Effects of (R,S)/(S,R)-4,5-bis(2-chloro-4-hydroxyphenyl)-2-imidazolines and (R,S)/(S,R)-2,3-bis(2-chloro-4-hydroxyphenyl)piperazines on estrogen receptor alpha level and transcriptional activity in MCF-7 cells

Ioanna Laïos a, Anny Cleerena, Guy Leclercqa,*, Denis Nonclercqb, Guy Laurentb, Miriam Schlenkc, Anja Wellnerc, Ronald Gustc

a Laboratoire J.-C. Heuson de Cancérologie Mammatiere, Université Libre de Bruxelles (U.L.B.), Institut Jules Bordet, Rue Héger-Bordet, 1 - 1000 Brussels, Belgium
b Laboratory of Histology and Experimental Cytology, Faculty of Medicine and Pharmacy, Université de Mons-Hainaut, Pentagone 1B, 6 Avenue du Champ de Mars, 7000 Mons, Belgium
c Institute of pharmacy, Free University of Berlin, Königin-Luise Strasse, 2+4, D-14195 Berlin, Germany

1. Introduction

Gene expression proceeds according to a well-defined program characterized by the formation of transient molecular complexes, each involved in a specific step of the transcriptional cycle. When bound to cognate response elements (ERE, PRE, GRE...) nuclear receptors recruit regulatory proteins until their conformation becomes inappropriate to accomplish a...
correct transcriptional cycle. Proteasomal degradation of such “senescent” receptors palliates this situation: it favors the action of neosynthesized receptors capable of accomplishing subsequent transcriptional cycles required for appropriate gene expression. Hence, the rate at which a receptor is synthesized and degraded is a factor of major importance for the onset of biological responses [1–5].

Current studies in our laboratories concern the estrogen receptor α (ERα), the importance of which is now well recognized in the development and treatment of breast cancer [6]. In the course of recent years, we have been focusing on the influence of estrogenic and antiestrogenic ligands on ERα turnover rate in the MCF-7 breast cancer cell line (receptor half-life: 3–4 h in the absence of ligand-induced stimulation). We found that most ligands decrease ERα half-life, except partial antagonists (i.e. tamoxifen, raloxifene) that stabilize ERα [7,8]. Ligand-induced conformational changes of ERα, established from X-ray crystallographic data, provide a molecular basis for this finding [9–12].

As yet, two main binding modes, each induced by a particular class of ligands, have been identified [13]. Planar agonists with hydroxyl groups separated by a distance of about 10.9–12.0 Å (i.e. 1,3,5-estratrienes, DES, genistein, ...), now classified as type I estrogens [13], are locked within the ligand binding domain (LBD) in a “closed” conformation that engulfs the ligand [9]. Partial antagonists derived from these compounds and bearing a basic side chain, e.g. raloxifene, maintain the binding pocket quite “open” [9,10]. Three residues, Glu-353, Arg-394 and His-524 contribute to the attachment of these ligands within the LBD by establishing H-bonds with the hydroxyl groups. 4-Hydroxytamoxifen (OH-Tam), as a further representative of this kind of antagonists, does not form H-bridges to His-524 when entrapped within the LBD. Only the 4-hydroxyphenyl residue of this compound is involved in H-binding, being H-bound to Glu-353 and Arg-394 while hydrophobic interactions of its C2-phenyl ring enhance complex stability. Hence, the anchorage of a ligand into the receptor LBD is achieved by various interactions (H– bond, van der Waals interactions) which most probably confer a large spectrum of conformations leading to the recruitment of different sets of co-regulators.

4,5-Diaryl-2-imidazolines and 2,3-diarylpiperazines (see Scheme 1) belong to so-called type II estrogens [13] because of the angular arrangement of their 1,2-diarylethane pharmacophore [14–17]. These compounds enhance ERα-mediated transactivation of ERE-driven reporter genes, but in contrast to

**Scheme 1**
most investigated agonists they do not at all compete with \([^{3}H]\) estradiol (E\(_{2}\)) in traditional binding assays [14–17]. This property is a consequence of the angular structure of these ligands: hydroxyl groups in the aromatic rings (O–O distance between 5.2 and 7.9 Å) cannot engage simultaneous interactions with Glu-353/Arg-394 and His-524, a condition which must be fulfilled for the displacement of E\(_{2}\) from its binding site. Supposing that Glu-353/Arg-394 are involved in the anchorage of the type II estrogens in the LBD, theoretical modeling studies identified Asp-351 and Thr-347 as potential alternative anchors for H– bonds [12].

