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Yeast reporter system for rapid determination of estrogenic activity

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Abstract

An in vitro test system for the determination of estrogens, xeno- and phytoestrogens, based on the activation of human estrogen receptor-α, has been examined for ability in monitoring environmental estrogens. The system consists of an expression plasmid for the human estrogen receptor-α and a reporter plasmid containing the lacZ gene under the control of the vitellogenin hormone response element. These plasmids have been transformed into *S. cerevisae*. Cultivation of yeast in the presence of estrogenic substances leads to activation of the estrogen receptor and induces the expression of the reporter lacZ. β-Galactosidase activity of the translated gene lacZ is a measure of the estrogenic activity of a compound. First, the selectivity of the system was compared to data available in the literature. Then the sensitivity of the system was checked. The detection limit is 0.1 ng 17-β estradiol or an equivalent activity per liter, if a sample can be concentrated 1000-fold. The system has been further characterized by selected compounds with known and unknown estrogenic activity.

Keywords: Yeast; Estrogen; Phytoestrogens; Xenoestrogens

1. Introduction

The question of whether compounds in food and beverages or chemicals in the environment can influence the hormonal systems of humans and animals has been discussed for many years. Evidence from animal experiments, wildlife studies, and reports of occupational and accidental human exposures indicate that natural compounds and chemical contaminants can adversely affect reproduction. Especially among fish, as well as vertebrates in general, there are many reports of anomalous development when exposed to xenobiotic and certain naturally occurring substances. In fish and in aquatic ecosystems, endocrine feedback regulates reproductive processes [1–3]. One of these processes, vitellogenesis or egg yolk synthesis, is crucial for oocyte maturation and subsequent ovarian maturation. Investigations showed that concentrations of 0.3 ng/l 17-α ethinylestradiol could interfere with the sexual development of aquatic wildlife [2]. On the other hand there are a plethora of reports showing a beneficial influence of compounds from food and beverages on the hormonal system of humans and animals [4–9]. The question arises as to which are the good estrogens and which are the bad ones [10].

Estrogenic activity in beer and hop extracts was described in the early 1950s [11]. Phytoestrogens were identified as the cause of the estrogenic activity [12–15]. These compounds were classified into five groups the flavones, flavanones, isoflavones, coumestans and dihydrochalcones [16–18]. The presence of 8-prenylnaringenin in hops may provide an explanation for the accounts of menstrual disturbances in female hop workers. This phytoestrogen can be also

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detected in beer, but the levels are low. The health effects of estrogenic active compounds in beer and other alcoholic beverages are still controversial [19–22]. Couwenbergs [19] reported significant hormone changes in males when consuming wine and beer. Other reports deny any health impact causing estrogenic activity present in beer [21,22]. Another concern is the food–drug interaction, which has been raised for grapefruit juice. Grapefruits contain different bioflavanoids e.g. naringenin, which inhibit various enzymatic processes. Because of this, various drugs cannot be combined with grapefruit juice [23].

The existence of compounds in plants influencing the endocrine system has been observed in the 1940s. Sheep grazing on red clover pastures showed breeding problems [24]. Later phytoestrogens in red clover were shown to be responsible for this effect. Currently extracts with high phytoestrogen content have been developed to treat menopausal symptoms of women [25]. Phytoestrogens in soybean-rich diets have been associated with the prevention of breast cancer, reduction of risk in coronary heart disease and beneficial influence on menopausal complaints [26–29].

Since a plethora of compounds in food, beverages and the environment have the ability to interact with the hormonal system, reliable methods are required to detect and to quantify the activity of these compounds. In vitro assays are necessary for screening, and together with chemical analysis these tests help to identify novel compounds. In this paper we concentrate on estrogenic activity, specially on the so-called selective estrogen receptor modulators (SERMs) [30]. The estrogen receptor belongs to the steroid–thyroid receptor superfamily and is comprised of five domains [31]. In its active form the receptor is bound to a protein complex composed of at least three proteins, hsp90, hsp70 and p60 [32]. Upon ligand binding the receptor is activated and the heat shock proteins are shed off. The receptor dimerizes and travels into the nucleus, binds to the hormone response element and initiates the transcription of the target gene. Several hormone response elements have been found [33]. Depending on the tissue and developmental status the expression of different target genes is controlled by different hormone response elements. Recently a second estrogen receptor has been found, called estrogen receptor β [34]. This receptor has a similar molecular architecture to the estrogen receptor α, but a slightly different selectivity for its ligands [25,35,36]. Also the distribution of this receptor in various tissues is different to the estrogen receptor α [35]. The biological significance of the occurrence of two different receptors is not yet fully understood.

The assessment of the interactive function of compounds on the hormone receptor and the related transactivation system can be carried out by in vitro transactivation systems. There are different ways that these compounds produce a biological response in the ER-dependent transactivation cascade. Compounds triggering the ER-dependent transactivation without reacting directly with the receptor may act in a cooperative manner with compounds which act through the receptor. The first mentioned compounds are only active in presence of the compounds which act through the receptor. As single entities, they have no biological significance. Katzenellenbogen et al. [37] have developed the model of the bi- and tripartite hormone pharmacology, which is a good model for interpretation of possible synergistic and antagonistic actions. In case of tripartite receptor pharmacology, a compound stabilizing the basal transcription machinery, the receptor and the cofactor will increase efficiency. Compounds destabilizing these complexes may act as antagonists. Therefore it is necessary to screen for estrogenic activities in the food, beverages or environment with systems mimicing the bipartite and tripartite receptor pharmacology.

