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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

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ABSTRACT

4,5-Diaryl-2-imidazolines (Im_s) and 2,3-diarylpiperazines (Pip_s) belong to the type II class of estrogens. These compounds enhance ERα-mediated transcription of ERE-driven reporter genes in MCF-7 cells but do not compete with [^{3}H]estradiol (E_2) for receptor binding, because of distinct anchoring modes. The present study examined whether the estrogenic action of Im_s and Pip_s is associated with a down regulation of ERα, as reported for conventional agonists. Im and Pip derivatives displaying a large spectrum of activities in three distinct ERE-dependent transactivation systems were selected for that purpose. ERα immunostaining as well as Western blotting analysis revealed that both classes of compounds down regulated ERα with an efficiency closely related to their transactivation potency. MG-132 abrogated this down regulation, pointing to a proteasomal degradation process. Im_s and Pip_s with strong transactivation potency also altered [^{3}H]E_2 binding parameters, leading to a progressive decrease of cellular estrogen binding capacity. This property occurred largely before ERα down regulation and persisted even in presence of MG-132, indicating that it did not result from ERα breakdown but rather from a conformational change of the receptor. The additional finding that the most active agonist tested in this study enhanced the capacity of a purified ERα recombinant to recruit LxxLL co-activators, while its inactive counterpart failed to do so confirmed this hypothesis. Altogether, our data indicate that the association of Im_s and Pip_s with ERα elicits similar responses to conventional agonists, even if they interact with distinct residues of the binding pocket.

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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

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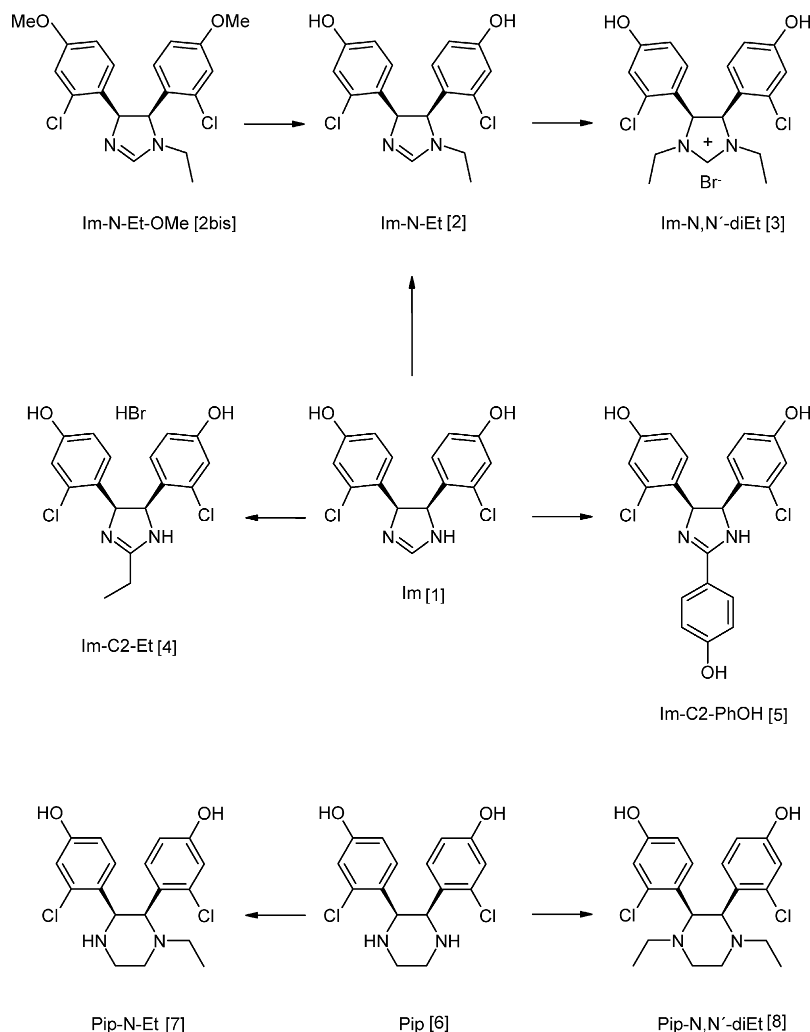
correct transcriptional cycle. Proteasomal degradation of such ''senescent'' receptors palliates this situation: it favors the action of neosynthesized receptors capable of accomplishing subsequent transcriptional cycles required for appropriate gene expression. Hence, the rate at which a receptor is 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_s) and 2,3-diarylpiperazines (Pip_s) regulate transcription by modifying ERα turnover rate like conventional ligands is a question of prime importance, e.g. for understanding the mechanisms of action of ERα. 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_s and Pip_s were synthesized according to [14]. [^{3}H]E_2 (~100 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_s and Pip_s 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 ERα recombinant*

