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Capacity of type I and II ligands to confer to estrogen receptor alpha an appropriate conformation for the recruitment of coactivators containing a LxxLL motif—Relationship with the regulation of receptor level and ERE-dependent transcription in MCF-7 cells

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ABSTRACT

Estrogen receptor α (ERα) belongs to the superfamily of nuclear receptors and as such acts as a ligand-modulated transcription factor. Ligands elicit in ERα conformational changes leading to the recruitment of coactivators required for the transactivation of target genes *via* cognate response elements. In many cells, activated ERα also undergoes downregulation by proteolysis mediated by the ubiquitin/proteasome system. Although these various molecular processes have been well characterized, little is known as to which extent they are interrelated. In the present study, we used a panel of type I (estradiol derivatives and "linear", non-steroidal ligands) and type II ("angular" ligands) estrogens, in order to identify possible relationships between ligand binding affinity, recruitment of LxxLL-containing coactivators, ERα downregulation in MCF-7 cells and related transactivation activity of ligand-bound ERα. For type I estrogens, there was a clear-cut relationship between ligand binding affinity, hydrophobicity around C-11 of estradiol and ability of ERα to associate with LxxLL motifs, both in cell-free condition and *in vivo* (MCF-7 cells). Moreover, LxxLL motif recruitment by ERα seemed to be a prerequisite for the downregulation of the receptor. By contrast, type II ligands, as well as estradiol derivatives bearing a bulky side chain at 11β, had much less tendency to promote ERα–LxxLL interaction or even behaved as antagonists in this respect, in agreement with the well known partial estrogenicity/antiestrogenicity of some of these compounds. Interestingly, some type II ligands which antagonized LxxLL motif recruitment were nonetheless able to enhance ERα-mediated gene transactivation.

1. Introduction

Estrogen receptor alpha (ERα) is a member of the nuclear receptor superfamily, the members of which function as ligand-regulated transcription factors [1]. Estrogens are essential for the development and the maintenance of the female reproductive system. Besides, ERα is also known to play a pivotal role in the etiology of hormone-dependent forms of breast cancer [2]. Hence, for the last 30 years or so there has been an intensive search for agents capable of modulating and/or inhibiting ERα-mediated cell proliferation. This has led to the identification of two main classes of ligands both containing two hydroxyl functions that contribute to their binding to the receptor (Tables 1 and 2) [3]. Type I ligands include estrogenic steroids and diphenolic structural analogs that similarly stimulate target tissues (*trans* stilbene derivatives such as diethylstilbestrol, isoflavones such as genistein, coumestanes such as coumestrol). These planar/linear agonists differentiate from angular weak agonists (type II) for which two subsets have been described: *cis* stilbene-like and *geminal* structures. Such angular ligands can in some cases antagonize the effect of strong type I estrogens [4]. Insofar as their pharmacological profile varies among different tissues, they are currently referred to as SERMs (Selective Estrogen Receptor Modulators).

All investigated type I ligands have been reported to fit within a cleft of the hormone binding domain (HBD) in such a way that their hydroxyl groups can form hydrogen bonds with a few amino acids

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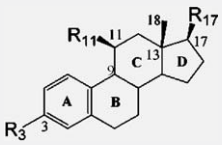
Table 1
Relative efficiency of steroidal ligands to associate with the E_2 binding site and induce the recruitment of co-regulators containing a LxxLL motif.

No.	Structure			E_2 binding (RBA)[a]	LxxLL recruitment (RRC)[a]
	17β estradiol derivatives 				
	R_3	R_{17}	R_{11}		
1 (E_2)	OH	OH	H	100	100
2	O–CH_3	OH	H	3	10
3	O–SO_2–O^-Na^+	OH	H	<<0.1	0
4	OH	H	H	10	<<0.01
5	OH	OH (α)	H	30	60
6α	OH	OH	OH (α)	0.3	1
6β	OH	OH	OH (β)	10	4000[b]
7	OH	OH	O–CO–CH_3	30	800
8	OH	OH	CH_3	100	1300
9	OH	OH	CH_2–Cl	100	600
10	OH	OH	C≡CH	30	400
11	OH	OH	$C_{(9)}$=$C_{(11)}$	10	200
12	OH	OH		10	Antagonist
13	OH	OH		10	Antagonist
14	OH	OH		3	No effect

[a] Ratio of concentrations of E_2 and investigated compounds to produce 50% of maximal effect. Values are expressed in percentages of E_2 efficiency to compete with [^{3}H]E_2 for ERα binding (RBA) or confer a conformation to ERα appropriate for binding to a plate coated with a peptide containing a LxxLL motif (RRC).
[b] Value most probably overestimated due to the low optimal RC value (see Fig. 6).

Table 2
Relative efficiency of non-steroidal ligands to associate with the E_2 binding site and induce the recruitment of co-regulators containing a LxxLL motif.

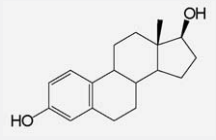
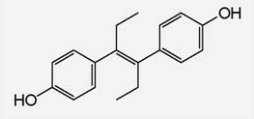
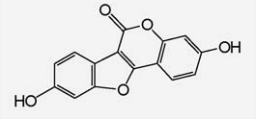
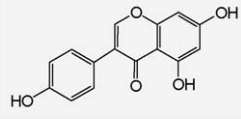
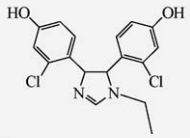
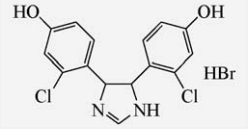
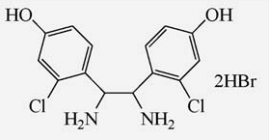
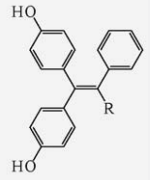
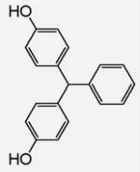
No.	Structure	E_2 binding (RBA)[a]	LxxLL recruitment (RRC)[a]
1 (E_2)		100	100
a. Non-steroidal "linear" estrogens (Type I)			
15		50	200
16		1	1
17		1	0.1

Table 2 (*Continued*)

No.	Structure		E_2 binding (RBA)[a]	LxxLL recruitment (RRC)[a]
b. Angular diphenolic estrogens (Type II)				
18			<0.01	0.1
19			<0.01	<<0.1
20			<0.01	<<0.1
		R		
21		–H	10	Antagonist
22		$-CH_3$	10	Antagonist
23		$-CH_2CH_3$ (bisphenol)	10	Antagonist
24		$-CH_2CH_2CH_3$	3	Antagonist
25			5	Antagonist

[a] Ratio of concentrations of E_2 and investigated compounds to produce 50% of maximal effect. Values are expressed in percentage of E_2 efficiency to compete with [^{3}H]E_2 for ERα binding (RBA) or confer a conformation to ERα appropriate for binding to a plate coated with a peptide containing a LxxLL motif (RRC).

(for E_2, 3-OH: Glu-353 and Arg-394; 17β-OH: His-524; Fig. 1) [5,6]. Although this property does not hold for type II ligands because of inappropriate orientation of their hydroxyl groups, interactions with Glu-353 and Arg-394 are conserved and concur to produce non-covalent ligand–receptor complexes. Stability of these complexes results from additional electrostatic interactions between the second phenolic ring of these ligands and Thr-347, a residue located in a subregion of the ligand binding domain (LBD) known to attract substituents in C-11 of estradiol (E_2) [6,7]. While hydrogen bonding between the hydroxyl of this phenolic ring and Thr-347 (Fig. 1) is of importance for ERα binding, hydrophobic interactions between the C-11 subregion of the steroid core and the LBD are largely dominant [8]. Such differences in binding modes between type I and II ligands have been reported to confer to ERα distinct conformations that should influence its ability to recruit coactivators containing a consensus LxxLL binding motif (L = Leucine, x = any amino acid) [9–13] such as those of the CBP/p300 or the SRC/p160 families known as acetyl-transferase enzymes and/or adaptator proteins for the transcription machinery recruitment (see [14–16]).