Furthermore it is known that the receptor has an additional binding site for compounds such as tamoxifen [38,39]. The binding site acts cooperatively with the binding site for the cognate hormone. These cooperative action may also contribute to synergistic or antagonistic action.

It is not clear if certain phytoestrogens act as agonists, partial antagonists or synergists. For instance naringenin has been described as weak agonist or even antagonist in complex test systems [40]. In bipartite test systems it is clearly negative. There are similar reports on genistein [41]. This phytoestrogen has a general activity in enhancing transcription [42]. As the estrogens, phytoestrogens and xenoestrogens act through transcription, a synergistic effect in complex mixture such as food, beverages or environmental sample is very likely (Fig. 1).

When we strictly follow the bi- and tripartite

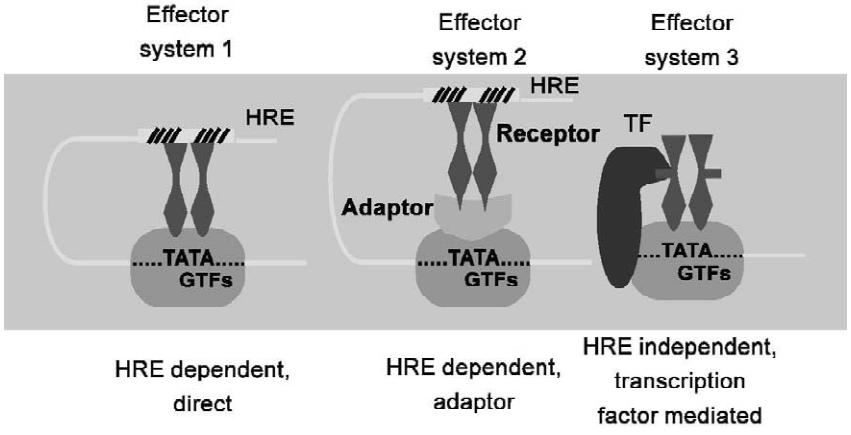

Fig. 1. Schematic drawing of how a tripartite and bipartite receptor pharmacology has been defined, according to Katzenellenbogen et al. [37]. In the effector system 1 the estrogen receptor directly activates the general transcription complex (GTFs) upon binding after binding to the hormone response element (HRE). In the effector system 2 an adapter protein is required to modulate GTFs. The adapter protein may up- or downregulate the transcription [72]. In the effector system 3 the receptor is interacting with the GFTs through another transcription factor (TF) and binding to the hormone response element is not required for modulate of GTFs.

receptor pharmacology, the synergistic action results in enhancement of efficiency not in potency. The potency is solely determined by the affinity of the hormone (ligand) to the receptor. Only compounds which change the receptor conformation so that the hormones become more tightly bound are capable of increasing the potency in a synergistic manner. Such compounds have not yet been identified.

Conformational change of the estrogen receptor so that the new conformation is capable of recruiting the basal transcription machinery is the current model to distinguish between agonists and antagonists. The antagonist binds to the receptor, but does not induce these conformational changes. The correlation between affinity and potency is the best way to examine whether a compound is an agonist or not. If

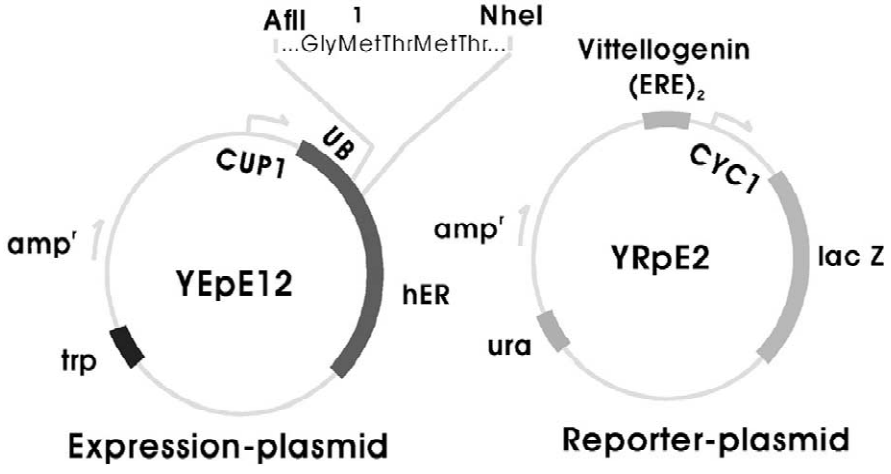

Fig. 2. Schematic drawing of the two-plasmid system in yeast [44]. The system consists of an expression plasmid (YEpE12) and a reporter plasmid (YRpE2). The expression plasmid contains an ampicillin resistance gen (amp^r), an tryptophan auxotrophy (trp) and the expression of the estrogen receptor (hER) is controlled by a copper inducible metallothionin promoter (CUP1). The receptor is expressed as ubiquitin fusion. The reporter plasmid (YRpE2) carries a uracil auxotrophy (ura), the hormone response element (Vitellogenin $(ERE)_2$), the CYC1 promoter and the reporter gene lacZ encoding for the β-galactosidase.

the affinity is higher than the potency in the transactivation assay then the compound is a partial antagonist. Preliminary experiments have shown that in environmental samples, e.g. wastewater, such antagonists are present.

