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Agonistic and Synergistic Activity of Tamoxifen in a Yeast Model System

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ABSTRACT. The background of agonist/antagonist behaviour of the non-steroidal antiestrogen tamoxifen is still not fully understood. Depending on cell type, its activities range from full agonistic to antagonistic in different tissues. We investigated the transactivational properties of tamoxifen in a basic yeast model system which reconstitutes ligand-dependent human estrogen receptor-α (hERα) gene activation. Tamoxifen exerted low agonist activity in this system compared to 17β-estradiol (E_2). Efficiencies and potencies of several isomers were calculated by fitting experimental data with a logistic dose–response function. *Cis-*, *trans-* and *cis-trans-*tamoxifen and *trans*-4-hydroxytamoxifen (4-OHT) showed comparable efficiencies and potencies in yeast. When subeffective doses of *trans-*, *cis-/trans-*, or *trans*-4-OH tamoxifen were combined with increasing 17β-estradiol concentrations, even a synergistic increase in efficiencies could be observed. Interestingly, the *cis*-isomer did not show this synergistic effect. Mutation of the N-terminus of the estrogen receptor changed the transactivational behaviour of tamoxifen and abolished the synergistic action with 17β-estradiol. Except for 4-OHT, the potencies of the investigated isomers, defined as ligand concentrations with half-maximal response, highly correlated with the binding affinities to hERα. Therefore, *cis-*, *trans-*, and *cis-/trans-*tamoxifen could be regarded as full agonists in yeast, while 4-OHT was regarded as a partial antagonist in yeast. Furthermore, these results indicate that the functional difference between *trans*-tamoxifen and *trans*-4-OHT is not due to their different affinities for the receptor protein. BIOCHEM PHARMACOL **59**;2:177–185, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. tamoxifen; antagonist; agonist; *Saccharomyces cerevisiae*; transcription; human estrogen receptor-α

Tamoxifen, a non-steroidal antiestrogen, is frequently used for treatment of hormone-dependent breast cancer [1–3]. The two structure isomers, *cis*-tamoxifen and *trans*-tamoxifen, differ in their biological function and are converted to several metabolites *in vivo*. The clinically used *trans* form is predominantly converted *in vivo* to *trans*-4-OHT† (Fig. 1), which shows markedly increased affinity for the estrogen receptor [1, 4]. Tamoxifen belongs to a family of class I antagonists [5] which show mixed agonist–antagonist properties depending on cell type and promoter context. This behaviour has been explained by cell-type specific factors that are critical for interaction of the tamoxifen–receptor complex with the basal transcription machinery [6–8]. In reconstituted estrogen receptor transactivation in yeast, tamoxifen acts as agonist [9] only at micromolar concentrations. The antagonistic property of tamoxifen has been explained by different modulation of the AF-1 and AF-2 domains, which are both required for full agonistic activity of 17β-estradiol, whereby AF-1 and AF-2 function in a synergistic manner [7, 8]. Just recently, it was demonstrated that 4-OHT differentially modulates the conformation of the ligand-binding domain compared to 17β-estradiol and thereby inhibits AF-2-mediated transactivation [10]. Therefore, antagonistic activity of tamoxifen can only be observed in cell types where AF-1 alone is not sufficient to fully recruit the transcription machinery.

In a bipartite transactivation system, as defined by Katzenellenbogen *et al.* [11], the potency of a ligand should correlate with the affinity to the receptor. This hypothesis can be employed to assess the agonistic properties of tamoxifen and its metabolites. The yeast system applied in these studies can be strictly considered as a bipartite transactivation system. We utilized ubiquitin-mediated overexpression of hERα [12–14] from a modified construct [15] compared to the one originally described [9, 16]. First, we were primarily interested in the transactivational properties of different tamoxifen isomers alone and their effects on 17β-estradiol-mediated responses at nanomolar levels. Additionally, with a mutant hERα where the N-terminal methionine was replaced by proline and the fusion to ubiquitin was therefore conserved [15, 17], some of the transactivational properties observed with wild-type hERα, but not the affinities of tamoxifen isomers, were altered compared to the wild-type receptor. Taken together, we

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† *Abbreviations:* 4-OHT, 4-hydroxytamoxifen; AF-1, transactivation function 1; AF-2, transactivation function 2; E_2, 17β-estradiol; hERα, human estrogen receptor-α; and LDR, logistic dose–response.