Even though there is a general consensus that the ability to recruit such coactivators determines the capacity of ERα to induce gene transactivation, no structure/activity study substantiating this concept has been reported so far. Similarly, there are no systematic data concerning a potential relationship between coactivator recruitment (or the absence of recruitment) and the capacity of a ligand to modulate ERα turnover, a factor known to influence receptor-mediated gene transactivation [17–19]. This gap in our knowledge has led us to conduct such a comparative study based on a panel of type I and type II estrogens. Of note, a part of our investigations focuses on several E_2 derivatives substituted in C-3, C-11 and C-17 since, as discussed above, these three reactive positions of the steroid core are known to play a prominent role in ligand binding and to influence the receptor conformations leading to an estrogenic response. Our experimental approach for this program relied on an ELISA-based assay allowing the quantitative measurements of ligand-activated ERα associated with immobilized LxxLL motif-containing peptide. In previous studies, the specificity of the assay was established by competition experiments showing that a peptide derived from the SRC-1 coactivator effectively suppresses receptor binding to LxxLL-coated plates through a complexation process [20].

2. Material and methods

2.1. Compounds

Type I ligands were purchased from Sigma (St Louis, MO) or Steraloids (Newport, RI) while type II [4,21] were obtained from Prof. R. Gust (Pharmacological Institute, Free University Berlin, Germany); 11β long chain derivatives of E_2 [22] were from Prof. J.-C. Blazejewski (University of Versailles, France). For assays, stock ethanol solutions of these compounds were diluted in buffer

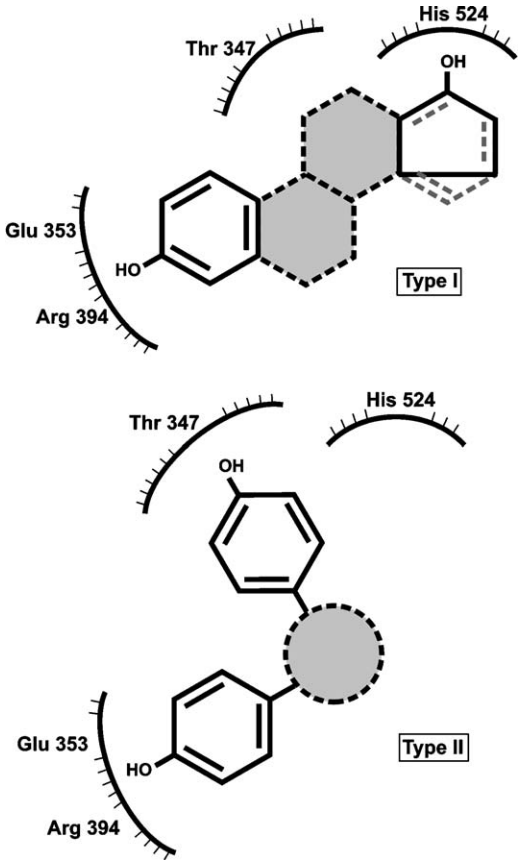


Fig. 1. Stabilizing interactions in (a) the type I estrogen/ERα complex (b) the type II estrogen/ERα complex.

(cell-free assays) or medium (cell culture) in order to have a final concentration of solvent below 0.1%. Routine biochemical reagents were from standard suppliers.

2.2. Cell-free assays with human ERα recombinant

Assays were conducted with a highly purified human ERα (hERα) recombinant obtained from Calbiochem (Euro-Biochem, Bierges, Belgium). Upon receipt, the protein was diluted (≈2.5 pmol/μL) in 50 mM Tris–HCl pH 7.5 containing 500 mM KCl, 2 mM DTT, 1 mM EDTA, 1 mM sodium orthovanadate and 10% glycerol. Diluted aliquots were stored at −80 °C.

2.2.1. Relative binding affinity of compounds for hERα

Binding affinity of the compounds for hERα was evaluated by a solid phase competition assay based on the adsorbing property of the receptor onto hydroxylapatite (HAP) at low ionic strength [23]. For that purpose, hERα diluted in a buffered bovine serum albumin (BSA) solution (1:500 in 10 mM Tris–HCl pH 8 containing 1 mg/mL BSA) was adsorbed onto HAP (Bio-Rad). After removal of unbound receptor by centrifugation, the HAP suspension was incubated during 24 h at 4 °C in the presence of 1 nM [^{3}H]E_2 (GE Healthcare Biosciences, Eindhoven, NL) with or without increasing amounts of the investigated compounds or unlabeled E_2 taken as a reference. Bound [^{3}H]E_2 was then extracted with ethanol and measured by liquid scintillation counting. Relative concentrations of investigated compounds and E_2 required to inhibit the binding of [^{3}H]E_2 by 50% yielded the relative binding affinity values (RBA = $[IC_{50}]_{E2}/[IC_{50}]_X \times 100$). Assays were performed twice, each time in duplicate. In each competition curve, variations between bound [^{3}H]E_2 values were extremely low (SD = 3%), suggesting a very high reproducibility of the data.

2.2.2. Ability of compounds to confer to hERα a conformation appropriate for recruitment of a LxxLL-containing peptide

The ability of hERα to associate with a capture LxxLL-containing peptide was assessed with an ELISA-based assay (Elisa NR peptide ERα-chemiluminescent assay from Active Motif, Rixensart, Belgium). hERα (5 ng in the diluent buffer) was incubated on ice with increasing concentrations of the investigated compounds (from 10 pM to 1 μM) for 30 min. These samples were then added to LxxLL peptide-coated multiwell plates and further incubated for 1 h at 20 °C; controls were run in parallel without any compound. Binding of hERα to the wells was finally assessed by successive exposures to an anti-ERα primary antibody, an HRP-conjugated secondary antibody and a chemiluminescent development reagent according to manufacturer's instructions. Light intensity was measured using an Infinite® 200 microplate reader (Tecan, Mechelen, Belgium). Assays were performed in triplicate and the recruitment capacities (RCs) values expressed as percentages ± SD of the optimal value established with 1 μM E_2.

2.3. Assays on MCF-7 cells

2.3.1. Cell culture

MCF-7 and MVLN cells (MCF-7 cells stably transfected with a vitERE-tk-Luc reporter plasmid [24]) were propagated at 37 °C (5% CO_2, humid atmosphere) in Earle's based minimal essential medium (EMEM; Invitrogen, Carlsbad, California) supplemented with Phenol Red, 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin (from Invitrogen) and 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT). Experiments were conducted in Phenol Red-free EMEM containing 10% charcoal-stripped FBS (estrogen-free medium, EFM).

2.3.2. Ability of compounds to confer to ERα a conformation appropriate for recruitment of an LxxLL-containing peptide

MCF-7 cells were cultured for 2 days in EFM (6-wells dishes) before treatment with increasing concentrations of investigated compounds. After treatment (usually 15 min), cell monolayers were washed twice with ice-cold PBS (40 mM Na_2HPO_4, 10 mM KH_2PO_4, 120 mM NaCl, pH 7.2) and the cells were lysed in 50 μL ice-cold RIPA (50 mM Tris–HCl, pH 7.5, 150 mM NaCl containing 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM NaF, 0.1 mM orthovanadate, 0.6 mM PMSF, 0.3 mM TPCK). Lysates were diluted by adding 200 μL of diluent buffer (reagent # 101251 of the Active Motif's ELISA kit) and clarified by fine needle aspirations followed by centrifugation. Ability of ERα from these samples (150 μL) to associate with LxxLL motif was then assessed as described above.