Here a test-system is described which strictly operates according to the bipartite receptor pharmacology.

2. Experimental

In a bipartite system the potency of a ligand correlates with the affinity to the receptor. Therefore the system is simply composed of the receptor, the hormone response element and the reporter gene which is under control of the response element. The basal transcription machinery is provided from the yeast system [43–45].

The transactivational activity of estrogens and estrogen-like substances is measured with a two-plasmid yeast system consisting of an expression and a reporter plasmid. The system has been described in Refs. [43–47] and the reliability and the capability to identify synergists have been assessed. The estrogen receptor gene is expressed under control of a metallothionin promoter and expression is induced by induction by copper salts. The reporter plasmid has a lacZ gene which is under control of the vitellogenin hormone response element. In presence of a hormone the estrogen receptor can bind the hormone response element and induces the expression of β-galactosidase (Fig. 2).

The expressed β-galactosidase is used as an quantitative measure for hormone activity. The β-galactosidase is determined by a chromogenic substrate. The developed color is measured photometrically and total protein is estimated by a modified Bradford method in parallel. The specific activity is expressed in Miller units [48]. They are defined as follows:

$$\text{Miller unit} = \frac{\text{OD 405 nm}}{\mu\text{g protein ml}^{-1}} \cdot \frac{1}{\Delta t} \cdot \frac{\text{Sample volume protein assay}}{\text{Sample volume β-gal assay}} \cdot 1000$$

where OD 405 nm is the optical density at a wavelength of 405 nm, and Δt is the incubation time at 37 °C in minutes. The activity is plotted against the dilution or the concentration and the data are fitted by a logistic dose–response function.

Expression of nuclear receptors, ligand recognition and transactivation of target genes consists of a cascade of complex biochemical reactions. Although sequential kinetic equations may be used to describe these complex events, the number of parameters prohibits accurate resolution. Therefore, we empirically derived a logistic dose–response equation to approximate the concentration-dependent effect of a ligand on transactivation [46]. Dose–response functions as well as ligand competition were approximated by logistic dose–response function using TABLE CURVE 2D software (SPSS). The function is described in Eq. (1):

$$Y = a + \frac{b}{1 + \left(\frac{x}{c}\right)^d} \quad (1)$$

where parameter a equals the baseline, b the plateau of the curve designated as the ligand efficiency. Parameter c gives the transition center and equals the ligand potency which is the concentration that causes 50% efficiency (Fig. 3).

By using this method we can accurately determine the change of the activity and when we add increasing concentrations of 17β-estradiol to our samples we easily get information on antagonists or synergists in our sample. Reduction of efficiency and potency will be an indication of the presence of antagonists. Increase of efficiency higher than additive effects are an indication of efficiency.

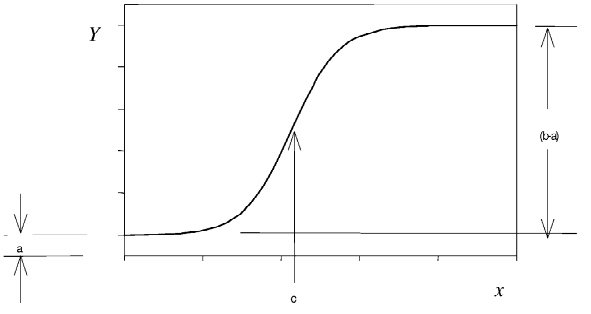

Fig. 3. Logistic dose–response function for fitting the data obtained by the yeast assay; a corresponds to the baseline; b the efficiency; c the potency and d the transition width.

2.1. Plasmids, vectors and transactivation assay

For all transactivation assays the yeast strain 188R1, a derivative of RS188 N [44] was used. The strain was transformed with YEpE12. Further, a β-galactosidase reporter plasmid YRpE2 was introduced [44,47]. Transformation of yeast cells was performed as described previously [47].

Transactivation assays were performed as described previously [47]. The transactivation-test was performed in 5-ml cultures (in 50-ml jars), therefore the overnight culture was diluted to $OD_{600} = 0.4$. hER expression was induced by addition of 10 μ*M* $CuSO_4$. For all preparations the same volume of DMSO was added to the yeast cultures. After inducing 4 h at 30 °C and 150 rpm the cells were extracted; 50 μl DMSO samples were used as a blank. A calibration curve was drawn with 17β-estradiol.

3. Results and discussion

To demonstrate the utility of a yeast two-plasmid system the sensitivity of the system was evaluated, then an attempt was made to further simplify for large scale screening assays and finally a large panel of compounds with estrogenic activity has been tested and compared to data from the literature.