Whether 4,5-diaryl-2-imidazolines (Im\(_{a}\)) and 2,3-diarylpipe-razines (Pip\(_{a}\)) regulate transcription by modifying ER\(_{a}\) turnover rate like conventional ligands is a question of prime importance, e.g. for understanding the mechanisms of action of ER\(_{a}\). This issue was addressed in the current studies conducted on MCF-7 breast cancer cells [18,19].

### 2. Materials and methods

#### 2.1. Chemicals and drugs

Investigated Im\(_{a}\) and Pip\(_{a}\) were synthesized according to [14]. \([^{3}H]\)E\(_{2}\) (\(\sim 100 \text{Ci/mM}\)) was purchased from GE Healthcare (Diegem, Belgium). E\(_{2}\) and OH-Tam were from Sigma (St Louis, MO), fulvestrant from Tocris Cookson (Bristol, UK), MG-132 from Calbiochem (La Jolla, CA). These chemicals as well as Im\(_{a}\) and Pip\(_{a}\) were solubilized in ethanol and stored at 4 °C. At time of experiments, these stock solutions were diluted in the culture medium to maintain ethanol at a maximal concentration of 0.1%.

#### 2.2. Human ER\(_{a}\) recombinant

Highly purified human ER\(_{a}\) recombinant (Calbiochem, San Diego, CA) was diluted (\(\sim 2.5 \text{pmole/ml}\)) in 50 mM Tris–HCl pH 7.5 containing 1 mg/ml BSA at time of receiving. Diluted samples were stored at –20 °C.

#### 2.3. Cell culture

MCF-7 cells were cultured at 37 °C in a cell incubator with humid atmosphere at 5% CO\(_{2}\) in Minimum Essential Medium (MEM, In Vitrogen, Carlsbad, California) supplemented with Phenol Red, 10% fetal bovine serum (FBS, HyClone, Logan, Utah), 2 mM l-glutamine, 100 U/ml penicillin G, 100 \(\mu\)g/ml streptomycin (In Vitrogen, Carlsbad, California). Experiments were conducted in Phenol Red-free MEM supplemented with 10% charcoal-stripped FBS (estrogen-free medium, EFM) as specified below.

#### 2.4. Influence of Im\(_{a}\) and Pip\(_{a}\) on ER\(_{a}\) transcriptional activity

##### 2.4.1. Luciferase assay with MCF-7 cells stably transfected with the reporter plasmid ERE\(_{a}\)luc\(_{+}\) (MCF-7-2a cells)

Assay was described earlier [20]. MCF-7-2a cells were seeded in 6-well plates containing EFM. After 24 h, Im\(_{a}\) and Pip\(_{a}\) were added to achieve concentrations ranging from 0.1 nM to 10 \(\mu\)M, E\(_{2}\) (0.1 pM to 10 \(\mu\)M) was used as reference. Fifty hours later, cells were lysed for the measurement of luciferase activity (Luciferase Assay System, Promega, Madison, WI). Induced light was expressed in relative light units (RLU). Protein content of each extract was measured and RLU were expressed per mg protein.

##### 2.4.2. Luciferase assay with U-2 OS cells transiently transfected with a plasmid encoding for ER\(_{a}\) (pSG5-ER\(_{a}\)) and the reporter plasmid (ERE\(_{a}\)luc\(_{+}\))

Assay was previously described [17]. Cells from an almost confluent monolayer were split and seeded in 10 cm Ø Petri dishes at a concentration of \(1 \times 10^{6}\) cells per dish prior to transfection. Transient transfection of the cells with 0.05 \(\mu\)g of receptor plasmid pSG5-ER\(_{a}\) and 5 \(\mu\)g of the reporter plasmid (ERE\(_{a}\)luc\(_{+}\)) was carried out using Fugene6 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. After 24 h the cells were treated for 21 h with either E\(_{2}\) or test compounds in appropriate amounts to achieve final concentrations ranging from 0.1 pM to 10 nM (E\(_{2}\)) or 0.1 nM to 10 \(\mu\)M (Im\(_{a}\) and Pip\(_{a}\)). The cells were then lysed and luciferase activity assayed as described above.