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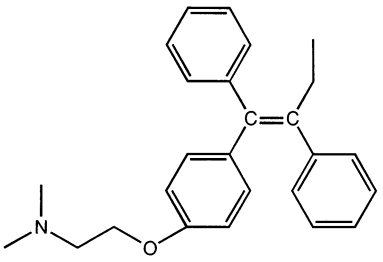
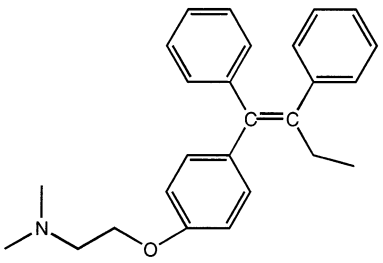
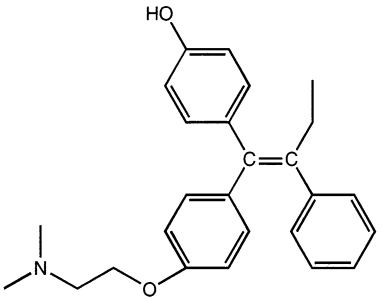


trans tamoxifen



cis tamoxifen



4 OH-trans tamoxifen

FIG. 1. Chemical structures of the investigated ligands.

report the following findings: (1) differential biological activities of *trans*-tamoxifen and *trans*-4-OHT are not due to different affinities for hERα; (2) non-hydroxylated tamoxifen isomers act as agonists in yeast while *trans*-4-OHT acts as partial antagonist; and (3), strikingly, nanomolar concentrations of *trans*-, *cis*-/*trans*-tamoxifen, and *trans*-4-OHT, which were non-active per se, synergistically enhanced the 17β-estradiol-mediated response. Possible explanations for this synergistic action will be discussed.

MATERIALS AND METHODS

Materials

Buffer components were purchased from Merck or Sigma (sodium molybdate and β-mercaptoethanol). Protease inhibitors (tosyl-L-lysine chloromethyl ketone, tosyl-L-phenylalanine chloromethyl ketone, and phenylmethylsulfonyl fluoride) and ligands (17β-estradiol and 4-hydroxytamoxifen) or chromogenic substrate (O-nitrophenyl-βD-pyrogalactoside, ONPG) were obtained from Sigma. *cis*-/*trans*-tamoxifen was a gift from Schering AG. The purified *cis* and *trans* forms of tamoxifen were gifts from Prof. Erich R. Schmid (Univ. of Vienna). 4-OHT (mainly the *trans*-isomer) was purchased from Sigma. Glass beads (0.25–0.5 mm diameter) were purchased from Merck, and [^{3}H]17β-estradiol and dextran-coated charcoal were from NEN-DuPont.

Strains and Plasmids

For all transactivation assays, the *Saccharomyces cerevisiae* strain 188R1, a derivative of RS188N [9], was used. This strain was transformed with YEpE12 encoding wild-type hERα in a ubiquitin fusion or YEpE14 encoding an N-terminal point mutation (Met → Pro) also in fusion with the human ubiquitin gene [15]. Both expression plasmids were transformed together with a β-galactosidase reporter plasmid YRpE2 [9, 16]. The double transformation procedure was performed according to a protocol of Hill *et al.* [18, 19].

Transactivation Assays

Transformed clones were selected on synthetic media plates and colonies were grown in synthetic liquid medium. Yeast cell growth was monitored at a wavelength of 600 nm with a spectrophotometer. Stock cultures were incubated overnight at 30 ± 1°, 180 rpm, cultures were diluted to $O.D._{600}$ = 0.5, and 5-mL aliquots were transferred into 50-mL sterile plastic tubes. hERα expression was induced by addition of 10 μM $CuSO_4$. Simultaneously, ligands were added to these 5-mL aliquots. All ligands were dissolved and diluted in DMSO. After 4 hr additional incubation, yeast cells were harvested and washed, and the pellets were disintegrated in lacZ buffer (100 mM sodium phosphate, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) using glass beads. Cell debris was removed by centrifugation at 10,000 *g* at 4° and the supernatants were recovered and assayed. The assay for β-gal activity was performed in microtiter plates as described previously [15]. The absorption was measured at 405 nm with an SLT EAR 400 AT plate reader (SLT). In parallel with each β-gal assay, total protein was estimated by the Bradford method using the Bio-Rad protein assay reagent. Bovine serum albumin (Serva) was used as a standard protein to generate a calibration curve. The specific enzyme activity was expressed in Miller units [20].