3.1. Sensitivity

The sensitivity of the yeast test system was tested by serial dilutions of 17β-estradiol studying different incubation times. To the induced yeast culture 17β-estradiol was added to reach a final hormone concentration between 0.001 to 1.0 n*M* and then incubated 2 h, 4 h and over night. Then the β-galactosidase activity was measured (Fig. 4).

The limit of detection was defined as the threefold standard deviation of the blank plus the mean value of the blank. In this case the solvent for dissolving 17β-estradiol was used as blank. Then a value of 0.1 n*M* (27.2 ng/l) was obtained as limit of detection. This corresponds to an actual amount of material of 0.5 pmol hormone per test tube (5 ml). If a compound can be enriched from a beverage, food or environmental sample by a factor of 1000 an estrogenic activity of 1 ng 17β-estradiol/l can be determined.

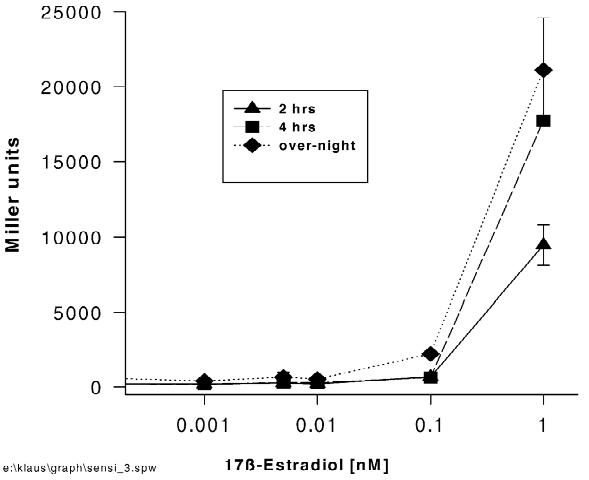

Fig. 4. Effect of incubation time on the β-galactosidase activity of a two-plasmid test system.

Table 1 shows that the prolongation of the incubation period does not significantly improve the limit of detection, since the blank is also increasing. Another possibility might be the augmentation of the sample volume. Here we are restricted by the toxicity of the solvent. DMSO as solvent is a good compromise. It has a good solubility power for the hormones and phytoestrogens and up to 1% DMSO in a yeast culture does not have any harmful effect on yeast.

3.2. Simplification of the test system

The test starts with a yeast preculture overnight and then the cells are diluted to a certain cell density and Cu^{2+} is added to induce the expression of the estrogen receptor. After a defined time the samples containing the hormone are added and after a further incubation period the cells are harvested by centrifugation, disintegrated, and from the clarified supernatant the β-galactosidase activity and the protein concentration are measured. At least eight different handling steps are required to perform the assay. In order to process large numbers of samples the test system is quite complex. Here an attempt was made to simplify the yeast disintegration. Besides the

Table 1
Influence of incubation time on the sensitivity of the transactivation efficiency

Incubation time	Blank (Miller units)	S.D. (σ) (Miller units)	Limit of detection mean$\pm 3\sigma$ (Miller units)	Limit of detection (nM)
2 h	225	164	719	0.10
4 h	233	104	313	0.06
Overnight	512	108	1805	0.08

mechanical disintegration, the chemical and enzymatical methods were also tested.

From transformation protocols it is known that with the addition of SDS and chloroform at a temperature of 30 °C the yeast cell wall becomes highly permeable. In addition to the incorporation of substances this effect can be also used for the release of compounds. According to the protocol of Johnston, who has combined the chemical and mechanical disintegration, an attempt was made to get sufficient release of β-galactosidase by the addition of SDS and chloroform. This method has the advantage that enzyme detection and disintegration are performed in the same reaction vessel. An economy of time is thus achieved, but the method has some severe disadvantages.

The sensitivity in all experiments was lower compared to the mechanical disintegration (Fig. 5). The presence of SDS in the extracts influences the protein determination and prevents quantification of protein by the simple method according to Bradford. Another assay for protein determination was not developed. For that reason only the optical density values at 405 nm of the β-galactosidase assay are shown in Fig. 5. Chloroform is a halogenated solvent and if possible, should be avoided.

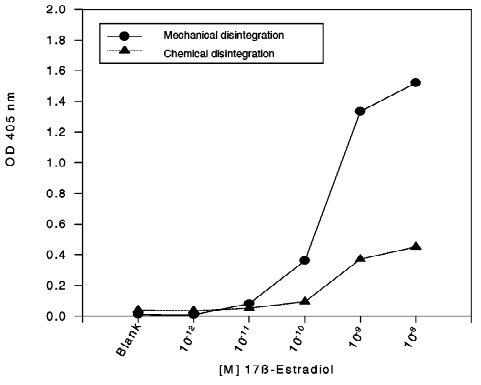

Fig. 5. Effect of chemical desintegration on the sensitivity of the test system.