Highly purified human ERα recombinant (Calbiochem, San Diego, CA) was diluted (~2.5 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 μg/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_s and Pip_s on ERα transcriptional activity*

2.4.1. *Luciferase assay with MCF-7 cells stably transfected with the reporter plasmid $ERE_{wtc}luc$ (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_s and Pip_s were added to achieve concentrations ranging from 0.1 nM to 10 μM, E_2 (0.1 pM to 10 μM) was used as reference. Fifty hours later, cells were lyzed 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 ERα (pSG5-ERα) and the reporter plasmid $(ERE)_2luc^+$*

Assay was previously described [17]. Cells from an almost confluent monolayer were split and seeded in 10 cm Ø Pétri dishes at a concentration of 1×10^6 cells per dish prior to transfection. Transient transfection of the cells with 0.05 μg of receptor plasmid pSG5-ERα and 5 μg of the reporter plasmid $(ERE)_2luc^+$ 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 μM (Im_s and Pip_s). The cells were then lyzed and luciferase activity assayed as described above.

2.4.3. *Luciferase assay with MCF-7 cells stably transfected with the reporter plasmid pVit-tk-Luc (MVLN cells)*

MVLN cells [21] 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 EFM containing Im_s and Pip_s (0.1 μM and 1 μM); control cells were maintained in the absence of any compound or exposed to 0.1 nM E_2. After 24 h of incubation, luciferase was assayed according to a previously described procedure [8]. Estrogenic activity of compounds was expressed in percentage of E_2-induced luciferase (100%).

2.5. *Influence of Im_s and Pip_s on ERα 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 Pétri dishes (500,000 cells *per* dish) containing EFM. After 3 days of culture, medium was removed and cells were exposed to Im_s and Pip_s (0.1 and 1 μM) 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 × buffer from In Vitrogen, Carlsbad, CA) proteins were boiled for 5 min. Each sample (15 μg) 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). ERα detection was performed with a

mouse primary antibody (F-10, 1:2000 dilution, overnight, 4 °C (Santa Cruz Biotechnology, Santa Cruz, CA)) and a control anti-actin antibody (1:5000 dilution, overnight, 4 °C (Chemicon, Temecula, CA)). ERα and actin bands were visualized with a peroxidase-labeled goat anti-mouse secondary antibody (1:1000 dilution, 2 h, room temperature) and a SuperSignal West Pico Chemiluminescent Substrate from Pierce.

2.5.1.2. *Immunofluorescence microscopy*. MCF-7 cells in EFM were plated at a density of 10,000 cells/cm^2 on sterile round glass coverslips in 12-well dishes. Two days after seeding cell cultures were fed with fresh EFM containing E_2 (1 nM) and selected Im_s (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 E_2 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 Na_2HPO_4, 10 mM KH_2PO_4, 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), 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 (Vectashield®, 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 immunostained cell preparations was documented by using a PC-driven digital camera (Leica DC 300F, Leica Microsystems AG, Heerbrugg, Switzerland). Microscopic fields were digitalized thanks to a software specifically designed for image acquisition and storage (Leica IM 50). Image adjustment and printing were achieved with appropriate softwares (Corel PHOTO-PAINT™ and CorelDRAW™, Corel Corporation, Ottawa, ON Canada).

2.5.2. [^{3}H]E_2 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 Im_s and Pip_s 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 [^{3}H]E_2 at 1 nM for evaluating the influence of the compounds upon the capacity of ERα to bind [^{3}H]E_2 (45 min of incubation; non-specific binding was established by a parallel incubation with a 500-fold excess of unlabeled E_2). Cells were then washed twice with PBS and the bound E_2 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 [^{3}H]E_2 accumulation in cells was calculated from the difference in cell-associated radioactivity after incubation in the absence or presence of unlabeled E_2.