#### 2.5. Influence of Im\(_{a}\) and Pip\(_{a}\) on ER\(_{a}\) level and \([^{3}H]E_{2}\) binding capacity in MCF-7

##### 2.5.1. ER level

#### 2.5.1.1. Western blotting. MCF-7 cells were plated in 10 cm Ø Petri dishes (500,000 cells per dish) containing EFM. After 3 days of culture, medium was removed and cells were exposed to Im\(_{a}\) and Pip\(_{a}\) (0.1 and 1 \(\mu\)M) for 24 h in a fresh medium with or without serum; control cells were maintained in culture without any compound (Control) or exposed to 10 nM E\(_{2}\). Cell cultures were then washed with TBS (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) and lyzed for 30 min at 4 °C in a lysis buffer (TBS with 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 clarified by fine needle aspiration and sonication (5 min at 4 °C) followed by a centrifugation (13,000 g, 20 min, 4 °C). The protein concentration of each sample was determined using BCA Protein Assay kit. After addition of loading buffer (LDS Sample 4 \(\times\) buffer from In Vitrogen, Carlsbad, CA) proteins were boiled for 5 min. Each sample (15 \(\mu\)g) was then loaded onto 4–12% SDS polyacrylamide gel and subsequently, electro transferred onto a nitrocellulose membrane (Amersham Biosciences, Roosendaal, NL). Non-specific sites were blocked with 5% non-fat dry milk in TBS–0.05% Tween 20 (3 h, room temperature). ER\(_{a}\) detection was performed with a...
immunoglobulins (EnVision™, Dako Belgium, Heverlee, Belgium) and a Dextran polymer conjugated 595 at the carboxy terminus of human ER (rabbit polyclonal antibody HC-20 raised against residues 576–595 at the carboxy terminus of human ER)

2.5.1.2. Immunofluorescence microscopy. MCF-7 cells in EFM were plated at a density of 10,000 cells/cm² on sterile round glass coverslips in 12-well dishes. Two days after seeding cell cultures were fed with fresh EFM containing E₂ (1 nM) and selected Im₂ (Im-N-Et (2) or Im-N-EtOMe (2bis), both at 1 μM), with or without MG-132 (10 μM). Cells were treated for 6 h with E₂ and imidazoline derivatives. Treatment with MG-132 was initiated 1 h before addition of the latter compounds. At the end of treatment, cell monolayers were washed with Dulbecco’s PBS and fixed with 4% paraformaldehyde in the same buffer. Following fixation, paraformaldehyde was changed for DPBS where cell cultures were stored at 4 °C until immunostaining. Demonstration of ERα by immunofluorescence was achieved as detailed in a previous publication [8]. In brief, cells monolayers were washed several times with PBS (PBS, 40 mM Na₂HPO₄, 10 mM KH₂PO₄, 120 mM NaCl, pH 7.2) containing 0.2% Triton X-100. For all subsequent incubation and washing steps Triton X-100 was included in buffer to ensure cell permeabilization. Cells were successively exposed to the following reagents: the primary antibody (rabbit polyclonal antibody HC-20 raised against residues 576–595 at the carboxy terminus of human ERα, Santa Cruz Biotechnology, Santa Cruz, CA), a Dextran polymer conjugated with both peroxidase and antibodies raised against rabbit immunoglobulins (EnVision™, Dako Belgium, Heverlee, Belgium), biotinylated swine anti-rabbit immunoglobulins antibody (Laboratory of Hormonology, Marloie, Belgium), a rabbit anti-peroxidase antiserum (Laboratory of Hormonology, Marloie, Belgium), biotinylated swine anti-rabbit immunoglobulins antibody (Dako) and Texas Red conjugated streptavidin (Vector Laboratories, Burlingame, CA). After final washing in PBS, the coverslips were mounted on glass slides using commercial anti-fading medium (Vectorshield®, Vector Laboratories). The cell preparations were examined on a Leitz Orthoplan microscope equipped with a Ploem system for epi-illumination. Excitation wavelength of 560 nm and emission wavelength of 590 nm were used for the observation of Texas Red fluorescence. The appearance of fluorescence was achieved as detailed in a previous publication [8].