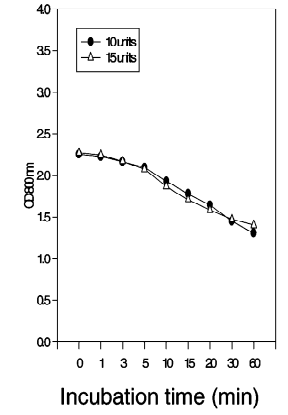

Fig. 6. Influence of enzyme concentration and incubation time on the lysis of the yeast cells.

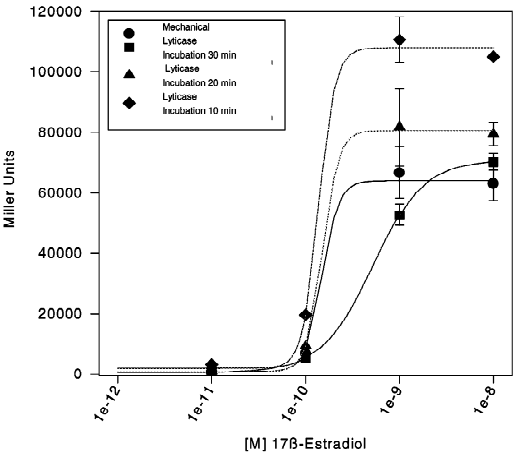

Fig. 7. Dose–response curves of the transactivation assay performed with enzymatic lysis; *a* is the baseline and *b* the efficiency.

The enzymatic disintegration was performed with lyticase, an enzyme hydrolyzing poly (β-1,3-glucose), and it is therefore suited for lysis of the glucan yeast cell walls. A certain amount of enzyme was added to the harvested yeast pellet, incubated and the suspension was then clarified by centrifugation. The influence of enzyme concentration and incubation time is shown in Fig. 6. The optical density was determined at 800 nm. At least 20 min are required for substantial lysis. Then serial dilutions of 17β-estradiol were added to the induced culture and instead of mechanical cell disrupture the cells were enzymatically lysed. The supernatant was assayed for protein and β-galactosidase activity (Fig. 7). These values were compared to the conventional procedure of mechanical disintegration. When the

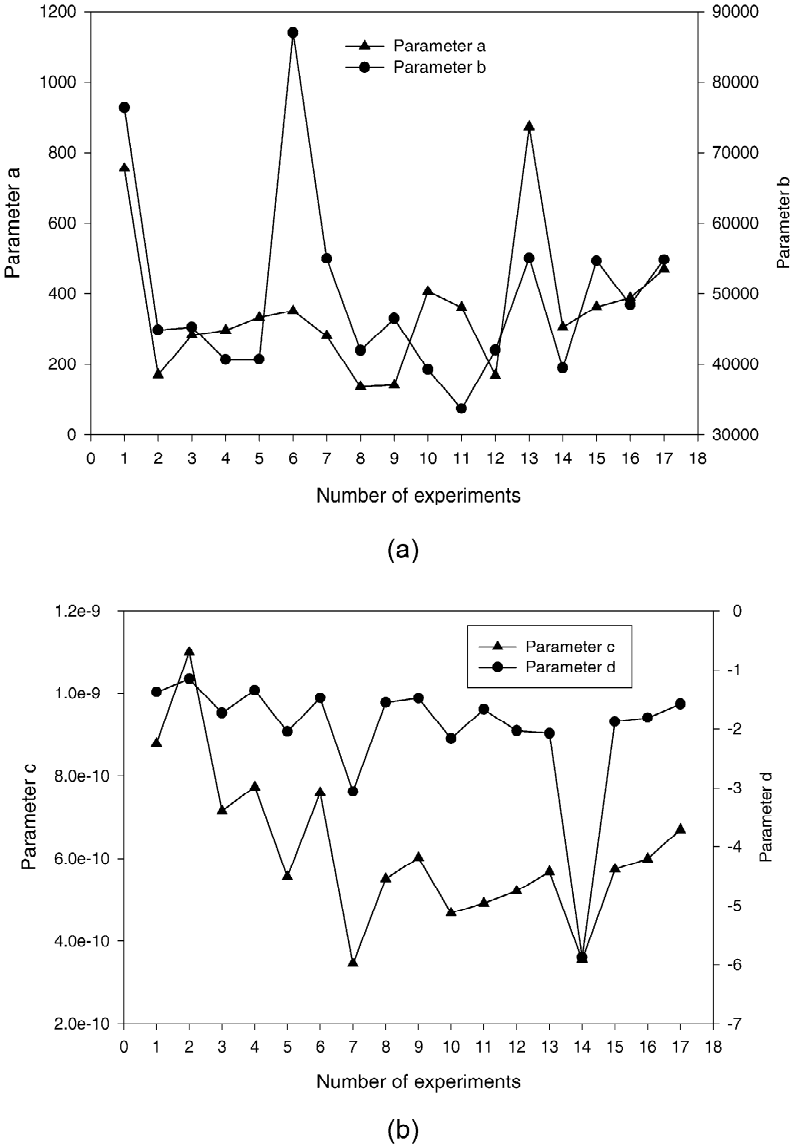

Fig. 8. Parameters of the dose–response curve of different batches to approximate the hormone dependent transactivation; *c* is the potency and *d* the transition width.

incubation time exceeds a certain length it seems that the β-galactosidase looses activity at lower expression level resulting a shallower dose–response curve. The lyticase method is extremely difficult to standardize.