2.3.3. Ability of compounds to bind to ERα ("whole cell assay")

The binding of test compounds to ERα in MCF-7 cells was evaluated by a [^{3}H]E_2 competitive assay, using E_2 as a reference compound (RBA = 100%) [25]. For that purpose, cells were exposed for 45 min to 1 nM [^{3}H]E_2 alone (control) or in the presence of increasing amounts of unlabeled E_2 or investigated compounds in serum-free medium; additional cell cultures were exposed to 500-fold excess unlabeled E_2 for assessment of non-specific binding (NSB). Bound [^{3}H]E_2 was measured by scintillation counting after extraction and the data (total − NSB) expressed in percentages of the control value in order to establish RBA values (*i.e.* $[IC_{50}]_{E2}/[IC_{50}]_X \times 100$).

2.3.4. Ability of compounds to regulate ERα level

MCF-7 cells were plated in 10 cm-diameter Petri dishes (500,000 cells per dish) containing EFM. After 3 days of culture, medium was removed and cells were exposed to increasing amounts of the investigated compounds for 4 h in a fresh medium;

control cells were maintained in culture without any compound (this short time period was selected to avoid an effect on ERα synthesis) [17]. Cells were then washed with TBS (50 mM Tris, pH 7.5, 150 mM NaCl) and lysed for 30 min at 4 °C in RIPA buffer (see above). ERα levels in these lysates were finally assessed by Western blotting following a procedure described previously [17]. Briefly, ERα was detected with a mouse monoclonal antibody (F-10, Santa Cruz Biotechnology, Santa Cruz, CA). Actin, used as a loading control, was detected with mouse monoclonal antibody MAB1501R from Chemicon International (Temecula, CA). Exposure to primary antibodies was followed by incubation in presence of a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Pierce, Rockford, IL). Peroxidase activity was revealed with Supersignal West Pico Chemiluminescent substrate from Pierce. ERα and actin bands were captured with a FLA-3000 digital camera (Fuji, Tokyo, Japan). Band intensities were quantified using AIDA software (Raytest, Straubenhardt, Germany). ERα data were normalized according to actin (ERα/actin) and expressed in percent of control (untreated cells). Ability of bisphenol (# 23) to antagonize E_2-induced downregulation of ERα was further assessed by immunofluorescence microscopy using an experimental protocol described previously [26].

2.3.5. Ability of compounds to enhance ERα-mediated transactivation

MVLN cells were cultured for 3 days in 6-well plates (plating density 100,000 cells/well) containing EFM. Medium was then removed and replaced by fresh medium containing increasing concentrations of the investigated compounds; control cell cultures were run in parallel in the absence of any compound. After 24 h of incubation, luciferase activity was assayed according to a previously described procedure [17]. Efficiency of compounds was expressed as percentages of luciferase activity induced by 10 nM E_2.

3. Results

3.1. Ability of estradiol and investigated derivatives to modulate the capacity of a hERα recombinant to recruit coactivators containing LxxLL motifs

3.1.1. Importance of ligand binding affinity for ERα

Interaction of each ERα-ligand complex with LxxLL-coated plates is likely to depend on various factors, including in particular the binding affinity of the ligand for the LBD. In other words, the extent of LxxLL motif recruitment by ERα should be related to the relative ligand binding capacity, even though the measurement of LxxLL recruitment and of ligand binding rely on very different procedures. To validate this view, we checked whether the recruitment curve established with increasing amount of E_2 correlated with the $[^3H]E_2/E_2$ competition curve. As expected, both curves mirrored each other (Fig. 2) with RC_{50} (3 nM) and IC_{50} (2 nM) values in the same order of magnitude.

We subsequently assessed whether this property holds for E_2 derivatives whose functions/regions of the steroid core known to influence ERα binding had been modified. The next sections refer to this study.

3.1.2. Importance of C-3 and C-17 hydroxyl groups of estradiol

In the binding pocket of ERα, Glu-353 and Arg-394, with the participation of water molecules, interact with the 3-phenolic function of E_2 while His-524, modeled as the ε tautomer, attracts its 17β-alcoholic group (Fig. 1) [5,27]. To assess the importance of these bridging properties on LxxLL-coactivator recruitment, we tested three E_2 derivatives bearing modified OH group at these critical positions (*i.e.* $-OCH_3/-OSO_2O^-Na^+$ for 3-OH, and –H for 17β-OH; compounds # 2, 3 and 4, respectively) (Table 1). As shown in Fig. 3a and b, these substitutions largely decreased the

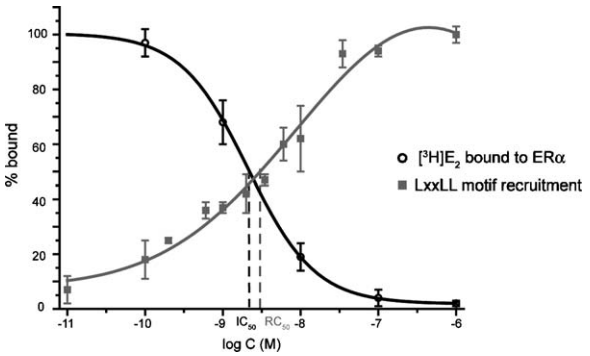


Fig. 2. Relationship between the capacity of E_2 to compete with $[^3H]E_2$ for binding to purified hERα recombinant and the binding of the receptor to the LxxLL-coated plate (recruitment). hERα recombinant was incubated with increasing amounts of E_2 and processed for each assay as described in Section 2.

capacity of the hormone to promote ERα binding to LxxLL-coated plates, reflecting the acidic role of phenol in the complex. Interestingly, stereochemistry change of the βOH at C-17 into the α configuration (# 5) decreased by 40% ERα association with LxxLL motifs while it decreased by 70% ligand binding to the receptor, stressing the importance of the hydrophobic steroidal core of the hormone in coactivator recruitment. Studies concerning substitutions in C-11 of E_2, reported below, support this view.

3.1.3. Influence of substitutions in C-11 of estradiol

The LBD is highly hydrophobic and therefore not well suited to accommodate E_2 derivatives in which an hydrogen in C-11 is substituted by a polar residue. In contrast, hydrophobic substituents are readily accepted if their size is not too large (see Table 1) and if they stabilize ERα-ligand complexes at high temperature [8].

Fig. 3c shows that the grafting of a hydroxyl in C-11 α or β of E_2 (compounds # 6α and 6β) modify its binding properties to the LxxLL-coated plate. While a decrease of the ability of hormone to promote high binding was observed at all concentrations for 11α-OH E_2, this property was solely recorded at concentrations ≥10 nM for 11β-OH E_2. The latter ligand was indeed more effective than E_2 at 1 nM indicating that regulation of LxxLL binding by the C-11 subregion of the steroid core is influenced by factors other than polarity, that antagonize its insertion within the LBD. In this regard, gas phase acidity study of these steroids has shown that 11β-OH E_2 presents a higher acidity than 11α-OH E_2 and E_2, indicating that particular electronic effects occur in 11β-OH E_2 [28]. Polarity appeared nevertheless of prime importance for LxxLL-containing co-regulators recruitment because acetylation of the 11β-OH function (# 7) resulted in a ligand able to induce "supraphysiological" receptor–LxxLL motif association.