Relative Binding Affinity

Yeast extracts for ligand competition assays were generated as described previously [21] and 20-μL extract was incubated with approx. 12 pmol of [2,4,6,7-^{3}H]17β-estradiol (NEN-DuPont) and increasing concentrations (10^{-13} –

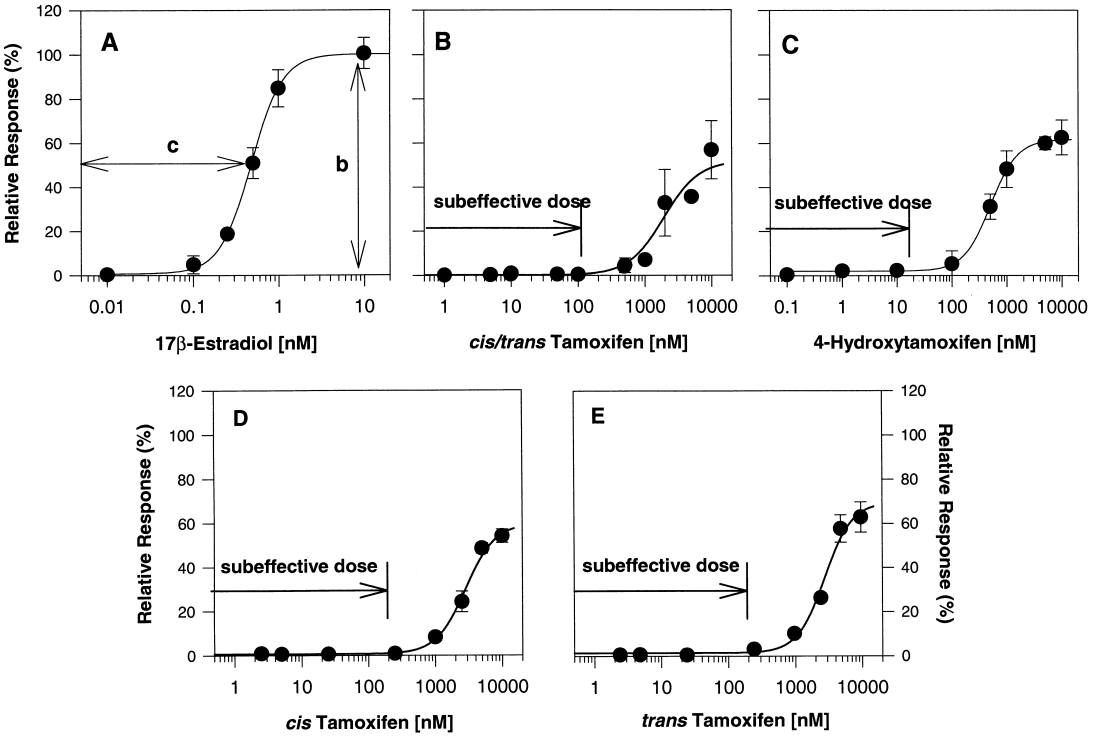


FIG. 2. Dose–response relationships of (A) 17β-estradiol, (B) *cis-/trans*-tamoxifen, (C) 4-hydroxytamoxifen, (D) *cis*-tamoxifen, and (E) *trans*-tamoxifen in a yeast hERα reporter assay. The subeffective dose arrow marks the concentration range where no significant β-galactosidase activity was detected. Yeast cells were simultaneously combined with copper sulfate (10 μM) to induce hERα expression and the respective ligands. Cultures were incubated for 4 hr at 30°. The response was normalized to the response of E_2, which served as an external control in each set of experiments. Error bars represent the standard deviation of quadruplets.

10^{-5} M) of non-labelled competing ligand for 16 hr at 4°. Unbound radioactivity was removed by addition of 300 μL dextran-coated charcoal suspension, centrifuged at 4000 *g* for 15 min at 4° and aliquots (100 μL) were used for liquid scintillation counting (Pharmacia/Wallay Oy).

Curve Fitting

Expression of nuclear receptors, ligand recognition, and transactivation of target genes consist 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. Dose–response as well as ligand competition data were approximated by LDR function using Table Curve 2D software (Jandel Scientific). The function is described as

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

where parameter *a* equals the baseline, *b* the plateau of the curve designated as the ligand efficiency, and parameter *c* gives the transition centre and equals the ligand potency which is the concentration that causes 50% efficiency. Absolute or normalized values from two or more indepen-

TABLE 1. Potencies and efficiencies of various ligands with human estrogen receptor-α (w.t.) measured by the yeast transactivation assay

Ligand	Potency [M] ±SE	Efficiency [Miller Units] ±SE	Relative response (%) ±SE
17β-Estradiol (E_2)	$4.8 \pm 0.3 \times 10^{-10}$	43,613 ± 1,856	100.0 ± 4.3
cis-/trans-Tamoxifen	$1.9 \pm 0.5 \times 10^{-6}$	28,486 ± 6,744	65.3 ± 15.5
17β-Estradiol (E_2)	$4.3 \pm 0.3 \times 10^{-10}$	45,262 ± 1,882	100.0 ± 4.2
cis-Tamoxifen	$2.8 \pm 0.2 \times 10^{-6}$	26,741 ± 1,170	59.1 ± 2.6
trans-Tamoxifen	$2.8 \pm 0.3 \times 10^{-6}$	30,948 ± 2,036	68.4 ± 4.5
17β-Estradiol (E_2)	$5.1 \pm 0.6 \times 10^{-10}$	34,882 ± 2,073	100.0 ± 5.9
4-Hydroxytamoxifen	$4.9 \pm 0.6 \times 10^{-7}$	20,237 ± 1,179	58.0 ± 3.4