3.3. Reproducibility

Currently the test system is used for large scale screening. All attempts to simplify the system failed. Here the assay is performed with the conventional mechanical disintegration of the cell. For every batch of samples a standard dose–response curve with 17β-estradiol was produced and fitted with the logistic dose–response function (Eq. (1)). In Fig. 8A and B the parameters *a*, *b*, *c* and *d* of the various sample batches are plotted.

Mean values of 354 ± 112 for *a*, 42495 ± 6270 for *b*, $4.732\cdot10^{-10}\pm8.67\times10^{-11}$ for *c* and -2.5 ± 0.34 for *d* were found. It seems that batch nos. 1, 7 and 8 contain outliers. In batch 1 parameters *a* and *b* seem to be significantly higher than the mean. In batch 7 the parameters *c* and *d* seem to be significantly lower. In Fig. 9 an average dose–response curve is shown.

All available data simplified of the outliers were used for calculation of the dose–response curve. For bioassays a high reproducibility and accuracy is obtained. Therefore, the system can be applied to large scale screening assays.

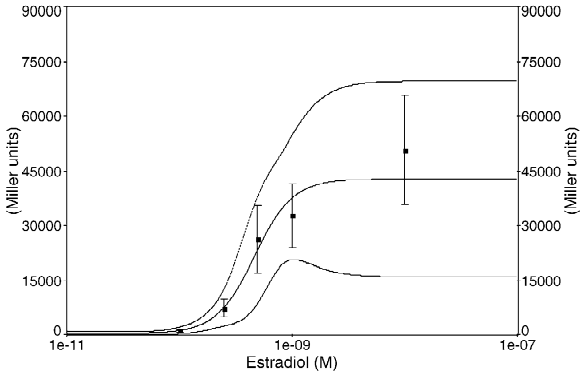

Fig. 9. Mean dose–response curve of the yeast two plasmid system. All cumulated data were used for the approximation.

3.4. Standardization of the system with pure compounds

Serial dilutions of pure compounds which may have estrogenic activity were tested. The compounds were diluted in DMSO and the efficiency and potency was determined and compared to data available from the literature. The summary of these results is shown in Table 2. Testosterone was used as a negative control; it is definitely not an estrogenic compound. Due to structural similarity between 17β-estradiol and testosterone, extremely high levels of testosterone are able to activate the estrogen receptor. Activation of estrogen receptor by testosterone in the submillimolar concentration range has no biological significance. Thus we consider potency values of the range of 10^{-4} *M* as biologically not relevant.

Due to a false report in Science in 1996 [49] the yeast test-system was discredited. Synergistic actions of various xenoestrogens have to be judged extremely cautiously. The false finding was not due to the lack of ability of such test systems to detect synergistic action, it was caused by the improper performance of the experiments [50]. Our laboratory [46] and others [51] have also tried to reproduce those findings and failed, although we could show that the system can measure cooperative antagonistic and synergistic effects [47]. We hope that we were able to show that yeast may be a reliable test-system for large scale screening assays.

For a lot of compounds we found a different potency to other workers. This fact can be explained by the applied test system. In the most cases a tripartite test system was applied. Therefore one cannot extract the information on pure estrogen receptor activation. In a previous paper we have shown that yeast responds similarly to mammalian cells [42]. In that case a liver cell was used as mammalian cell and steroid metabolism had to be taken into account. In most cases authors do not provide efficiency data. Such data are also very interesting, since the efficiency provides information of the stabilization of the transcription complex. Unfortunately comprehensive data are not available in the literature.

In future it will be necessary to decipher the effects of the various ligands on the receptor, the

Table 2
Comparison of the transactivational capacity of various phytoestrogens and xenoestrogens