According to this concept, hydrophobic substitutions in the C-11β stereochemistry should confer to ERα a conformation favoring extensive LxxLL recruitment. In agreement with this view, we found that $-CH_3$ (# 8), $-CH_2Cl$ (# 9) and $-C{\equiv}CH$ (# 10) substituents enhanced ligand-induced association of ERα with LxxLL motifs (Fig. 3d). Interestingly, distinct LxxLL binding profiles were recorded for these three ligands showing that the chemical nature of the substituent influence the ability of the receptor to interact with LxxLL motifs. The observation that the introduction of a double bond between C-9 and C-11 (# 11) produced a shift in ERα–LxxLL binding (as compared to the curve generated by E_2), similar to that produced by the grafting of $-CH_2Cl$ of $-C{\equiv}CH$ groups, revealed that it is the whole region around C-11 which exerts an influence on LxxLL recruitment (Fig. 3f).

In contrast to these small substituents, bulky hydrophobic side chains grafted at 11β (# 12, 13 and 14) totally abrogated the basal LxxLL binding capacity of ERα (Fig. 3e). Hence, such E_2 derivatives,

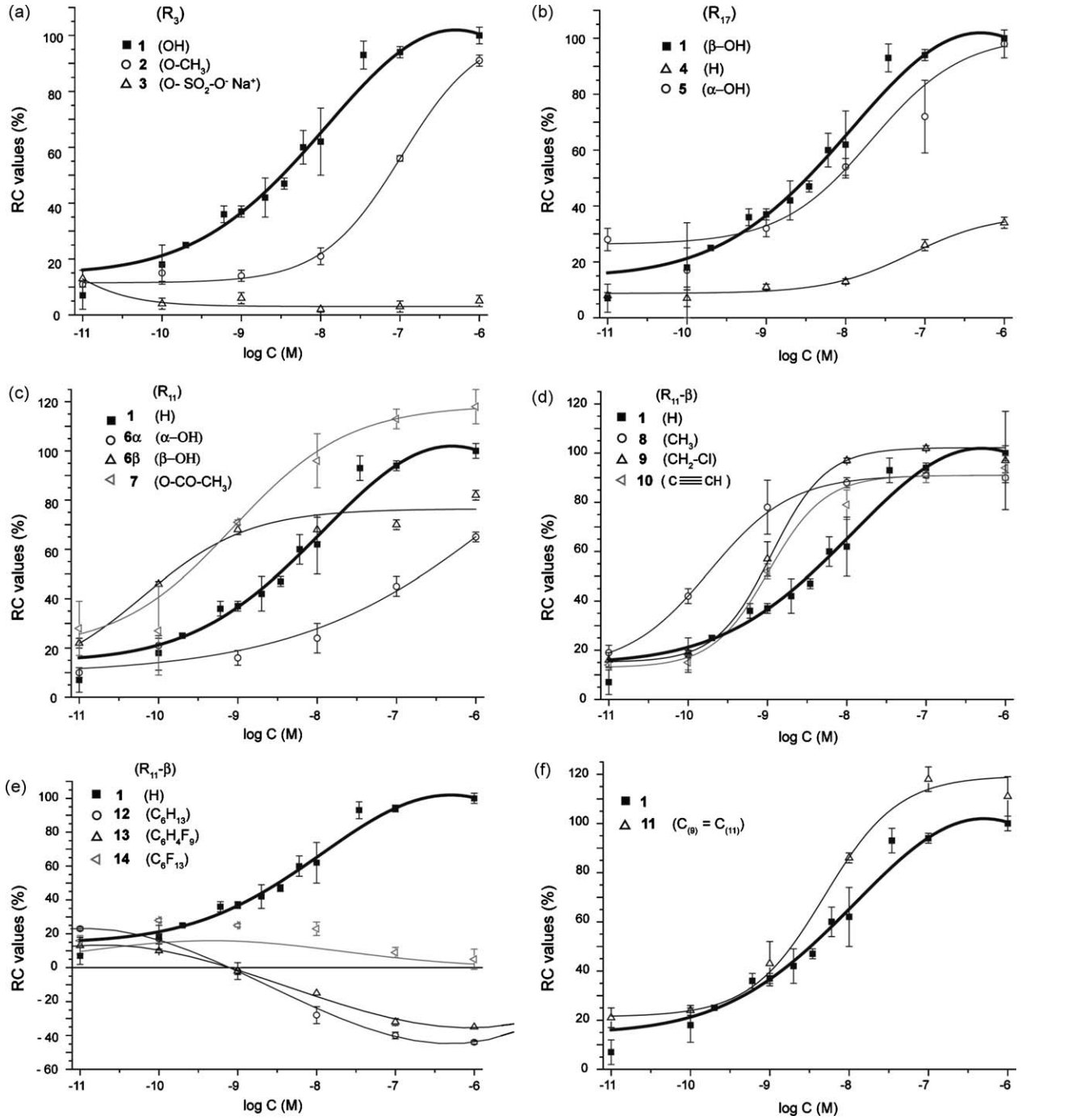


Fig. 3. Influence of substitution onto E_2 on its capacity to enhance the binding of hERα recombinant to the LxxLL-coated plate. hERα recombinant was incubated with increasing amounts of compounds and processed for LxxLL binding assay. *Hydroxyl substitutions*: panel on the left (a) refers to 3-OH, panel on the right (b) to the 17β-OH. *Substitutions at* C-11: panels refer to the linkage of (c) a polar group, (d) an hydrophobic group or (e) a long side chain. Panel (f) refers to the introduction of a double bond in C_9–C_{11} onto the steroid.

usually not well accommodated by the LBD [8,22], must be considered as LxxLL recruitment antagonists, a property confirmed by the fact that a $-C_6H_{13}$ derivative – tested as a prototype – blunted the enhancing effect of E_2 on ERα–LxxLL association.

3.2. Ability of non-steroidal estrogens to modulate the capacity of ERα to recruit coactivators containing LxxLL motifs

3.2.1. Type I ligands

Diethylstilbestrol (# 15), whose binding affinity for ERα largely exceeds that of coumestrol (# 16) and genistein (# 17) enhanced the binding of the receptor to LxxLL-coated plates with a much higher efficiency as compared to the latter compounds (Table 2 and Fig. 4), suggesting a direct relationship between ligand affinity and capacity to induce receptor–LxxLL association. Hence, "linear" estrogens, the phenolic functions of which interact with Glu-353, Arg-394 and His-524 may be assimilated to steroidal estrogens in terms of coactivator recruitment.

3.2.2. Type II ligands

None of the "angular" ligands tested in this study enhanced the binding of ERα to LxxLL-coated plates, with the notable exception of

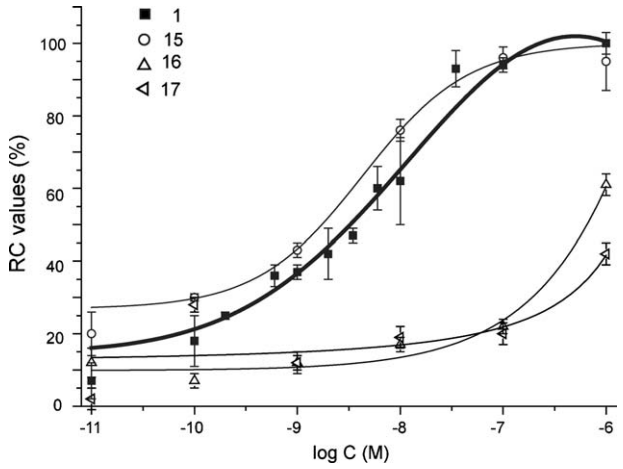


Fig. 4. Capacity of non-steroidal type I estrogens to enhance the binding of hERα recombinant to the LxxLL-coated plate. hERα recombinant was incubated with increasing amounts of compounds and processed for LxxLL binding assay.

a diphenolic imidazoline derivative (# 18) for which a weak ability to induce LxxLL recruitment had already been reported [29] (Table 2, Fig. 5a and b). Moreover, *gem* diphenolic ligands (# 21–25) antagonized ERα binding to LxxLL motifs in the presence as well as in the absence of E_2, as clearly demonstrated with bisphenol (# 23) and its analogs (Fig. 5c). This antagonism could not be ascribed to a lack of hydrogen bonding with His-524 in view of the fact that substitution of 17β-OH of E_2 by a hydrogen atom (# 4) failed to show such a property. Hence, hydrogen interaction with Thr-347 seems directly responsible of the antagonistic property of these ligands. Thus, type II angular ligands totally distinguish themselves from type I linear ligands in terms of coactivator recruitment.