The values represent parameters obtained from representative logistic dose–response curve fits ± standard error (SE) shown in Fig. 2. Each set of titration experiments contained a 17β-estradiol titration as external standard and each concentration step was done in duplicate.

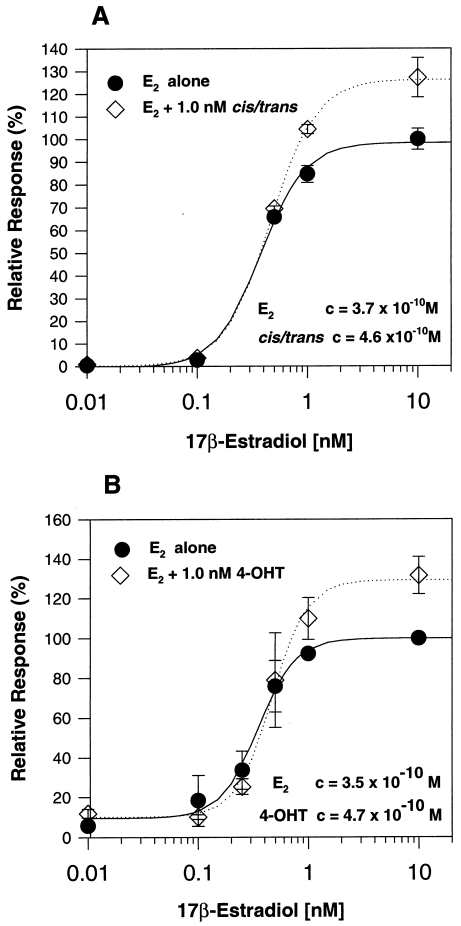


FIG. 3. Synergistic increase in ligand-dependent transactivation of mixtures between (A) *cis-/trans*-tamoxifen and 17β-estradiol and (B) 4-hydroxytamoxifen and 17β-estradiol. Increasing concentrations of 17β-estradiol were combined with subeffective concentrations of tamoxifen. Normalized data of two or more independent experiments were combined. Error bars represent the standard deviation of quadruplets.

dent experiments were fitted. Each ligand concentration step was done in duplicate for transactivation assays or triplicate for competitive ligand-binding assays.

RESULTS

Transactivational Properties of Different Tamoxifen Isomers in Yeast

We employed a two-plasmid system consisting of an hERα expression plasmid and a reporter plasmid containing vitellogenin ERE_2 to study agonistic properties of *cis-/trans*-tamoxifen, as well as the purified *cis-* and *trans*-isomers and the biologically more active metabolite 4-OHT (Fig. 1). The tamoxifens were tested alone and in combination with 17β-estradiol. The reporter gene product β-galactosidase gave a measure for ligand-dependent transactivation. The effect of increasing concentrations of 17β-estradiol, *cis-*, *trans-*, *cis-/trans*-tamoxifen, and 4-OHT on the reporter enzyme activity can be seen from Fig. 2. As expected, 4-OHT was more potent than *cis-/trans*-tamoxifen. 4-OHT exerted half-maximal response at a concentration of 5×10^{-7} M and the *cis/trans* mixture at 2×10^{-6} M, a concentration approximately four times higher. In addition, we tested the transactivational potential of purified *cis-* and *trans*-isomers. In our system, *trans*-tamoxifen was as active as the *cis* form, taking into account that we cannot exclude isomerization. All dose–response relationships were approximated by the LDR function. Parameters *b* (efficiency) and *c* (potency) are given in Table 1.

To assess possible antagonism or synergistic agonism between E_2 and tamoxifen, we combined increasing concentrations of E_2 with subeffective levels of tamoxifen. We considered 0.1, 1.0, and 10 nM concentrations of tamoxifen as subeffective levels: no significant transactivation could be observed with these concentrations (Fig. 2). Combining 0.1 nM *cis-/trans*-tamoxifen with increasing concentrations of 17β-estradiol indicated a small increase in efficiency that was not clearly significant. Mixing 1.0 nM *cis-/trans*-tamoxifen as well as 4-OH tamoxifen showed an increase in efficiency of 15–40% as evaluated by the LDR function (see Fig. 3). Increasing the tamoxifen concentration to 10 nM did not further enhance efficiency (data not shown). In contrast, addition of subeffective concentrations of tamoxifens to 17β-estradiol did not alter overall ligand potency (parameter *c* in Eqn. 1), as defined above (Table 2 and Fig. 3).