For kinetic analysis, instead of a single concentration of [^{3}H]E_2, increasing concentrations (0.05–1 nM) of the labeled hormone were used for assessing the effect of the compounds on E_2 binding parameters (Scatchard plot analysis).

2.6. Ability of Im_s 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 diluent buffer) was incubated at 4° C either with E_2 at 1 nM, fulvestrant at 0.1 μM or selected Im_s (**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 diluent 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 Im_s and Pip_s 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.

Table 1 – ERE-dependent transcription in various cell culture systems

Compound	U2 OS cells, transiently transfected with the plasmid pSG5-ERα and the receptor plasmid $(ERE)_2Luc^+$	MCF-7-2a, stably transfected with the reporter plasmid $ERE_{wtc}luc$	MVLN cells, stably transfected with the reporter plasmid pVit-tk-Luc (% E_2 induction)*	
	EC_{50} [μM] or % E_2 induction at 10 μM*		0.1 μM	1 μM
Estradiol	2.0×10^{-6}	3.8×10^{-5}	100**	100**
Im [**1**]	0.029	0.63	28**	80**
Im-*N*-Et [**2**]	0.00038	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: $(RLU_x - RLU_{CTR}/RLU_{E2} - RLU_{CTR}) \times 100$.
** Statistical analysis: ANOVA, $p < 0.05$ *vs.* control Dunnett's *post hoc* test (data refer to the means of four values from two independent experiments).

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 Im_s and Pip_s 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 Im_s and Pip_s enhanced luciferase gene expression with an efficiency matching EC_{50} 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 Im_s (*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 Pip_s, which might limit gene transcription at high drug concentration could explain this paradoxical observation.

Differences between compounds were more clear-cut with U2 OS cells (Table 1). This might be due to the presence in MCF-7 cell variants (MCF-7-2a and MVLN cells) of endogenous ERα signaling systems which might respond differently to various ligands. Under such circumstances variations in the amounts of ERα/drug complexes at the ERE could be expected. Nevertheless, fulvestrant and OH-Tam at 0.1 μM decreased the capacity of active Im_s and Pip_s at 1 μM to induce luciferase (MVLN cells), definitely establishing the involvement of ERα in the transactivation processes (Fig. 1). As expected, the residual activity of *N*-ethyl imidazoline **2** in presence of 0.1 μM antiestrogen was totally abrogated when the antagonists were given at equimolecular concentrations (data not shown).

After having checked the consistency of the agonist activity of these selected Im and Pip derivatives in different models of ERα-mediated transcription, we proceeded to examine their impact on ERα regulation and conformation.

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

ERα immunoblotting performed on lysates from MCF-7 cells treated with investigated Im_s and Pip_s 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 Im_s and Pip_s 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

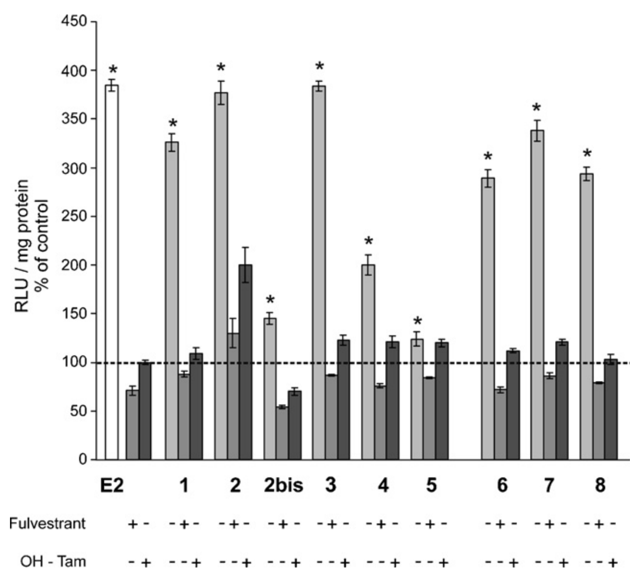


Fig. 1 – Antagonistic effect of fulvestrant or OH-Tam on Im and Pip-induced ERE-dependent transcription. MVLN cells were exposed for 24 h to Im and Pip derivatives (1 μM) in the absence or presence of fulvestrant or OH-Tam (both at 0.1 μM). Untreated cells (Control) as well as cells exposed to 0.1 nM E_2 were cultured in parallel. Cells were then processed for luciferase measurement. Data refer to means of six values ± S.D. (established in two independent experiments). Asterisks point to significant increases of Im and Pip-induced transcriptions (ANOVA, $p < 0.05$ *vs.* control, Dunnett's *post hoc* test), fulvestrant and OH-Tam significantly reduced these increases except for the weakest agonist 5 (ANOVA, $p < 0.05$ *vs.* compounds, Tukey's *post hoc* test).