3.3. Recruitment of coactivators containing an LxxLL motif by ERα in MCF-7 cells

Exposure of MCF-7 cells to E_2 produced in ERα a rapid conformational change favoring the recruitment of LxxLL-containing coactivators, as shown by measuring in cell extracts the extent of ERα binding to LxxLL-coated plates (Fig. 6). Enhanced ERα binding, which culminated within the first minutes of incubation, became undetectable after 4 h. Thus, experiments aiming to assess whether observations under cell-free conditions can be extended to living cells were based on short-term (15 min) incubations of cells with the ligands. After this treatment duration, the LxxLL recruitment curve established with increasing amounts of E_2 displayed a profile quite similar to that observed for the recombinant receptor, although the RC_{50} value was about 10 times higher (Fig. 6 and Table 3), probably reflecting the existence of cellular factors (*i.e.* endogenous receptor co-regulators) limiting the binding of ERα to LxxLL-coated plates. The fact that this property was not recorded for the hydrophobic 11β-substituted derivatives (# 9, 8 and 10) of E_2 (Table 3) suggests that the stability of ERα ∼ ligand complexes modulates the impact of these factors. In agreement

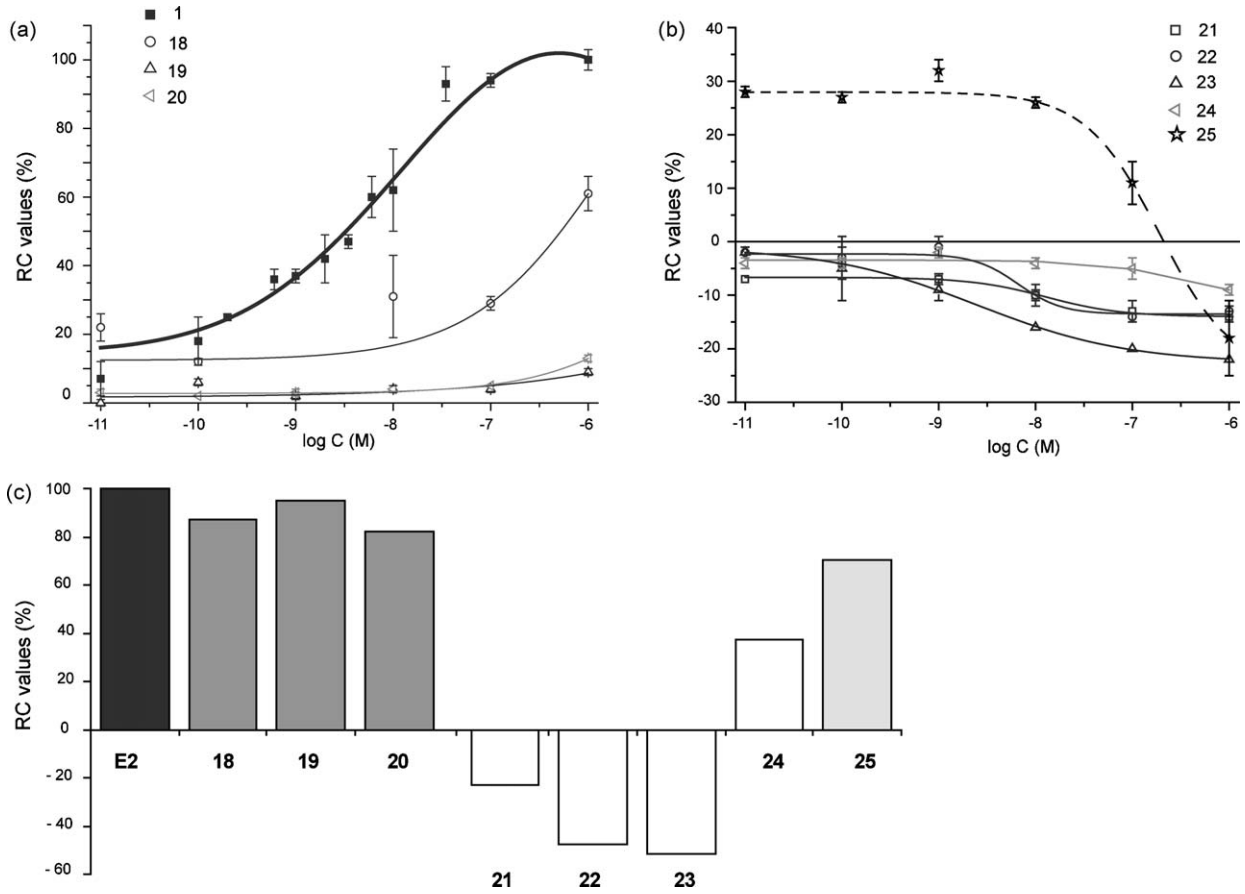


Fig. 5. Capacity of angular type II ligands to modulate the binding of hERα recombinant to the LxxLL-coated plate. hERα recombinant was incubated with increasing amounts of compounds and processed for LxxLL binding assay. Upper part: the panel on the left (a) refers to "*cis* diphenolic" compounds, the panel on the right (b) to "*gem* diphenolic" compounds. The figures evidence that former compounds are weak agonists while the latter are antagonists. Lower part (c): strong antagonists (# 21–23) at 100 nM abrogate the promoting effect of 1 nM E_2 while weak antagonists (# 24, 25) display a moderate inhibition, weak agonists (# 18–20) are ineffective.

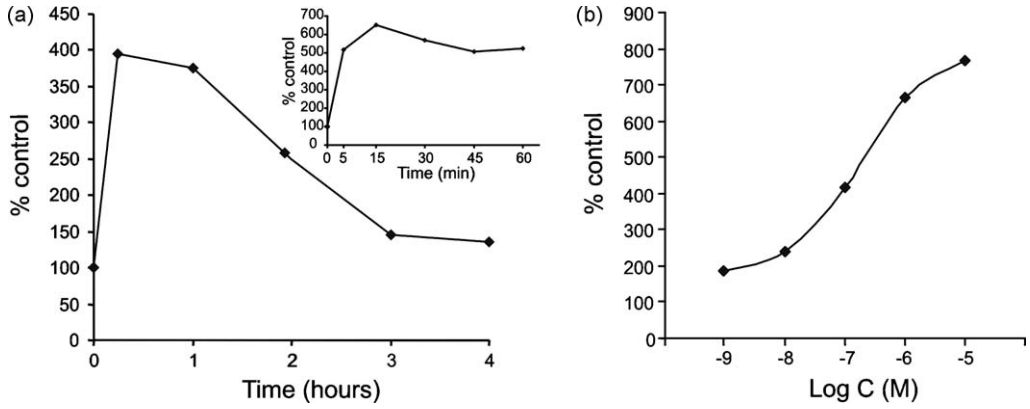


Fig. 6. Capacity of E_2 to modulate the LxxLL motif recruitment in MCF-7 cells. (a) Kinetics of recruitment: cells were incubated with 0.1 μM E_2 for various periods (from 15 min to 4 h; inset: for 5–60 min). (b) Dose-effect study: cells were treated for 15 min with increasing concentrations of E_2 (from 1 nM to 10 μM). Values are expressed as percentage of untreated cells (control). Figure refers to an experiment performed (a) twice and (b) five times (identical results).

with this view, we found that the inhibitory effect of 11β-OH E_2 on receptor–LxxLL interactions was exacerbated in cell culture experiments, leading to an almost total suppression of the recruitment capacity of the receptor. As expected, substitutions of 3 or 17β-hydroxyls of E_2 (# 2, 3, 4 and 5) also abrogated its recruitment ability (weak effect at 10 μM).