2.5.2. [³H]E₂ binding capacity (whole cell assay)

MCF-7 cells were cultured for 2 days in 24-well plastic dishes under standard growth conditions (40,000 cells/well). Medium was then replaced by serum-free MEM, containing Im₂ and Pip₂, at 0.1 and 1 μM with or without MG-132; control cells were maintained in this medium without any compound. After a short (15 or 30 min) and prolonged (3 h) incubation, the medium was replaced by a fresh one containing [³H]E₂ at 1 nM for evaluating the influence of the compounds upon the capacity of ERα to bind [³H]E₂ (45 min of incubation; non-specific binding was established by a parallel incubation with a 500-fold excess of unlabeled E₂). Cells were then washed twice with PBS and the bound E₂ extracted from the monolayer with 250 μl ethanol at −20 °C (1 h). Aliquots of 200 μl were then transferred to scintillation vials containing 3.8 ml scintillation counter Ecoscint H (National Diagnostic, Atlanta, GE) for radioactivity counting. Specific [³H]E₂ accumulation in cells was calculated from the difference in cell-associated radioactivity after incubation in the absence or presence of unlabeled E₂.

For kinetic analysis, instead of a single concentration of [³H]E₂, increasing concentrations (0.05–1 nM) of the labeled hormone were used for assessing the effect of the compounds on E₂ binding parameters (Scatchard plot analysis).

2.6. Ability of Im₂ to confer an ERα conformation appropriate for the recruitment of LxxLL-containing co-activators (ERα ELISA NR peptide)

Binding of ERα to an LxxLL containing peptide was assessed with an ERα Elisa NR peptide kit (Active Motif, Rixensart, Belgium). Briefly, highly purified ERα recombinant (10 ng in the diluted buffer) was incubated at 4 °C either with E₂ at 1 nM, fulvestrant at 0.1 μM or selected Im₂ (2 or 2bis both at 0.1 and 1 μM) for 30 min. Samples were then added to LxxLL coated wells; controls were maintained in the absence of any compound. In the whole assay, binding of ERα to the wells was measured by colorimetry (difference between absorbance at 450 and 600 nm) using anti-ERα primary antibody and HRP-conjugated secondary antibody according to manufacturer’s instructions. The same experiment was performed with a MCF-7 nuclear extract (from Active Motif; 12.5 μg in diluted buffer).

2.7. Statistical analysis

Data were reported as means ± S.D. and statistical analysis was performed by ANOVA. Dunnett’s post hoc test was used to compare treated conditions to the untreated condition (control) and Tukey’s post hoc test was performed for multiple comparisons between groups. The level of statistical significance was arbitrary set at 0.05. All analyses used SPSS software (Paris, France).

3. Results

3.1. Enhancing effect of selected Im₂ and Pip₂ on ERE-dependent transcription

Compounds listed in Table 1 were selected to conduct our experimental program aimed at examining whether the transactivation properties of Im and Pip estrogens are associated with a change of ERα turnover rate in MCF-7 cells. Reported capacity to induce expression of target genes in U2 OS and MCF-7-2a cells (previous studies conducted in Berlin [14–17]) oriented the selection of experimental compounds.
However, in order to avoid any inappropriate extrapolation of such studies, ERα mediated transcription induced by the compounds under investigation was tested again under conditions close to the one used for assessing ERα degradation. MCF-7 cells stably transfected with a luciferase reporter construct (Vit-tk-Luc, MVLN cells [21]) were used for that purpose.

Concentrations of Imα and Pipα which could produce a significant effect according to reported data [14–17] (i.e. 0.1–1 μM) were chosen. When given to MVLN cells at these concentrations, selected Imα and Pipα enhanced luciferase gene expression with an efficiency matching EC50 values established in U2 OS and MCF-7-2a cells (Table 1) (these values were unknown at the time of experimentation to satisfy single blind conditions).