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 E_2 [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_s and Pip_s decreased the capacity of ERα to bind $[^3H]E_2$ (whole cell binding assay; Fig. 4A, upper panel). In this regard, Im-*N*-Et **2** was the strongest modifier again. This loss of estrogen binding capacity could not be ascribed to a proteasomal 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 $[^3H]E_2$ accumulation was recorded when the cells were incubated with the labeled hormone in presence of investigated Im_s and Pip_s. Actually, only a 1000-fold excess of **7** and **8** produced a loss of $[^3H]E_2$ labeling (Fig. 4B). Non-specific interference of these two compounds in the assay might be advocated to explain this

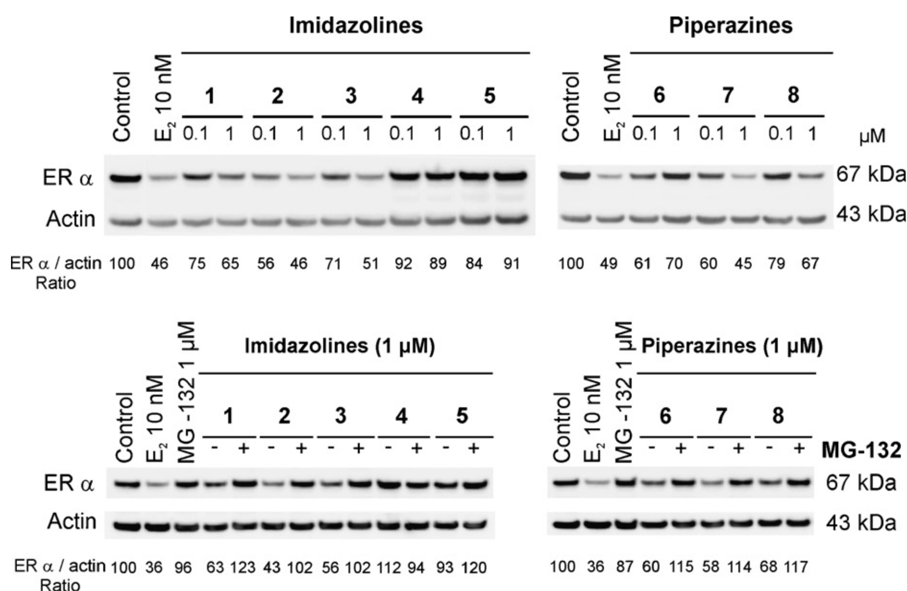


Fig. 2 – Effect of Im and Pip derivatives on ERα level. MCF-7 cells were exposed for 24 h to E_2 (10 nM) or Im and Pip derivatives (0.1 and 1 μM) (upper panel). In parallel, cells were incubated for 4 h with Im_s and Pip_s (1 μM) in the absence or presence of MG-132 (1 μM) (lower panel). Untreated cells (Control) were maintained in culture without any ligand. Equal amounts of solubilized protein (20 μg) were subjected to Western blotting. Representative data of an experiment which was performed twice. The peculiar behavior of 6 (weaker effect at 1 μM) is anecdotal since it was not recorded in other similar experiments; all other compounds gave reproducible results.