ERα from MCF-7 cells treated with DES bound to the LxxLL-coated plates like the receptor from E_2-treated cells. By contrast, other non-steroidal type I ligands had almost no effect on receptor–LxxLL interactions, most probably because of their low affinity for ERα in cultured cells (Table 3). As expected, compounds antagonizing hERα interaction with LxxLL motifs in cell-free conditions (# 12, 23 and 25) produced a similar effect in cell extracts.

On the other hand, kinetic studies of the ERα–LxxLL peptide interaction induced by E_2 and its 11β-CH_2Cl derivative revealed identical profiles. Thus, any ERα ~ ligand complexes potentially capable of recruiting LxxLL-motif coactivators progressively disappeared. This property may reflect a competition with cellular coactivators, a strong ERα association with an insoluble target, an alteration of its LxxLL binding motif or a proteasomal degradation of the receptor (these various possibilities being non exclusive) [30,31]. On the other hand, concerning the partial estrogenicity of bisphenol (# 23), we found that this compound failed to display any ability to recruit LxxLL motifs even after 4 h of cell treatment. Hence, the agonistic activity of this ligand [32] seems to be unrelated to a delayed recruitment of LxxLL motif-harboring coactivators.

3.4. Relationship between ligand-induced recruitment of coactivators containing LxxLL motif and ERα level in MCF-7 cells

Early studies from our laboratory have shown that E_2-induced depletion (downregulation) of ERα in MCF-7 cells is evident after 4 h of hormone treatment [17]. As shown in Fig. 7a, after this duration of exposure, E_2 concentrations ranging from 0.1 nM to 1 μM induced a similar extent of ERα decrease (~30–40%). Thus in the case of E_2, there was no quantitative relationship between the ligand-induced downregulation of ERα and the degree of ERα association with LxxLL motifs. This apparent absence of relationship was further illustrated by the behavior of 11β-substituted E_2 derivatives (# 9, 8 and 10) as well as the C9=C11 analog (# 11) which at 10 nM produced ERα downregulation with an efficiency similar to that of E_2, even though they were more efficient at recruiting the LxxLL motif (Fig. 7b and Table 3) (note that at higher concentrations of these compounds did not produce more pronounced downregulation). However, the low sensitivity of Western blotting (see SD of the data) could preclude the detection of small ERα level changes which might occur during

Table 3
Relationship between ligand-induced recruitment of co-regulators containing a LxxLL motif, ERα downregulation and ERE-dependent transcription.

Ligand	RBA[a]	LxxLL-recruitment[b]	ERα downregulation[c]	ERE-dependent transcription[d]
		Minimal concentration (Log C) producing ~50% change		
Steroidal				
1 (E_2)	100	**−7**	−10	−11
9	100	−10/−9	−10	−12/−11
8	100	−10/−9	−10	−11
10	100	−9	−10	−11
11	40	−9/−8	−9	−10
6β	4	>−5	−10	−11
14	1	No effect	−8	−9/−8
12	10	Antagonism	Upregulation at −8	weak increase at −10/no effect at −8
Non-steroidal				
15	80	−7	−10	−11/−10
18	<0.01	−6	−9	−9/−8
16	3	No effect	−8	−9/−8
17	1	No effect	−7	−7
23	2	Antagonism	Weak decrease at −9/no effect at −7	−9
25	0.1	Antagonism	−6	−7

[a] Binding affinity for ERα ("whole cell assay") established by measuring the capacity of a ligand to compete with [^{3}H]E_2 for uptake in MCF-7 cells.
[b] Ability of ligands to confer an ERα conformation for a LxxLL motif-containing coactivators in MCF-7 cells. Assay performed on cell extracts from cells preincubated for 15 min with the ligands.
[c] Downregulation established by Western blotting from MCF-7 cells treated with ligands for 4 h.
[d] Ligand-induced luciferase induction in MVLN cells.

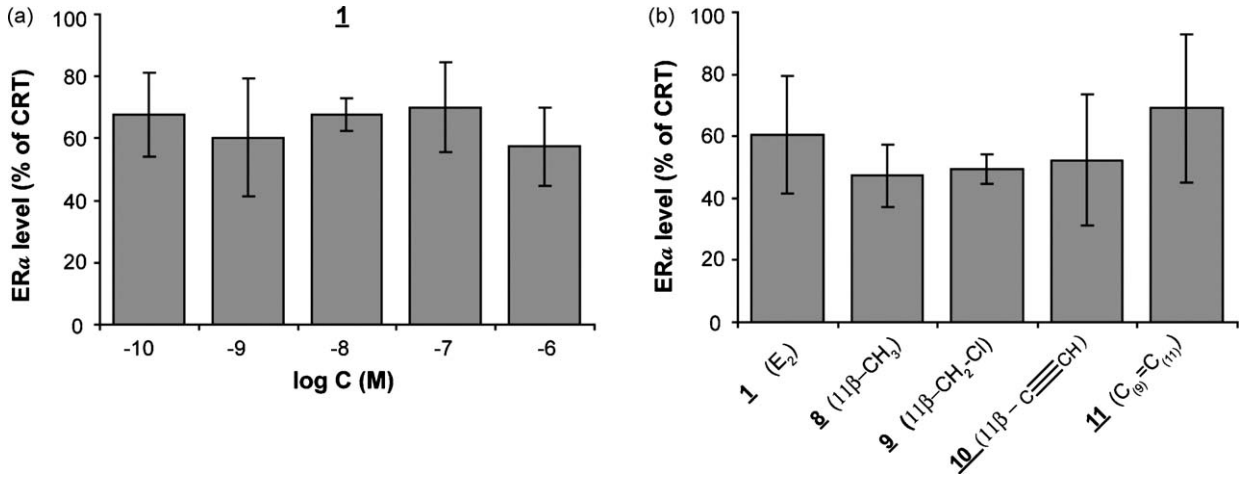


Fig. 7. Effect of E_2 and C-11β derivatives with strong LxxLL recruitment potency on ERα level in MCF-7 cells. Western blotting data established with extracts from cells incubated for 4 h with the compounds were normalized according to actin and expressed in percentage of ERα level in control, untreated cells. Panel (a) refers to increasing concentrations of E_2 and (b) to C-11β derivatives at 10 nM.

the 15-min time period used for the assessment of LxxLL binding, obscuring thereby a potential relationship between recruitment of LxxLL-containing coactivators and ERα downregulation. Indeed, because of the limited sensitivity of Western blotting, downregulation of ERα can only be evidenced after several hours of drug exposure [17]. Nevertheless, such a relationship may exist in view of the fact that, within the exception of 11α-OH E_2, compounds provoking an extensive LxxLL recruitment downregulated ERα with a higher efficiency than compounds which were less active in this respect (Table 3). This view is supported by the additional finding that a 100-fold excess of bisphenol, which impedes the recruitment of LxxLL-containing peptides (# 23) abrogated E_2-induced ERα downregulation (immunofluorescence: see Fig. 8; Western blotting: residual ERα level 43% → 108%). Note also in this context that the 11β-C_6H_{13} derivative of E_2 (# 12), which similarly antagonized this recruitment, was able to stabilize the receptor when added at concentrations ≥10 nM, producing an upregulation similar to that described for tamoxifen [17].