The (R,S)/(S,R)-4,5-bis(2-chloro-4-hydroxyphenyl)-2-imidazoline 1 induced ERE-dependent transcription in these three cell culture systems. An optimization of the effect of this compound was achieved by N-alkylation, thus confirming the paramount importance of hydrophobic interactions in the binding of this type of ligand [15,17]. The N-ethyl substituted imidazoline 2 displayed the highest recorded potency to induce the expression of the luciferase gene. N,N'-diEt 3 showed nearly the same efficacy as Im 1, although it represents a permanent cationic structure. Essential contacts within the LBD appear, however, performed by H-bonds comparable to those produced by other estrogens. O-Methylation of Im-N-Et 2 resulted, indeed, in a compound unable to significantly enhance luciferase gene expression (Im-N-EtOMe 2bis). Substituents at C2 of Im 1 (C2-Et 4, C2-PhOH 5) also strongly decreased transactivation capacity. The reason of this observation is quite unclear, but it might be the consequence of steric repulsions resulting in an inadequate orientation within the LBD.

(R,S)/(S,R)-2,3-bis(2-chloro-4-hydroxyphenyl)piperazines were less active than the respective 2-imidazolines. Ethylation of Pip 6 enhanced its effect on luciferase gene expression although to a largely lower extent than that found with Imα, (N-Et 7 > N,N'-diEt 8). Surprisingly and in contrast to all other compounds, Pip 6 elicited a same extent of transactivation at 0.1 and 1 μM suggesting that it had reached its maximal activity at the lower concentration. Observed toxicity of Pipα, which might limit gene transcription at high drug concentration, could explain this paradoxical observation.

| Table 1 – ERE-dependent transcription in various cell culture systems |
|-------------------------|------------------------|-------------------------|------------------------|
| Compound | EC50 [μM] or % E2 induction at 10 μM | MCF-7-2a, stably transfected with the reporter plasmid (ERE)2Luc+ | MVLN, stably transfected with the reporter plasmid pVit-tk-Luc (% E2 induction)* |
| Estradiol | 2.0 x 10⁻⁶ | 3.8 x 10⁻⁵ | 100 | 100 |
| Im [1] | 0.029 | 0.63 | 28 | 80 |
| Im-N-Et [2] | 0.0038 | 0.015 | 90 | 94 |
| Im-N-EtOMe [2bis] | 17% | 10% | 9 | 18 |
| Im-N,N'-diEt [3] | 0.011 | 0.62 | 66 | 95 |
| Im-C2-Et [4] | 0.14 | 29% | 5 | 15 |
| Im-C2-PhOH [5] | 2.2 | 20% | 4 | 0 |
| Pip [6] | 3.9 | 32% | 32 | 32 |
| Pip-N-Et [7] | 0.051 | 0.21 | 13 | 73 |
| Pip-N,N'-diEt [8] | 0.10 | 15% | 14 | 32 |

The table confronts data established by the German and Belgian teams (i.e. U2 OS and MCF-7-2a cells vs. MVLN cells) according to specific experimental protocols described in Section 2.

* Induction potency: (RLUx<sub>RLUCTR</sub>/RLUE2) < 0.05 vs. control Dunnett’s post hoc test (data refer to the means of four values from two independent experiments).

ERα immunoblotting performed on lysates from MCF-7 cells treated with investigated Imα and Pipα, at 0.1 and 1 μM revealed that these compounds are able to cause receptor down regulation like conventional agonists (Figs. 2 and 3A). N-Ethylation (2 versus 1 and 7 versus 6) which had the strongest impact on ERα-mediated transactivation had also the most marked effect on receptor level. The same result was obtained with N,N'-diethylation (3 versus 1 and 8 versus 6), albeit to a lower extent. This suggested a close relationship between ERα-mediated gene transactivation and receptor down regulation. In agreement with this view, substitutions that decreased the ability of Imα and Pipα to stimulate transcription suppressed the down regulating effect of ligands on ERα level (2bis, 4 and 5). Of note, in cells exposed to Im-N-Et (2) and its O-methylated

**3.2. Ability of Im and Pip derivatives to down regulate ERα**

ERα immunoblotting performed on lysates from MCF-7 cells treated with investigated Imα and Pipα, at 0.1 and 1 μM revealed that these compounds are able to cause receptor down regulation like conventional agonists (Figs. 2 and 3A). N-Ethylation (2 versus 1 and 7 versus 6) which had the strongest impact on ERα-mediated transactivation had also the most marked effect on receptor level. The same result was obtained with N,N'-diethylation (3 versus 1 and 8 versus 6), albeit to a lower extent. This suggested a close relationship between ERα-mediated gene transactivation and receptor down regulation. In agreement with this view, substitutions that decreased the ability of Imα and Pipα to stimulate transcription suppressed the down regulating effect of ligands on ERα level (2bis, 4 and 5). Of note, in cells exposed to Im-N-Et (2) and its O-methylated
form (2bis) – taken as prototypes of active and inactive compounds, respectively – the examination of ERα after immunofluorescence staining gave results similar to those obtained by Western blotting (Fig. 3B), ruling out the possibility of artifacts in the latter approach.