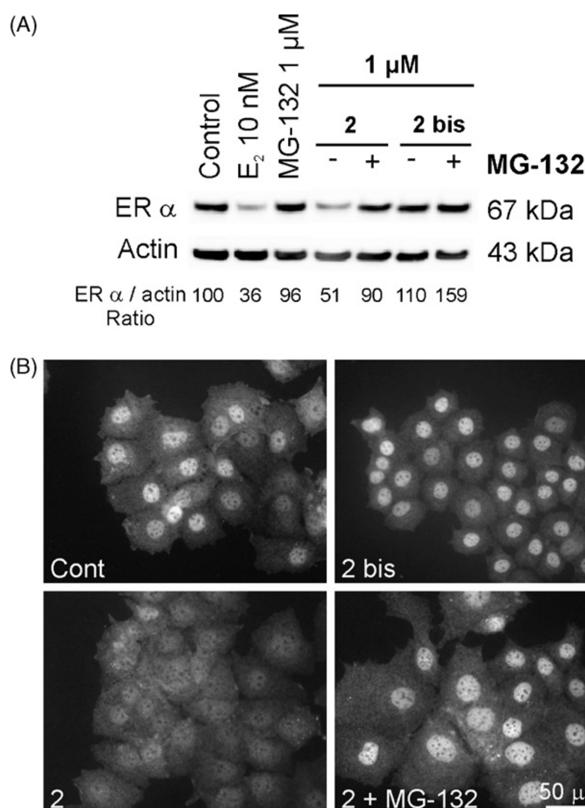


Fig. 3 – Effect of Im-N-Et (2) and Im-N-EtOMe (2bis) on ERα level. (A) Western blot analysis. MCF-7 cells were exposed for 4 h to Im-N-Et (2), Im-N-EtOMe (2bis) (both at 1 μM), E_2 at 10 nM or MG-132 at 1 μM alone or in combination. Control: no drug addition. Culture samples were processed for ERα evaluation by Western blotting as specified in Fig. 2. (B) Immunofluorescence microscopy. Cells were treated for 6 h with 2 or 2bis. The effect of MG-132 on ERα immunofluorescence decrease induced by 2 was demonstrated by adding the proteasome inhibitor 1 h before the latter drug. Control: untreated cell culture processed in parallel. ERα was demonstrated by immunofluorescence microscopy with HC-20 antiserum, as detailed in Methods. Texas Red labeling.

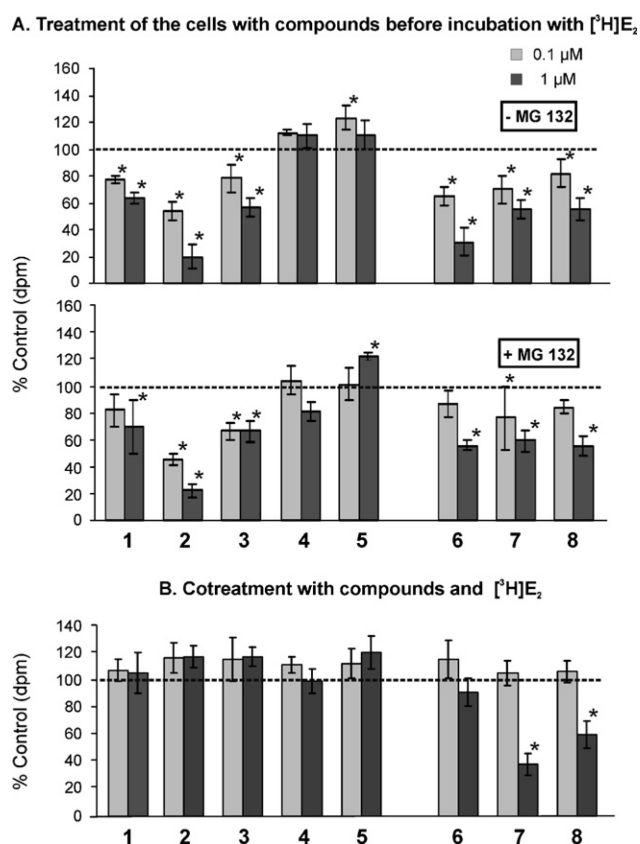


Fig. 4 – Effect of Im and Pip derivatives on cellular [^{3}H]E_2 accumulation. Panel A: MCF-7 cells were maintained for 3 h with Im and Pip derivatives (0.1 and 1 μM) in the absence or presence of MG-132 (1 μM) before incubation with 1 nM [^{3}H]E_2 for 45 min. Asterisks point to significant differences (ANOVA, $p < 0.05$ *vs.* control or MG-132, Dunnett's *post hoc* test). Panel B: cells were exposed in parallel to 1 nM [^{3}H]E_2 in the absence or presence of Im_s and Pip_s (0.1 and 1 μM). Asterisks point to significant differences (ANOVA, $p < 0.05$ *vs.* control, Dunnett's *post hoc* test). In each case, specific cellular accumulation of [^{3}H]E_2 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.

property since they did not markedly differ from other Im_s and Pip_s with similar transactivation potency and ability to regulate ER level.

