilized in the assay medium and that assay medium was filter-sterilized to 0.2 µm. The reaction products of CPRG, RFG and ONPG were detected photometrically at 540 nm, 750 nm and 405 nm, respectively.

Assay procedure

Yeast assay medium (100 ml) was inoculated with 50 µl yeast cells from a stationary phase culture and 20 ml were aliquoted in DURAN-bottles of 100 ml, as described by Routledge and Sumpter (1996). The oestrogenic activity of CPRG was tested in a dose–response experiment, using CPRG concentration series. Therefore DURAN-bottles with a ten-fold dilution series of CPRG (1.64×10^{-3} to 1.64×10^{-6} M) were incubated with assay medium in a shaking warm water bath at 28°C. After 48 h incubation in the presence of CPRG, 1 ml sub-samples of these cultures were taken and transferred into test tubes, centrifuged, washed several times with assay medium to remove the CPRG and resuspended in 1 ml fresh assay medium containing a β-galactosidase substrate. The following substrates were chosen to quantify the β-galactosidase activity in these subsamples: CPRG, RFG, and ONPG. The final concentrations (1.64×10^{-4} M) in the assay media were the same for CPRG, RFG and ONPG. These samples (200 µl) were transferred into the wells of a microtitreplate (Novolab) and incubated (iEMS Incubater/Shaker, Labsystems) at 32°C. The colour developments of the CPRG, RFG, and ONPG metabolized products in the wells were measured after 7 h incubation at the corresponding wavelengths of 540, 750 nm and 405 nm, respectively. (iEMS Reader/Dispenser MF, Labsystems). The oestrogenic activities of chlorophenol red and phenolphthalein were tested in an identical way except for the fact that the β-galactosidase activity was measured after 24 h at 32°C. An additional assay at a lower pH (3.72) was performed to evaluate the oestrogenicity of chlorophenol red. The standard buffer in the assay medium was therefore replaced with a citrate buffer with the same molarity. All results presented are the average of four independent assays.

Results and discussion

The yeast oestrogenic assay using CPRG as the reporter enzyme substrate, described by Routledge and Sumpter (1996), is very sensitive. In typical experimental set-ups, reported results for E2 (lowest detection limit of 10^{-11} M) and some known xenoestrogens (e.g. bisphenol A, nonylphenol) were confirmed (not shown). However, high blank values were obtained after prolonged incubation for up to 6–7 days instead of 4 days or after 2 days in the presence of higher initial yeast cell densities, relative to the experimental conditions in the RS-assay (OD_{620} at the start of the experiment in the wells of at least 0.1) (results not shown). It was postulated that an unknown factor modulated the oestrogen-dependent induction mechanism of the assay. Attempts to identify that factor immediately focused on CPRG and/or its reaction product chlorophenol red, because the latter has a high structural similarity to phenolphthalein (figure 1) that has been described as an oestrogenic compound (Vethaak and Opperhuizen 1996). Another product with a similar structure, namely phenol red has also been described as an oestrogenic compound (Berthois *et al.* 1986). Chlorophenol red only differs from phenolphthalein by the presence of two Cl-residues, one hydroxyl group and a sulphon group (figure 1). Phenol red and chlorophenol red only differ by the presence of two Cl residues.

The oestrogenic potential of CPRG was investigated in a dose–response experiment. Three different β-galactosidase substrates (see Materials and Methods) were used to visualize the effect. The results show that at CPRG concentrations lower than 1.65×10^{-5} M, oestrogenic effects were not detected. At 1.65×10^{-4} M CPRG and higher, production of β-galactosidase could be visualized, in a CPRG dose-dependent way. These observations could be made with the three tested β-galactosidase substrates (CPRG, RFG and ONPG; figure 2). The lowest concentration of CPRG (1.65×10^{-4} M) showing a clear oestrogenic response is also the standard CPRG substrate concentration in the RS-assay. It seems obvious to conclude from these assessments that CPRG is causing the high blank values after prolonged incubation of the RS-assay. However, we also investigated the possibility that chlorophenol red (the β-galactosidase reaction product of CPRG) had oestrogenic characteristics. At the same time the

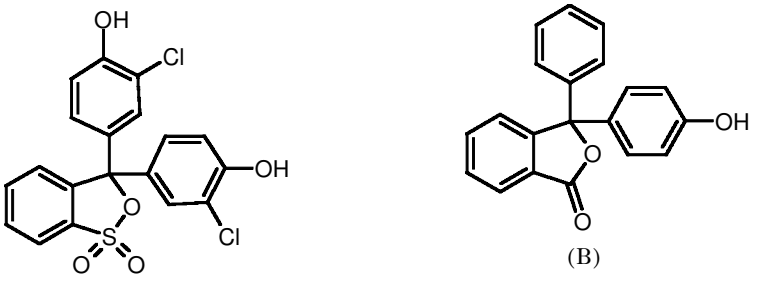

Figure 1. Chemical structure of chlorophenol red (A) and phenolphthalein (B).