As revealed by ERα immunoblotting as well as by ERα immunofluorescence staining, the down regulation of the receptor caused by active Im and Pip derivatives was abrogated by the proteasome inhibitor MG-132 (Figs. 2 and 3), as usually found with E2 [8]. This indicated that ERα decrease resulting from exposure to these compounds occurs via the ubiquitin–proteasome pathway, similar to the down regulation induced by conventional ligands.

3.3. Ability of compounds to modulate ERα binding capacity

At 0.1 and 1 μM, pre-exposure of MCF-7 cells to Im and Pip decreased the capacity of ERα to bind [3H]E2 (whole cell binding assay; Fig. 4A, upper panel). In this regard, Im-N-Et2 was the strongest modifier again. This loss of estrogen binding capacity could not be ascribed to a proteosomal degradation of the receptor since it persisted in the presence of MG-132 (Fig. 4A, lower panel). Moreover, it occurred within the first hour of treatment, thus largely before ERα agonist-induced down regulation (Fig. 5). Any residual effect of compounds incompletely eliminated by culture rinsing before assay was highly unlikely since no major decrease of [3H]E2 accumulation was recorded when the cells were incubated with the labeled hormone in presence of investigated Im and Pip. Actually, only a 1000-fold excess of 7 and 8 produced a loss of [3H]E2 labeling (Fig. 4B). Non-specific interference of these two compounds in the assay might be advocated to explain this
property since they did not markedly differ from other Im and Pip derivatives with similar transactivation potency and ability to regulate ER level.

Measurement of [3H]E2 binding parameters by Scatchard plot analysis of MCF-7 cells preincubated with the strongest modifier Im-Et2 (1 μM) revealed that this compound slightly affected the binding affinity of the receptor for the labeled hormone (increase of \(K_d\) value), as well as its binding capacity (decrease of the number of binding sites) (Table 2). A similar effect was also recorded for E2 (0.1 nM), used as a reference. Hence, the decrease of estrogen binding capacity induced by Im and Pip derivatives is most probably related to an ERα conformational change like that produced by E2, even if these compounds interacted with other residues than those involved in the binding of the hormone to the LBD. The assessment of the ability of Im and Pip to confer a conformation suitable for interaction with the LxxLL motif of co-activators confirmed this statement.

### 3.4. Ability of Im-N-Et 2 to confer an ERα conformation appropriate for the recruitment of LxxLL co-activators

Ability of the strongest agonist Im-N-Et 2 to promote the recruitment of LxxLL-containing co-activators was analyzed by an ELISA based procedure (Fig. 6). Incubation of a highly purified preparation of ERα recombinant (hER) with 2 (1 μM) increased its binding to the LxxLL coated plate, as \(E_2\) did at 10 nM. O-methylated 2bis was totally ineffective in this regard. In each case, specific cellular accumulation of [3H]E2 was established by an incubation with a 500-fold excess of unlabeled \(E_2\). Data refer to means of four values ± S.D. from two independent experiments.
could be ascribed either to the presence in the nuclear extract of an activated ERα pool able to interact with LxxLL motifs in a ligand-independent manner or a potential interference of endogenous LxxLL co-regulators. Of note, 2 failed to enhance binding at 0.1 μM while it stimulates ERα transactivation at this concentration (Table 1). Such a lack of congruence has already been described between assays performed with whole cells and assays performed with cell extracts or recombinant receptor. Actually, ER-mediated responses (i.e. growth rate change, reporter gene transcription, receptor level variation) in intact cells often occur at concentrations lower than would be expected from estrogen binding assays. Absence of the entire ERα machinery in the latter systems may explain this property.