Measurement of [^{3}H]E_2 binding parameters by Scatchard plot analysis of MCF-7 cells preincubated with the strongest modifier Im-Et **2** (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 E_2 (0.1 nM), used as a reference. Hence, the decrease of estrogen binding capacity induced by Im_s and Pip_s derivatives is most probably related to an ERα conformational change like that produced by E_2, 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_s and Pip_s 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. Reproduction of this experiment with a nuclear extract from MCF-7 cells gave a similar outcome, although **2** appeared less efficient with this ERα preparation (ERα binding, basal/E_2: hER = 100/228; nuclear extract = 396/585). This lower efficiency

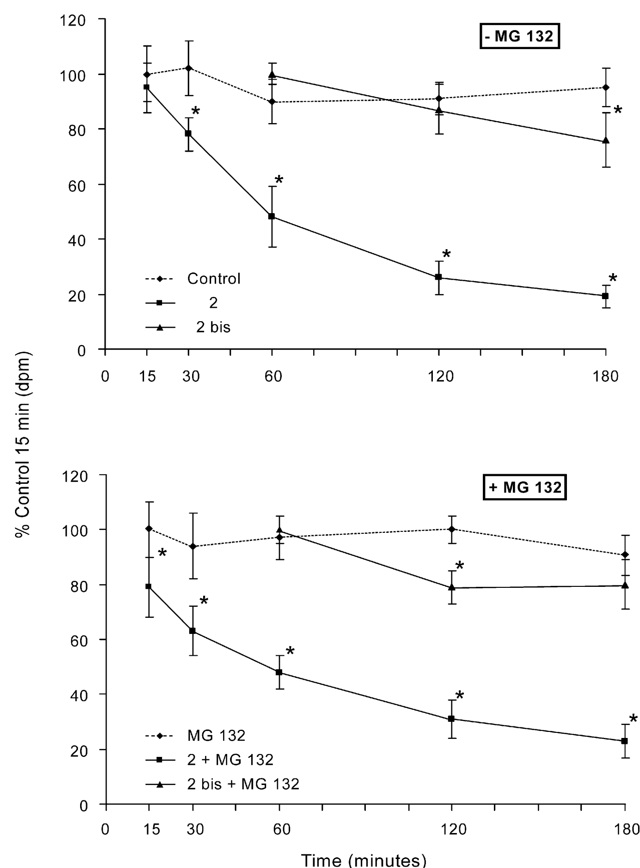


Fig. 5 – Effect of Im-N-Et (2) and Im-N-EtOMe (2bis) on cellular [^{3}H]E$_2$ accumulation. MCF-7 cells were maintained for 15 min to 3 h with 2 or 2 bis (1 μM) in the absence or presence of MG-132 (1 μM) before incubation with 1 nM [^{3}H]E$_2$ for 45 min. Specific cellular accumulation of [^{3}H]E$_2$ was established by an additional incubation with a 500-fold excess of unlabeled E$_2$. Data refer to means of six values ± S.D. from two independent experiments. Asterisks point to significant decreases of [^{3}H]E$_2$ accumulation (ANOVA, $p < 0.05$ *vs.* control or MG-132 at same time of incubation, Tukey's *post hoc* test).

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 E$_2$. 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: E$_2$ > apo ≫ fulvestrant) [22].

Table 2 – Effect of E$_2$ and Im-*N*-Et *2* on cellular [^{3}H]E$_2$ accumulation

Time of exposure of the cells to the compounds	Binding parameters	
	K_d [a]	n [a]
Control		
15 min	0.09	929 (100) [b]
30 min	0.08	867 (100)
E$_2$ (0.1 nM)		
15 min	0.18	766 (82)
30 min	0.13	486 (56)
Im-*N*-Et **2** (1 μM)		
15 min	0.14	888 (96)
30 min	0.14	656 (76)

MCF-7 cells were incubated for 15 or 30 min with E$_2$ or **2**, control cells were maintained in parallel in the absence of any ligand. Cells were then incubated with increasing amounts of [^{3}H]E$_2$ to measure the influence of ligands on the ability of the cells to specifically accumulate [^{3}H]E$_2$ (whole cell assay). Binding parameters were established by Scatchard plot analysis; data refer to the mean of three values from two independent experiments.
[a] Values expressed: K_d in nM, n in fmole/mg protein.
[b] Percentage.