Ligand	Potency (*M*)	Efficiency (%) (compared to 17β-estradiol)	Relative potency in other test-systems	Test system[a]	Reference
β-Sitosterol	$>10^{-6}$	<0.001	10^4		Rosenblum et al., 1993 [53]
			$6\cdot10^4$	YES	Fang et al., 2000 [54]
			10^4	E-Screen	Gutendorf et al., 2001 [55]
			$7\cdot10^4$	T-47D	Mellanen et al., 1996 [56]
17β-Estradiol	$4.5\cdot10^{-10}$	100			
2-Hydroxybiphenyl	$7.4\cdot10^{-5}$	43	$2\cdot10^6$	YES	Routledge and Sumpter, 1997 [57]
			$5\cdot10$	E-Screen	Soto et al., 1995 [58]
			$5\cdot10^6$	YES	Vinggard et al., 2000 [59]
			$2\cdot10^6$	YES	Miller et al., 2001 [60]
2-sek. Butylphenol	$8.6\cdot10^{-4}$	8	No report		
2-*tert*.-Butylphenol	$1.9\cdot10^{-5}$	9	No activity	E-Screen	Soto et al., 1995 [58]
2,4-(Dichlorphenoxy)-acetic acid	No activity	No activity	No activity	E-Screen	Soto et al., 1995 [58]
3-Butylhydroxyanisol	$4.3\cdot10^{-4}$	19	10^6	YES	Jobling et al., 1995 [61]
			$8\cdot10^4$	E-Screen	Soto et al., 1995 [58]
			$>10^6$	YES	Miller et al., 2001 [60]
3-Hydroxybiphenyl	$1.0\cdot10^{-5}$	59	10^6	YES	Routledge and Sumpter, 1997 [57]
3-*tert*.-Butylphenol	$1.0\cdot10^{-4}$	14	No activity	E-Screen	Soto et al., 1995 [58]
4-Hydroxybiphenyl	$1.3\cdot10^{-6}$	81	10^5	YES	Routledge and Sumpter, 1997 [57]
			10^4	E-Screen	Soto et al., 1995 [58]
			$5\cdot10^4$	YES	Vinggaard et al., 2000 [59]
			10^4	YES	Miller et al., 2001 [60]
4-Nonylphenol	$2.4\cdot10^{-7}$	41	10^6	YES	Routledge and Sumpter, 1997 [57]
			10^3	E-Screen	Soto et al., 1995 [58]
			$5\cdot10^4$	YES	Fang et al., 2000 [54]
			$3\cdot10^5$	YES	Vinggaard et al., 2000 [59]
			10^4	YES	Payne et al., 2000 [28]
			$8\cdot10^4$	E-Screen	Gutendorf et al., 2001 [55]
			$3\cdot10^4$	YES	Garcia-Reyero et al., 2001 [62]
4-Octylphenol	$2.1\cdot10^{-7}$	63	10^4	YES	Routledge and Sumpter, 1997 [57]
			10^6	YES	Jobling et al., 1995 [61]
			10^2	E-Screen	Soto et al., 1995 [58]
			$3\cdot10^4$	YES	Fang et al., 2000 [54]
			$7\cdot10^5$	YES	Vinggaard et al., 2000 [59]
			$5\cdot10^c$	YES	Miller et al., 2001 [60]
			$6\cdot10^4$	YES	Payne et al., 2000 [28]
4-*tert*.-Butylphenol	$1.2\cdot10^{-5}$	30	10^7	YES	Routledge and Sumpter, 1997 [57]
			10^4	E-Screen	Soto et al., 1995 [58]
			$3\cdot10^6$	YES	Miller et al., 2001 [60]
Atrazin	$>10^{-5}$	>2	No activity	E-Screen	Soto et al., 1995 [58]
			No activity	E-Screen	Balaguer et al., 1999 [63]
Biochanin A	$9.9\ 10^{-7}$	51	10^4	Hela Cells	Miksicek 1994 [17]
			$4\cdot10^4$	Ishikawa cells	Liu et al., 2001 [64]
Biphenyl	$2.6\cdot10^{-4}$	22	No report		
Bisphenol A	$2.4\cdot10^{-6}$	60	10^4	YES	Milligan et al., 1998 [65]
			$1.5\cdot10^4$	YES	Gaido et al., 1997 [66]
			$1.4\cdot10^3$	YES	Matthews et al., 2001 [67]
			$4\cdot10^4$	E-Screen	Gutendorf et al., 2001 [55]
			$2\cdot10^4$	YES	Fang et al., 2000 [54]
			$4\cdot10^4$	YES	Vinggaard et al., 2000 [59]
			10^4	YES	Miller et al. [60]