Further mixtures of purified *cis-* and *trans*-tamoxifen isomers with 17β-estradiol were tested in a similar manner. Again, subeffective doses (Fig. 2, D and E and Table 1) were added to increasing concentrations of 17β-estradiol. Although the transactivational properties of the *cis-* and *trans*-isomers were almost identical to those observed in the previous experiment, the synergistic effect on efficiency in the presence of increasing concentrations of 17β-estradiol was limited to *trans*-tamoxifen. *Cis*-tamoxifen was ineffective in this regard. When both pure components were

TABLE 2. Synergistic effect of *cis-/trans-* and 4-hydroxytamoxifen on 17β-estradiol-dependent transactivation

Tamoxifen [nM]	Potency [M] ±SE	Efficiency [Miller Units] ±SE	Rel. Response (%) ±SE
E_2 (standard *cis/trans*)	$3.7 \pm 0.6 \times 10^{-10}$	43,826 ± 3,033	100.0 ± 6.9
E_2 + 0.1 nM *cis/trans*	$4.4 \pm 0.5 \times 10^{-10}$	51,917 ± 2,581	118.5 ± 5.9
E_2 + 1.0 nM *cis/trans*	$4.2 \pm 0.3 \times 10^{-10}$	64,711 ± 2,229	147.7 ± 5.1
E_2 (standard 4-OHT)	$5.8 \pm 0.7 \times 10^{-10}$	41,781 ± 3,685	100.0 ± 8.8
E_2 + 1.0 nM 4-OHT	$6.6 \pm 0.6 \times 10^{-10}$	52,458 ± 3,436	125.6 ± 8.2

The values ± standard error (SE) for potency and efficiency, parameters *c* and *b*, respectively, were obtained from a logistic dose–response fit of a representative set of experiments. The titrations contained 17β-estradiol as external standard which was set to 100%.

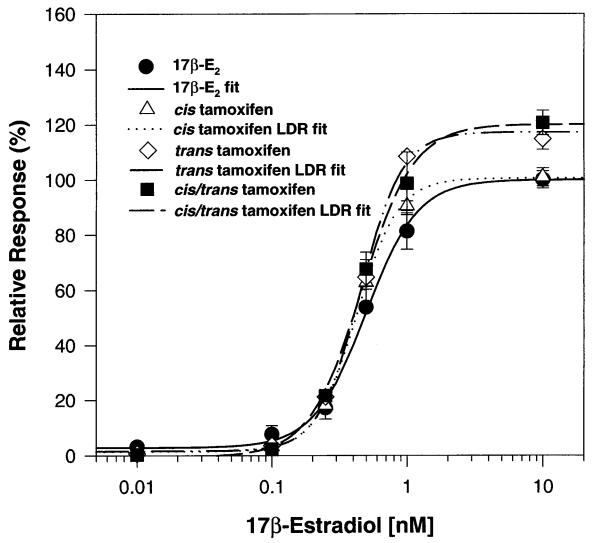


FIG. 4. Effect of purified *cis-* and *trans*-tamoxifen isomers (1.0 nM each) on 17β-estradiol-dependent transactivation. A representative experiment with 17β-estradiol alone as external control is shown. Error bars represent the standard deviation of quadruplets.

mixed at a ratio of 1:1, a transactivation efficiency comparable to *trans*-tamoxifen was produced (Fig. 4).

Effect of Conserved N-terminal Ubiquitin Fusion

In a previous report [15], we showed that mutation of the N-terminal Met→Pro leads to a ubiquitin–ERα fusion protein that is cleaved inefficiently by the endogenous yeast ubiquitin carboxy-terminal hydrolases. Interestingly, this fusion changed expression levels and DNA-binding properties, while the E_2-dependent transactivation level (estimated as β-gal activity) was comparable to wild-type hERα. However, in more detailed studies [22], a 2-fold shift in potency of *cis-*/*trans*-tamoxifen was observed with the Pro–ER mutant compared to wild-type receptor. Interestingly, the potency of 4-OHT with the proline mutant was unaffected and in the same range as observed with wild-type hERα (Table 3 and Fig. 5). A significant change in E_2-dependent efficiency with subeffective doses of *cis-*/*trans-* or 4-hydroxytamoxifen could not be detected. These results raised the question of whether these effects were due to changes in ligand-binding affinities or somehow related to the conservation of the N-terminal ubiquitin fusion. Therefore, we determined the binding affinities of tamoxifen relative to E_2 by competitive radioligand binding assays.