Regardless of the nature of ERα preparation, fulvestrant (1 μM) abrogated the enhancement of LxxLL binding induced by 2, confirming that this compound could change the receptor conformation like E2. This property of 2 could be reasonably extrapolated to other active compounds investigated in the current study. Note that in this assay hER binds to the LxxLL coated-plate even in the absence of any agonist (basal binding largely exceeds binding found with fulvestrant), in agreement with the observation that the apo-receptor recruits LxxLL coactivators (order of coactivator recruitment: E2 > apo/C29/fulvestrant) [22].

4. Discussion

According to a commonly held point of view, the agonistic activity of estrogens and estrogen-like substances parallels their capacity to compete with [3H]E2 in ERα binding assays. The biological properties of Im and Pip derivatives described here – a few compounds of a peculiar class of type II estrogens unable to competitively inhibit [3H]E2 binding [14–17] – do not fit into this view. Interactions of these ligands with appropriate residues of LBD, albeit distinct from those participating to the stable anchorage of E2 (i.e. Glu-353/Arg-394; His-524[9–13]), appear sufficient to trigger specific intra- and inter-peptidic interactions required for the association of the receptor with LxxLL motifs of co-activators, generating in fine gene transactivation.

When given to MCF-7 cells, investigated Im s and Pips progressively decrease the capacity of ERα to bind [3H]E2, as already reported for strong agonists and antagonists [23,24]. The fact that Imα and Pipα fail to compete with [3H]E2 for ERα binding shows that the loss of receptor binding capacity cannot not be ascribed to a simple saturation of the LBD but is more likely the result of a major conformational change. Co-regulator recruitment associated with such a conformational change may perhaps favor a closure of the ligand binding pocket with a concomitant entrapment of Im and Pip [25]. According to this view, occasional presence of these ligands within the LBD may turn into long term occupation,

---

**Table 2 – Effect of E2 and Im-N-Et 2 on cellular [3H]E2 accumulation**

<table>
<thead>
<tr>
<th>Time of exposure of the cells to the compounds</th>
<th>Binding parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kd</td>
</tr>
<tr>
<td>Control</td>
<td>0.09</td>
</tr>
<tr>
<td>15 min</td>
<td>0.09</td>
</tr>
<tr>
<td>30 min</td>
<td>0.08</td>
</tr>
<tr>
<td>E2 (0.1 nM)</td>
<td>0.18</td>
</tr>
<tr>
<td>15 min</td>
<td>0.13</td>
</tr>
<tr>
<td>30 min</td>
<td>0.13</td>
</tr>
<tr>
<td>Im-N-Et 2 (1 μM)</td>
<td>0.14</td>
</tr>
<tr>
<td>15 min</td>
<td>0.14</td>
</tr>
<tr>
<td>30 min</td>
<td>0.14</td>
</tr>
</tbody>
</table>

MCF-7 cells were incubated for 15 or 30 min with E2 or 2, control cells were maintained in parallel in the absence of any ligand. Cells were then incubated with increasing amounts of [3H]E2 to measure the influence of ligands on the ability of the cells to specifically accumulate [3H]E2 (whole cell assay). Binding parameters were established by Scatchard plot analysis; data refer to the mean of three values from two independent experiments.

b Values expressed: Kd in nM, n in fmole/mg protein.

Percentage.

---

Fig. 5 – Effect of Im-N-Et (2) and Im-N-EtOMe (2bis) on cellular [3H]E2 accumulation. MCF-7 cells were maintained for 15 min to 3 h with 2 or 2 bis (1 μM) in the absence or presence of MG-132 (1 μM) before incubation with 1 nM [3H]E2 for 45 min. Specific cellular accumulation of [3H]E2 was established by an additional incubation with a 500-fold excess of unlabeled E2. Data refer to means of six values ± S.D. from two independent experiments. Asterisks point to significant decreases of [3H]E2 accumulation (ANOVA, p < 0.05 vs. control or MG-132 at same time of incubation, Tukey’s post hoc test).
making impossible a displacement by \[^{3}H\]E\textsubscript{2} [26]. Drastic alteration of the LBD topology due to the dissociation of a chaperone such as Hsp-90 from the oligomeric complex containing ER\textsubscript{a} may be advanced as an alternative explanation to our observations [27]. Synthesis of radio-labeled Im and Pip derivatives may help to elucidate the mechanism by which they induce a loss of E\textsubscript{2} binding capacity. Although the biological consequence of the latter phenomenon is not established yet, we speculate that it could provoke a rapid desensitization of the cells to any subsequent stimulus which might antagonize the initial signal produced by Im/Pip binding. Hence, such a loss of receptor binding capacity would confer an irreversible character to the cascade of events triggered by ligand binding, a property of prime importance to initiate dynamic molecular processes required for gene transactivation [3–5]. Subsequent proteasomal degradation of the receptor may logically complete this physiological mechanism.