Table 2. Continued

Ligand	Potency (*M*)	Efficiency (%) (compared to 17β-estradiol)	Relative potency in other test-systems	Test system[a]	Reference
Benzylbutylphthalate	$2.0 \cdot 10^{-2}$	62	$8 \cdot 10^{6}$	YES	Jobling et al., 1995 [61]
			10^{4}	E-Screen	Soto et al., 1995 [58]
			$3 \cdot 10^{5}$	YES	Fang et al., 2000 [54]
Bis-(2-Ethylhexyl)phthalate	No activity	No activity	No activity	YES	Jobling et al., 1995 [61]
Coumestrol	$9.6 \cdot 10^{-9}$	149	10^{2}	Hela cells	Miksicek 1994 [17]
			$9 \cdot 10^{4}$	E-Screen	Gutendorf et al., 2001 [55]
			$2 \cdot 10^{2}$	YES	Fang et al., 2000 [54]
			$2 \cdot 10^{2}$	YES	Milligan et al., 1999 [68]
			40	Ishikawa cells	Milligan et al., 1999 [68]
Curcumin	No activity	No activity	No report		
Daidzein	10^{-5}	14	$9 \cdot 10^{3}$	Ishikawa cells	Liu et al., 2001 [64]
			$2 \cdot 10^{3}$	Ishikawa cells	Milligan et al., 1999 [68]
			$7 \cdot 10^{3}$	YES	Milligan et al., 1999 [68]
Desethylatrazin	No activity	No activity	No report		
Desisopropylatrazin	No activity	No activity	No report		
Dieldrin	10^{-5}–10^{-6}	1	10^{6}	E-Screen	Soto et al., 1995 [58]
			10^{4}	Leiomyoma cells	Hodges et al., 2000 [69]
Dibutylphthalate	No activity	No activity	10^{6}	YES	Jobling et al., 1995 [61]
			$4 \cdot 10^{6}$	YES	Vinggaard et al., 2000 [59]
Diethylphthalate	$1.7 \cdot 10^{-4}$	23	No activity	E-Screen	Soto et al., 1995 [58]
Dimethylphthalate	No activity	No activity	No activity	E-Screen	Soto et al., 1995 [58]
Diosmin	No activity	No activity	No activity		
EndosulfanA	10^{-5}	<5	10^{6}	E-Screen	Soto et al., 1995 [58]
			$5 \cdot 10^{2}$	Leiomyoma cells	Hodges et al., 2000 [69]
EndosulfanAB	10^{-5}	10	10^{6}	E-Screen	Soto et al., 1995 [58]
EndosulfanB	10^{-5}	<5	10^{6}	E-Screen	Soto et al., 1995 [58]
			$5 \cdot 10^{2}$	Leiomyoma cells	Hodges et al., 2000 [69]
Ethinylestradiol	$1.7 \cdot 10^{-10}$	100	1	E-Screen	Soto et al., 1995 [58]
			1	YES	Fang et al., 2000 [54]
			0.8	E-Screen	Gutendorf et al., 2001 [55]
Estriol	10^{-9}	100	10	E-Screen	Soto et al., 1995 [58]
			$1.6 \cdot 10^{2}$	YES	Fang et al., 2000 [54]
			14	E-Screen	Gutendorf et al., 2001 [55]
			$3 \cdot 10^{2}$	YES	Garcia-Reyero 2001 et al. [62]
Estron	10^{-9}	100	10	E-Screen	Soto et al., 1995 [58]
			10	YES	Fang et al., 2000 [54]
			100	E-Screen	Gutendorf et al., 2001 [55]
Genistein	$9 \cdot 10^{-7}$	107	$4 \cdot 10^{3}$	Ishikawa cells	Liu et al., 2001 [64]
			$2.5 \cdot 10^{2}$	Ishikawa cells	Milligan et al., 1999 [68]
			$4 \cdot 10^{3}$	YES	Milligan et al., 1999 [68]
			$8 \cdot 10^{3}$	E-Screen	Gutendorf et al., 2001 [55]
			$2 \cdot 10^{3}$	YES	Fang et al., 2000 [54]
			$2 \cdot 10^{3}$	YES	Payne et al., 2000 [28]
Formononetin	$2 \cdot 10^{6}$	76	$9 \cdot 10^{4}$	Ishikawa cells	Liu et al., 2001 [64]
Lindan	$1.6 \cdot 10^{-5}$	5	No activity	E-Screen	Soto et al., 1995 [58]
			No activity	E-Screen and Hela cells	Balaguer et al., 1999 [63]
Linuron	No activity	No activity	No activity	E-Screen	Soto et al., 1995 [58]
Naringenin	10^{-5}	8	$5 \cdot 10^{3}$	Hela cells	Miksicek 1994 [17]
Parathion	No activity	No activity	No activity	E-Screen	Soto et al., 1995 [58]
Podocarpic acid	10^{-5}	30	10^{3}	Hela cells	Miksicek 1994 [17]

Table 2. Continued

Ligand	Potency (M)	Efficiency (%) (compared to 17β-estradiol)	Relative potency in other test-systems	Test system[a]	Reference
Phloretin	No activity	No activity	10^3	Hela cells	Miksicek 1994 [17]
Phthalic acid	No activity	No activity	No report		
Resveratrol	No activity	No activity	10^5	Transfected MCF-7	Gehm et al., 1997 [70]
Sarsasapogenin	No activity	No activity	10^3	Hela cells	Miksicek 1994 [17]
Tamoxifen (*trans*-4-hydroxy)	$5\cdot10^{-7}$	60	Antagonist		Osborne et al., 1993 [71]
			10^4	YES	Fang et al., 2000 [54]
Tamoxifen (*cis*)	$2–3\cdot10^{-6}$	60	No report		
Tamoxifen (*cis*/*trans*)	$2\cdot10^{-6}$	52	Antagonist		Osborne et al., 1993 [71]
			$2\cdot10^4$	YES	Fang et al., 2000 [54]
			10^5	E-Screen	Gutendorf et al., 2001 [55]
Tamoxifen (*trans*)	$2–3\cdot10^{-6}$	70	Antagonist,	E-Screen	Osborne et al., 1993 [71]
Trifluralin	No activity	No activity	No activity	E-Screen	Soto et al., 1995 [58]
Zearalenone	$2.9\cdot10^{-8}$	79	10	E-Screen	Soto et al., 1995 [58]
			10	Hela Cells	Miksicek 1994 [17]
			$\times10^2$	YES	Fang et al., 2000 [54]

[a] YES, yeast estrogen screen; E-Screen, estrogen screen with MCF7 cells.

coactivators and repressors as already started by Routledge at al. [52] Additionally, we want to mention here that this test-system only measures the estrogen receptor activation. Estrogen enzyme modulation and estrogen storage are other very important biological processes not taken into consideration here.

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