Relative Binding Affinities

As shown in Table 4, the competitive behaviour of 17β-estradiol, *cis-*/*trans*-tamoxifen, and 4-OHT with wild-type hERα and with Pro–ER expressed in yeast was essentially the same. Relative binding affinities were estimated from LDR fitting parameter *c*. The findings that the affinity of 4-OHT to hERα was in the range of 17β-estradiol and that the *cis*/*trans* mixture had 2–3 orders of magnitude lower affinity were consistent with earlier reports. Furthermore, these results demonstrated that (1) affinity changes did not account for differences in transactivational behaviour and (2) affinity and potency of 4-hydroxytamoxifen were much less correlated compared to the *cis-*/*trans*-tamoxifen mixture or both purified isomers.

DISCUSSION

The agonistic or antagonistic activity of tamoxifen is dependent on cell-specific factors such as promoter context, the presence of co-activators and co-repressors of gene regulation, and metabolizing enzymes [23–26]. The level of complexity is considerably increased by the fact that different metabolites show contrary responses [2]. To shed more light on the basic aspects of tamoxifen isomer action, we chose to perform cell-based transactivation assays in yeast. Although human estrogen receptors function in *Saccharomyces cerevisiae* [27], antagonists exert considerable agonistic potential in this lower eukaryotic organism [9]. This behaviour can be attributed to a lack of several co-factors which are responsible for cell-type-specific modulation of transcription. However, tamoxifen shows agonistic and partial and full antagonistic activity in different mammalian tissues [2, 28]. In our yeast studies, tamoxifen consistently acted as agonist at micromolar concentrations (Fig. 2). *trans*-4-OHT was significantly more potent compared to *cis-*, *trans-*, and *cis-*/*trans*-tamoxifen, which indicates that hydroxylation of these isomers either did not occur or did so only at a very low rate.

Ligands with certain affinities for the receptor will compete for binding sites. They will produce an additive response which is dependent on their agonistic/antagonistic behaviour and efficiencies. In the present study, a subeffective dose of tamoxifen was able to further increase 17β-

TABLE 3. Potencies and efficiencies of various ligands with the Pro-ER mutant measured by the reconstituted yeast transactivation assay

Ligand	Potency [M] ±SE	Efficiency [Miller Units] ±SE	Rel. Response (%) ±SE
17β-Estradiol (E_2)	$5.5 \pm 1.0 \times 10^{-10}$	51,054 ± 4,960	100.0 ± 9.7
cis-/*trans*-Tamoxifen	$3.6 \pm 0.6 \times 10^{-6}$	24,872 ± 3,507	48.7 ± 6.9
4-Hydroxytamoxifen	$4.9 \pm 0.1 \times 10^{-7}$	34,200 ± 325	67.0 ± 0.6

The values represent parameters obtained from logistic dose–response curve fits ± standard error (SE) of representative experiments shown in Fig. 5.

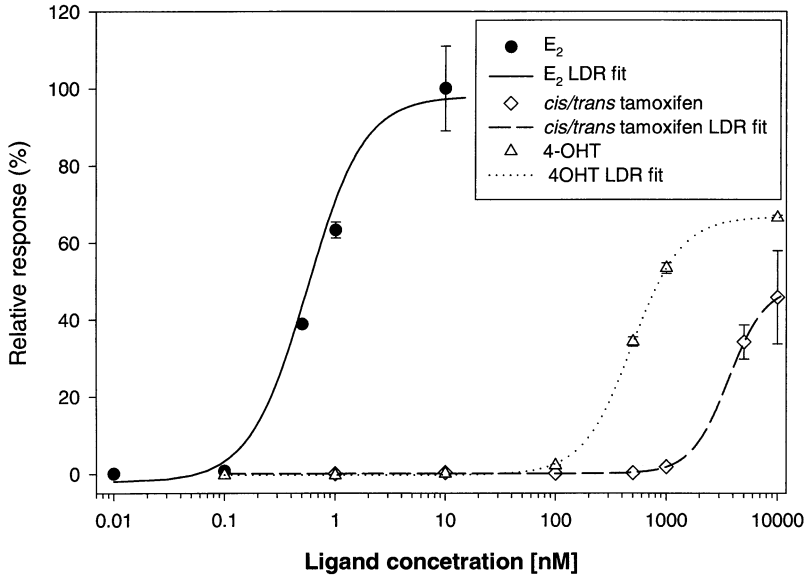


FIG. 5. Dose–response relationships of 17β-estradiol, *cis-/trans*-tamoxifen, and 4-OHT with the proline mutant of the estrogen receptor. Error bars represent the standard deviation of quadruplets.