While loss of estrogen binding capacity and ER\textsubscript{a} proteasomal degradation may have the same biological impact, they are independent phenomena. Indeed, the decrease of \[^{3}H\]E\textsubscript{2} binding capacity induced by a ligand occurs largely before ER\textsubscript{a} breakdown and, moreover, is neither abrogated by MG-132 (present observations and data reported in [24]) nor suppressed by partial antiestrogens such as hydroxytamoxifen [28]. Such a loss of binding capacity also occurs in cells exposed to strong antagonists [23], showing clearly that it is not attributable to a particular class of transactivation regulators. In support of this concept, we recently reported that a synthetic peptide corresponding to a regulatory motif of ER\textsubscript{a} similarly decreased the capacity of MCF-7 cells to accumulate selectively \[^{3}H\] E\textsubscript{2} [29,30].

To our knowledge, only one drug displaying some structural analogies with a coumarinic estrogen (estrothiazine) has been reported to increase ERE-dependent transcription in MCF-7 cells without affecting ER\textsubscript{a} turnover [31]. Hence, present data support the concept that in these cells most agonists confer to ER\textsubscript{a} a conformation appropriate for its targeting to the ubiquitin–proteasome pathway [32]. Moreover, they reveal that the docking mode of such ligands within the LBD is not of prime importance to elicit receptor activation and degradation. By contrast, the recruitment of LxxLL co-activators associated with these ligand-induced conformational changes would play a major role. Investigations aimed at correlating the ability of experimental compounds to provoke the recruitment of LxxLL-containing co-activators with their ability to induce ER\textsubscript{a} elimination have not been carried out so far. We think that such structure/activity relationship studies may help to progress in the understanding of the mechanism regulating ER-mediated transcription.

Altogether, our data indicate that the capacity of a ligand to confer a conformation appropriate for LxxLL co-activators is mandatory in order to produce ERE-dependent transcription and the associated change in ER\textsubscript{a} turnover rate. Assessment of such a capacity would be largely more reliable than conventional competitive \[^{3}H\]E\textsubscript{2} binding assays for the selection of active receptor modulators. Therefore, systematic measurement of binding of ER\textsubscript{a} to LxxLL coated plates, as illustrated here, seems to be of great interest in drug screening programs.

**Acknowledgments**

This work was supported by Belgian research grants from the Belgian “Fonds de la Recherche Scientifique Médicale” (grant no. 3.4512.03), the “Fonds Lambeau-Marteaux”, the MEDIC Foundation as well as the Deutsche Forschungsgemeinschaft (grants Gu285/3-1 and Gu285/3-2). The technical assistance of S. Bergemann and I. Schnautz is acknowledged. G. Laurent is a Senior Research Associate of the Belgian “Fonds de la Recherche Scientifique-FNRS”.

**Fig. 6** – Influence of Im-\textsubscript{N}-Et (2) and Im-\textsubscript{N}-EtOMe (2bis) on the capacity of ER\textsubscript{a} to associate with LxxLL containing peptide. Purified recombinant ER\textsubscript{a} was incubated with 2 and 2bis (0.1 and 1 \( \mu \text{M} \)) alone or in combination with fulvestrant (0.1 \( \mu \text{M} \); upper panel). As controls, similar ER\textsubscript{a} preparations were processed in parallel without ligand. ER\textsubscript{a}-LxxLL peptide complexation was then assayed by the ER\textsubscript{a} Elisa NR peptide procedure as described in Methods. Data refer to the mean value (±S.D.) of three independent experiments. Asterisks point to significant differences (ANOVA, \( p < 0.05 \) vs. control, Dunnett’s post hoc test). Experiments conducted with MCF-7 nuclear extracts (n = 2; bottom panel) gave data whose variation around the means did not exceed 10%.
REFERENCES