Unexpectedly, 11β-OH E_2 downregulated ERα with an efficiency as strong as E_2. The lack of capacity of this compound to promote an ERα conformation appropriate for the recruitment of LxxLL co-regulators, as well as its limited binding to the receptor suggest a mechanism of receptor downregulation different from that induced by other steroids.

Previous studies have revealed that cycloheximide (CHX) at 50 μM abrogates E_2-induced ERα degradation by the ubiquitin–proteasome system [33–35]. Our investigations extended this finding to all ligands able to induce LxxLL-motif recruitment (data not shown). Downregulation induced by the E_2 derivative C_6F_{13}-substituted in 11β (# 14), unable to enhance the ERα–LxxLL

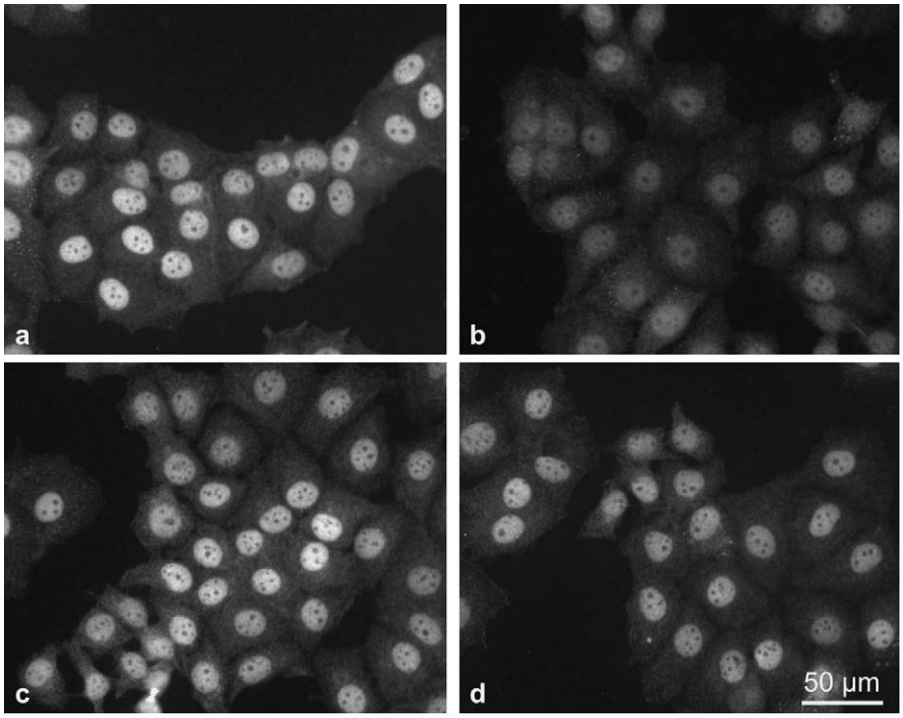


Fig. 8. Demonstration by immunofluorescence that bisphenol suppresses the down regulation of ERα induced by E_2. MCF-7 cells were exposed to drugs for a period of 6 h and processed for immunofluorescence microscopy as described in Section 2. (a) No drug, (b) 1 nM E2, (c) 100 nM bisphenol, (d) E_2 and bisphenol in combination. ERα was revealed by using polyclonal antibody HC-20 raised against the receptor. Texas Red labeling.

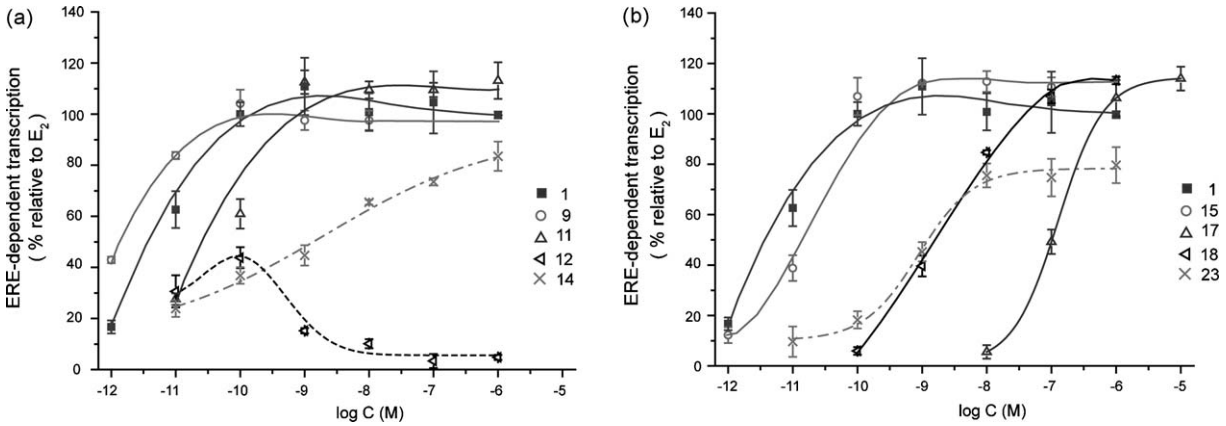


Fig. 9. Influence of (a) steroidal and (b) non-steroidal ligands on ERE-dependent transcription. MVLN cells were incubated for 24 h with increasing amounts of a set of representative ligands varying in their ability to modulate the binding of ERα in our LxxLL binding assay (—: agonists; –·–·–: no effect; – – –: antagonists). Luciferase activity was then assayed in cell extracts as described in Section 2.

peptide interaction, was also sensitive to CHX (% residual ERα level −/+ CHX: 43/95), excluding any major implication of LxxLL-coactivator recruitment in the action of CHX.

3.5. Relationship between ligand-induced recruitment of coactivators containing LxxLL motif and ERE-dependent transcription in MCF-7 cells stably transfected with a vitERE-tk-Luc plasmid (MVLN cells)

Steroidal and non-steroidal ligands able to enhance the binding of ERα to the LxxLL-coated plate increased transcription of the luciferase gene (reporter gene) with an efficiency closely related to their ability to down regulate the receptor (see Table 3 and representative compounds in Fig. 9). Hence, as already reported [18,19], proteasomal degradation of ERα appeared as an important step in ERα-mediated transcription induced by compounds sharing some structural homology with E_2. We believe, therefore, that physiological ERα-induced gene transactivation might be relevant to a process involving LxxLL-motif coactivator recruitment followed by the proteasomal degradation of the receptor.

Paradoxically, 11β C_6F_{13}-E_2 (# 14), which failed to modify the binding of ERα to the LxxLL-coated plate, and compounds that antagonized this binding (# 12, 23, 25) increased luciferase expression. This indicates that recruitment of LxxLL-containing coactivators is not absolutely required for ERE-dependent transcription (Table 3 and Fig. 9). ERα downregulation seems of higher importance in this regard since enhancement of luciferase expression was recorded at ligand concentrations which decreased receptor level. The additional observation that 11αOH E_2, which down regulated ERα by a mechanism most probably independent of LxxLL-motif recruitment, increased luciferase expression supports this view.

At concentrations which failed to decrease ERα level, 11βC_6H_{13}-E_2 (# 12) lost its capacity to enhance transcription, while bisphenol (# 23) remained able to induce transcriptional activity (Fig. 9), in agreement with the ability of these compounds to either up regulate or stabilize the receptor. The fact that bisphenol was reported to antagonize E_2-induced ERE-dependent transcription [4] clearly indicates that this compound interferes with the normal action of the hormone, most probably by abrogating the capacity of the hormone to confer a conformation appropriate for the recruitment of LxxLL-motif-containing co-regulators.