estradiol-mediated transactivation. Therefore, we interpret the behaviour of subeffective doses of tamoxifen with 17β-estradiol as synergistic in nature. The *cis-* and *trans*-isomers showed similar agonist properties when tested alone (Fig. 2), but only the *trans* form was able to act synergistically in combination with 17β-estradiol. The synergistic action could be recovered when both isomers were mixed at a 1:1 ratio (Fig. 4). *cis-* and *trans*-tamoxifen isomers could not be distinguished according to their agonistic/antagonistic activity as in other systems [2]. Remarkably, however, they differed in their ability to synergistically enhance 17β-estradiol-mediated transactivation efficiency. In general, due to the high affinity of 4-OHT, one could expect a shift in potency upon its addition to increasing 17β-estradiol concentrations, which did obviously not occur under the conditions applied. The striking finding that subeffective doses of *trans-*, *cis-/trans-* and 4-OH-tamoxifen synergistically enhanced transactivational efficiency (Figs. 3 and 4) could be explained by (1) stabilizing effects on receptor half-life or DNA–receptor complex formation, (2) an influence on receptor mRNA stability [29, 30], (3) activation of an hERα-independent signal pathway, e.g. by an increase in receptor phosphorylation [31–33], or (4) by a direct influence on the transcription machinery. In a bipartite model of ligand action, efficiency is determined by the effectiveness of the conformation of the ligand–receptor complex. The biological response is proportional to the affinity of the ligand for the receptor. Tamoxifen could also act by enhancing receptor stability or ligand-induced binding of receptor to response elements on the reporter plasmid. Co-operative effects between liganded ERα and different response elements [34–37] as well as stabilization of receptor–DNA complexes by antiestrogens have been described. As mentioned above, variable receptor–ligand binding domain occupation could account for variations in potencies and/or efficiencies; however, if this were the case, different ligand affinities should be reflected by these results. Furthermore, tamoxifen could influence the recruitment of co-activators and/or co-repressors, leading to a modulated transcription efficiency.

TABLE 4. Relative binding affinities (RBA) of wild-type human estrogen receptor and a proline mutant for tamoxifen as compared to 17β-estradiol

Ligand	50% Competition [M]	RBA
Wild-type		
17β-Estradiol (E_2)	$4.7 \pm 1.3 \times 10^{-10}$	1
cis-Tamoxifen	$1.7 \pm 1.1 \times 10^{-7}$	362
trans-Tamoxifen	$1.7 \pm 0.7 \times 10^{-7}$	362
cis-/trans-Tamoxifen	$6.1 \pm 1.8 \times 10^{-7}$	1298
4-Hydroxytamoxifen	$2.1 \pm 0.2 \times 10^{-9}$	4.5
Proline Mutant		
17β-Estradiol	$5.7 \pm 1.1 \times 10^{-10}$	1
cis-/trans-Tamoxifen	$3.4 \pm 1.2 \times 10^{-7}$	597
4-Hydroxytamoxifen	$6.8 \pm 4.2 \times 10^{-10}$	1.2

Ligand competition assays were performed as described in Materials and Methods. RBA values correspond to 50% competition of a respective ligand with [^{3}H]17β-estradiol.

Recently, stabilizing effects of both 17β-estradiol and tamoxifen on estrogen receptor mRNA have been reported [29, 30]. For several reasons, this explanation seems reasonable in the light of our findings: (1) the synergistic effects of *trans-*, *cis-/trans*-tamoxifen, and 4-OHT were similar, although their affinities and potencies were significantly different. This indicates a mechanism independent of direct receptor transactivation; (2) *cis*-tamoxifen was comparable to *trans*-tamoxifen in terms of transactivation, but non-effective at subeffective doses; (3) ligand potency remained unchanged following addition of different tamoxifen isomers; and (4) no increased synergistic effect could be observed at higher tamoxifen concentrations. Indirect ac-