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 [^{3}H]E$_2$ 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 [^{3}H]E$_2$ 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 E$_2$ (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 Pip$_s$ progressively decrease the capacity of ERα to bind [^{3}H]E$_2$, as already reported for strong agonists and antagonists [23,24]. The fact that Im$_s$ and Pip$_s$ fail to compete with [^{3}H]E$_2$ 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$_s$ and Pip$_s$ [25]. According to this view, occasional presence of these ligands within the LBD may turn into long term occupation,

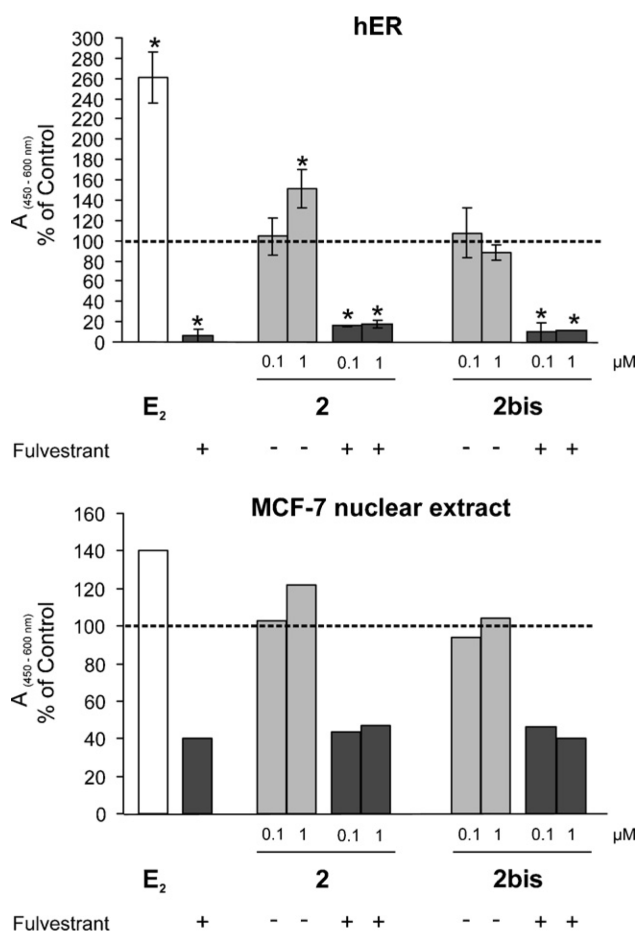


Fig. 6 – Influence of Im-N-Et (2) and Im-N-EtOMe (2bis) on the capacity of ERα to associate with LxxLL containing peptide. Purified recombinant ERα was incubated with 2 and 2bis (0.1 and 1 μM) alone or in combination with fulvestrant (0.1 μM; upper panel). As controls, similar ERα preparations were processed in parallel without ligand. ERα-LxxLL peptide complexation was then assayed by the ERα 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%.

making impossible a displacement by $[^3H]E_2$ [26]. Drastic alteration of the LBD topology due to the dissociation of a chaperone such as Hsp-90 from the oligomeric complex containing ERα 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_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 ERα proteasomal degradation may have the same biological impact, they are independent phenomena. Indeed, the decrease of $[^3H]E_2$ binding capacity induced by a ligand occurs largely before ERα 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 ERα similarly decreased the capacity of MCF-7 cells to accumulate selectively $[^3H]$ E_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 ERα turnover [31]. Hence, present data support the concept that in these cells most agonists confer to ERα 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 ERα 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 ERα turnover rate. Assessment of such a capacity would be largely more reliable than conventional competitive $[^3H]E_2$ binding assays for the selection of active receptor modulators. Therefore, systematic measurement of binding of ERα to LxxLL coated plates, as illustrated here, seems to be of great interest in drug screening programs.

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