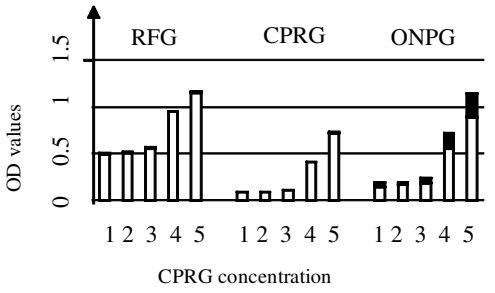

Figure 2. Dose–response relationship of CPRG in the recombinant yeast oestrogenic assay as measured with three different β-galactosidase chromogenic substrates (RFG, CPRG, ONPG). 1, 2, 3, 4 and 5 represent CPRG concentrations, respectively: blank, 1.65×10^{-6}, 1.65×10^{-5}, 1.65×0^{-4}, 1.65×10^{-3} M. Open bars: indicate values of four replicates; closed bars represent standard deviation. OD values were measured at wavelengths specific for the three β-galactosidase substrates (see Material and Methods).

oestrogenicity of phenolphthalein was re-evaluated. Phenolphthalein clearly confirmed its oestrogenicity in the assay (figure 3). In contrast chlorophenol red did not show any measurable response. Chlorophenol red is an ionic compound and, in the standard assay conditions, is present as its basic form. In order to test the influence of the acidic form, the assay was repeated in a different buffer system containing citrate buffer at pH 3.7. The acidic form of chlorophenol red is yellow. The underlying assumption was that a difference in charge might influence the compound's ability to penetrate the cell membrane providing a better interaction with the oestrogen receptor. Again, no oestrogenic effect was measurable.

The results presented here allow two hypothetical models for the measured oestrogenic effects to be formulated. In the first model CPRG interacts directly with the hER and acts as oestrogenic compound in the assay. In the second model chlorophenol red (CPR) would still act as the oestrogenic compound, despite the negative results in the yeast oestrogenic assay. CPRG might enter the yeast cell where it is metabolized to chlorophenol red by a low level of unspecifically or constitutively produced β-galactosidase reporter enzyme. The produced chlorophenol red may bind to the oestrogen receptor, resulting in the induction of *LacZ* transcription and the subsequent production of β-galactosidase. In other words CPRG would act indirectly as an oestrogenic compound as a result of an auto-induction cycle initiated by an initial low level of β-galactosidase and accelerated

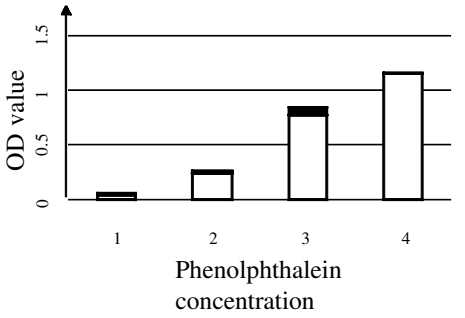

Figure 3. Dose–response relationship of phenolphtalein in the recombinant yeast oestrogenic assay using CPRG as chromogenic substrate. OD values were measured at 540 nm. 1, 2, 3 and 4 represent the following phenolphtalein concentrations: 1.65×10^{-6}, 1.65×10^{-5}, 1.65×10^{-4} and 1.65×10^{-3} M. Open bars indicate values of four replicates; closed bars indicate standard deviation. The blank, in which no CPR was produced, is not presented.

by the produced chlorophenol red during its short stay in the cell. In this model chlorophenol red would be the oestrogenic compound rather than CPRG, although no direct evidence for this assumption is in place, as it was shown that chlorophenol red did not induce β-galactosidase production. CPR would be taken up as CPRG and be passively or actively secreted after metabolization. Actually CPR has been found to be present predominantly in the culture supernatant. The yeast cells, gathered by centrifugation in a pellet, never showed any red staining by CPR (result not shown).

A necessary assumption in this explanation is that chlorophenol red does not enter the yeast cells. It is known that small structural differences can already drastically alter permeability in yeast cells. The difference in anti-oestrogenicity of tamoxifen and hydroxyltamoxifen has been attributed to their differential ability to enter the yeast cell (Tran *et al*. 1997). So the difference in oestrogenicity between CPRG and CPR on the one hand and phenolphtalein and CPR on the other hand, could originate in their differential ability to penetrate the yeast cell wall and would form the basis for the observed differences in oestrogenicity between these three compounds. Obviously this model awaits further experimental evidence. At present it is being investigated whether or not the use of CPRG would interfere in the assay as described by Routledge and Sumpter (1996), resulting in erroneous interpretation of results. It is anticipated that this would only be the case if CPRG and oestrogenic compounds acted synergistically. The possibility that pseudo-oestrogenic compounds would act synergistically has been suggested (Arnold *et al*. 1996) and rejected (Ashby *et al*. 1997, Ramamoorthy *et al*. 1997). Thus positive oestrogenic signals obtained after prolonged incubation of the assay have to be interpreted with caution. In order to avoid any possible interference of CPRG, the assay is currently being redesigned.

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