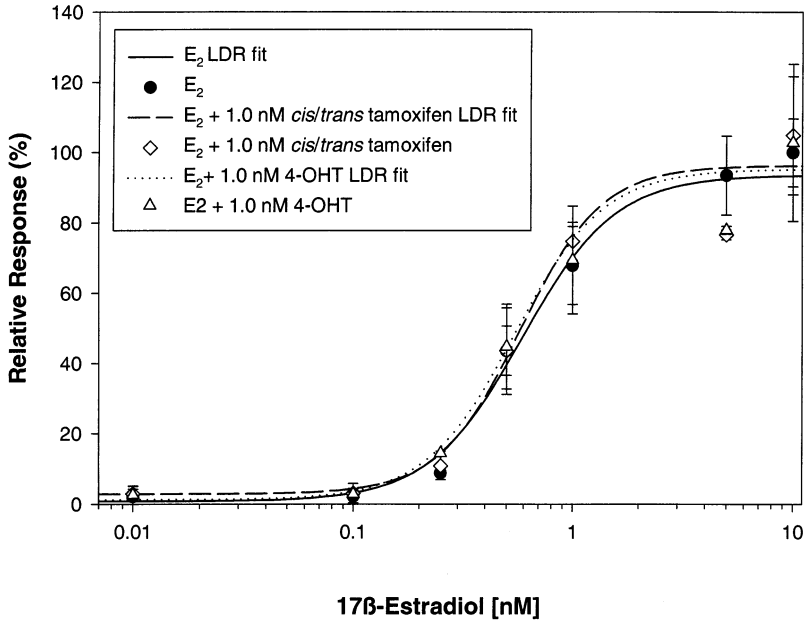


FIG. 6. Transactivation of mixtures between different concentrations of 17β-estradiol and 1.0 nM *cis-/trans*-tamoxifen or 1.0 nM 4-OHT with the proline mutant of hERα. The data were combined from different experiments which were normalized to the respective external standard 17β-estradiol alone. Error bars represent the standard deviation of quadruplets.

tivation of hERα by hyperphosphorylation could also account for an increase in transactivational response, measured as β-galactosidase activity [38]. Castano *et al.* [39] have demonstrated that serine 118 in the A/B domain is susceptible to phosphorylation by kinases activated by different signal pathways and plays a role in transactivation efficiency. Independently of the above-described effects, synergism could be due to direct stabilization of the basal transcription machinery. These mechanisms could be excluded, since tamoxifen acted according to the bipartite receptor pharmacology (potency was correlated to affinity of the ligand) and 4-OHT possesses lower potency than expected from the affinity to the receptor. If tamoxifen stabilized the basal transcription machinery, higher potency than expected from affinity data would have been produced.

Katzenellenbogen *et al.* [11] summarized the following characteristics for bipartite receptor pharmacology. The receptor binds a ligand and subsequent steps lead to a biological response. Partial antagonists bind to the receptor, but the biological response is not fully developed. Full antagonists interact with the receptor but do not produce a biological response. In yeast test systems as used in this investigation, the biological response is simulated by a reporter plasmid with a vitellogenin A2 estrogen response element which controls the expression of a β-gal reporter gene. If a ligand is able to bind to the receptor and to induce a conformational change leading to transcription of the reporter gene, it can be considered as an agonist. Since *cis-/trans*-tamoxifen does not develop its antagonistic behaviour in yeast test systems and its affinity to the estrogen receptor correlates with the potency measured in transactivation assays (compare Fig. 2 and Table 3), isomers taken individually or in combination can be regarded as full agonists. The affinity of 4-OHT is in the same order of magnitude as that of 17β-estradiol, but does not exert comparable potency. 4-OHT can therefore be regarded as a partial agonist or antagonist in yeast. Taken together, these results suggest that selective uptake of tamoxifen (isoforms), as proposed previously for antiestrogens in yeast [40], is unlikely.

To enhance the expression levels and quality of hERα, we expressed the receptor as an ubiquitin fusion protein which also facilitated modification of the amino-terminus. When the N-terminal amino acid was mutated to proline, the ubiquitin fusion could not be cleaved efficiently by endogenous yeast ubiquitin carboxy-terminal hydrolases [15, 22]. Interestingly, this mutant showed changes in transactivational behaviour with *cis-/trans*-tamoxifen but not with 4-OHT. The potency of 17β-estradiol remained largely unchanged. Additionally, the synergistic effect of *cis-/trans*-tamoxifen or 4-OHT could not be observed with the concentrations applied (Fig. 6). Metzger *et al.* [41] demonstrated that the AF-1 domain is essential for full transactivation. They identified three discrete activating domains near the amino-terminus which act independently in yeast. Our observations underscore the importance of the A/B domain for agonistic/antagonistic activity of tamoxifen. Taking these results together would lead one to conclude that the synergistic effect observed was directly or indirectly mediated through the AF-1 domain. Again, for the Pro–ER mutant this may mean steric hindrance, changes in phosphorylation, or less productive DNA–receptor complexes. Spatz *et al.* [42] also reported a synergistic effect between tamoxifen and 17β-estradiol in mammalian systems (HeLa cells and red oesteoblastic sarcoma).

The receptor was overexpressed and transactivation was assessed by a CAT reporter system. In MCF-7 cells, on the other hand, they could not observe synergism. In the context of these observations, we believe that synergism between 17β-estradiol and subeffective doses of antagonists may be a common phenomenon, one not limited to simple yeast systems. However, the target(s) and exact mechanism(s) of action have yet to be identified.

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