4. Discussion

The present study evaluates the ability of various ERα ligands to induce the recruitment of LxxLL-containing coactivators by the receptor and examines a possible relationship between this recruitment, ERα level and ERα-mediated gene transactivation. Our data clearly show that both steroidal and planar/linear type I estrogens enhance LxxLL motif recruitment with an efficiency related to their binding affinity. By contrast, angular type II estrogens have much less tendency to promote receptor binding to LxxLL motifs or even act as antagonists in this respect. This is in agreement with the usual classification of these non-steroidal compounds as partial estrogens/antiestrogens, as opposed to pure antagonists which exclusively abrogate the recruitment of LxxLL-containing coactivators [36].

The insertion of E_2 (and probably of most type I estrogens) into the LBD of ERα provokes a rapid shift of its helix H12 which seals the ligand binding cavity [5]. In this new conformation, H12 covers the binding pocket and plays a major role in stabilizing ligand-receptor complexes. In contrast, type II estrogens behave differently inasmuch as they maintain the pocket in an "open conformation", this resulting in less stable complexes. The latter conformation being generated by ligands belonging to various chemical families, one may reasonably postulate that it is associated with a large panel of H12 helix orientations explaining the agonistic/antagonistic profile of these compounds in terms of LxxLL-coactivator recruitment.

Hydrogen bridges involved in E_2 binding (3-OH and 17β-OH), which contribute to the displacement of H12 [5], are found here to be essential for optimal LxxLL recruitment. We show in this study that small hydrophobic substituents in C-11β, as well as the introduction of a double bound between C-9 and C-11, enhance the recruitment of LxxLL motifs. This observation suggests that hydrophobic interactions between the LBD and substituents around the C-11 position of the ligand strengthen the affinity of the receptor toward LxxLL coactivators. Polar residues, as well as long hydrophobic side chains not readily accommodated by the LBD largely limit this promoting effect. However, the 11β-OH E_2 shows an unexpected behavior that could be relevant to electronic effects [28]. In fact, the nature of the interactions between ligand substituents and the LBD most likely influence the affinity of ERα for coactivators since (–CH_3) *versus* (–CH_2Cl and –C≡CH) gave distinct LxxLL binding profiles. Hence, steric hindrance and/or electronic delocalization may also play a role. In this context, it should be stressed that C-11β hydrophobic substituents often increase E_2 binding to ERα at 25 °C [8]. This property, most probably due to a higher plasticity of the LBD at this temperature, was associated with an increase (–CH_3, –CH_2Cl, –C≡CH) or a decrease (–C_6H_{13}) of LxxLL recruitment capacity, stressing its relevance to the agonistic as well as antagonistic character of a ligand.

Stereochemistry change of βOH at C-17 of E_2 significantly decreases its binding affinity for ERα. Our data reveal that such a change would not markedly affect the ability of the hormone to recruit LxxLL-containing coactivators, in agreement with the known estrogenicity of 17α-E_2. On the contrary, α/β configuration of a hydroxyl at C-11 strongly affects the recruitment of a LxxLL motif in a way closely related to the decrease of binding affinity.

According to our data, Glu-353/Arg-394 and His-524, which contribute to the binding of type I ligands through hydrogen bonds, appear to be similarly implicated in LxxLL-coactivator recruitment. This property does not hold for Thr-347, even though this residue participates to the interaction of type II ligands with the LBD. Indeed, bisphenol displays an evident antagonistic activity on LxxLL motif recruitment. Thus, H-bonds between the aforementioned residues and the phenol/alcohol groups of these two classes of ligands govern the coactivator recruitment capacity of the receptor. It should be noted in this context that the spatial orientation of these hydroxyl groups is also of importance, as revealed by the distinct LxxLL recruitment capacity of steroidal estrogens and the type I estrogens (e.g., genistein and coumestrol).

Influence of substitutions in position 11β of E_2 on LxxLL-motif recruitment could not similarly be explained because of the lack of information concerning the interactions of such substituents with specific residues of the LBD. While their insertion in the subpocket containing Thr-347 may be advocated, a "7α subpocket" could not be excluded in view of the fact that E_2 may rotate around a 3OH–17βOH axis [37]. This 7α subpocket, essentially hydrophobic, is known to accept alkyl substituents which generate ligands with RBA values overpassing that of E_2 [38]. Hence, this mode of insertion within the LBD appears particularly well suited for our $-CH_3$, $-CH_2Cl$ and $-C{\equiv}CH$ derivatives of E_2, in contrast to the Thr-347 insertion mode which is more appropriate for ligand with aromatic substituents [38]. Hydroxyl groups being hardly accepted by this 7α subpocket, 11β-OH would be rejected away from the latter with a concomitent decrease of the capacity of ERα to recruit LxxLL motifs.

The acquisition of an ERα conformation appropriate for LxxLL motif recruitment seems to be necessary for its proteasomal degradation in MCF-7 cells. This property does not hold for ERE-dependent gene transactivation, since bisphenol, which antagonizes LxxLL motif recruitment, enhances the transcription of reporter genes at concentrations which upregulate the receptor. The fact that this ligand suppresses the stimulatory effect of E_2 on LxxLL motif recruitment by ERα suggests that its ability to enhance transactivation in estrogen-free conditions results from a mechanism involving a distinct class of coactivators. Another possibility would be the induction of intermolecular interactions *via* the expression of LxxLL-like motifs, which would generate a competitive inhibition in our LxxLL binding assay. To our knowledge, this model, which explains the antagonistic property of SERMs (*i.e.* the LLEML motif of H12 that fits between H3 and H5 [39]), has never been proposed in the context of type II agonists probably because of the lack of any description of auto-activating mechanism of ERα.

In agreement with previous observations, CHX was found to systematically abrogate the proteasomal degradation of ERα induced by the ligands which enhanced ERE-dependent transcription in MCF-7 cells, even the 11β-C_6F_{13} derivative of E_2 which failed to promote LxxLL-motif recruitment. Hence, the absence of CHX effect on the downregulation of ERα induced by the pure antiestrogen RU 58668 [34,35] which contains a functionalized side chain in C-11β like this C_6F_{13} derivative, would be related to the antiestrogenic activity of this compound. This interpretation similarly holds for the ERα downregulation induced by ICI 182,780, which is also insensitive to the action of CHX [35]. All these considerations indicate that newly synthesized protein(s) operate in concert with the activated receptor to induce its degradation, in a process independent of LxxLL-containing coactivator recruitment.

In MCF-7 cells, E_2-induced LxxLL-coactivator recruitment was fast and transient. Although the underlying mechanism has not been investigated as yet, this observation is consistent with the existence of repeated cyclic interactions between ERα and cognate partners during target gene transactivation, a concept developed by several authors [18,19,30,40,41]. Whether the membrane/cytoplasmic receptor pool, which participates to early estrogenic responses [31,42,43], is implicated in this dynamic LxxLL-coactivator recruitment is unknown. Investigating such a possibility seems to us of primary importance. Use of ligands that selectively interact with this pool [44–47] might be an adequate approach to initiate such a study.

In conclusion, our study shows that ERα-mediated transcription induced by type I estrogens depends upon LxxLL-coactivator recruitment. In contrast, type II estrogens are largely less dependent of this recruitment, a fact which may explain their mixed estrogenic/antiestrogenic properties. Moreover, same hydrophilic and hydrophobic interactions appear to govern the stability of receptor-ligand complexes, the attraction of LxxLL motifs and associated biological responses.

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