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# Role of the proteasome in the regulation of estrogen receptor α turnover and function in MCF-7 breast carcinoma cells

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**Abstract**

Estrogen receptor α (ER) turnover in MCF-7 cells was assessed by pulse chase analysis and measurement of ER steady-state level. In untreated cells, degradation of $^{35}$S-labeled ER was characterized by a slow phase followed by a more rapid decline. Without ligand, ER elimination was totally compensated by synthesis which maintained receptor homeostasis. Estradiol ($E_2$) and the pure antiestrogen RU 58,668 abolished the slow phase of ER breakdown and enhanced the degradation of neosynthesized ER, producing a low ER steady-state level. By contrast, the partial antiestrogen OH-Tam was ineffective in this respect and caused ER accumulation. Regardless of the conditions, ER breakdown was abolished by proteasome inhibition (MG-132). ER ligands decreased cell capacity to bind [$^3$H]$E_2$, even in the presence of MG-132, indicating that the regulation of ER level and $E_2$ binding capacity occurs through distinct mechanisms. MG-132 partially blocked the basal transcription of an ERE-dependent reporter gene and modified the ability of $E_2$ to induce the expression of the latter: the hormone was unable to restore the transactivation activity measured without MG-132. RU 58,668 and OH-Tam failed to enhance the inhibitory action of MG-132, suggesting that a loss of basal ER-mediated transactivation mainly affects the stimulatory effect of estrogens. Overall, our findings reveal that ER steady state level, ligand binding capacity and transactivation potency fit in a complex regulatory scheme involving distinct mechanisms, which may be dissociated from each other under various treatments.


*Keywords:* Proteasome; Estrogen receptor; Turnover; MCF-7 cells

## 1. Introduction

Estrogen receptors α and β belong to the steroid/thyroid hormone superfamily of nuclear receptors, which act as ligand-dependent transcription factors [1]. Implication of the α isoform (here referred to as ER) in the onset and the development of breast cancer is now well established. Besides, high ER concentration in a primary tumor is considered as a good prognostic factor, generally predictive of a favorable response to antiestrogen administration (e.g. tamoxifen, raloxifene) [2]. Hence, the study of the mechanisms regulating ER expression in mammary tumors appears of prime importance, with the perspective of maintaining tumor sensitivity to hormonotherapy by stabilizing the receptor at a high level. Such investigations have been pursued for many years in several laboratories including ours. MCF-7 breast cancer cells were often selected for these studies since they express substantial amounts of receptor and respond to the mitogenic and growth inhibitory effects of estrogens and antiestrogens, respectively [3].

Among factors potentially able to modify ER expression (growth factors, cytokines, fatty acids, hormones, etc.), cognate ligands have been reported to play a crucial role. Ex-

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posure of MCF-7 cells to estrogens (17β-estradiol ($E_2$), diethylstilbestrol, etc.) rapidly represses ER gene transcription leading to a decrease of ER mRNA [4–7]. By contrast, all investigated antiestrogens prove to be ineffective on ER transcription [5,8,9], indicating a major regulatory difference between estrogen agonists and antagonists. Some ER ligands can also favor the ubiquitination of the receptor [10], provoking its rapid degradation *via* the proteasomal pathway [11–13] (down regulation). Yet, tamoxifen and related compounds (partial antiestrogens) are poorly efficient in this regard [10,14], and cause ER accumulation (up regulation) [8,15–17] since they do not affect ER synthesis while they strongly interfere with its breakdown. By contrast, pure antiestrogens, which do not stabilize ER, lead to a rapid elimination of the latter [10,18,19].

Interestingly, only 10% of the hormone-binding sites detected in estrogen-responsive cells need to be occupied in order to modulate the overall ER population, suggesting the existence of an amplification process [20]. The finding that ligands can induce the degradation of ER even when its hormone-binding site is blocked with an irreversible inhibitor (i.e. tamoxifen aziridine [21]) is in agreement with this view. Compounds released in the extracellular compartment as a result of ligand-receptor interactions may play a role in this amplification mechanism since conditioned media from cell cultures exposed to estrogens or antiestrogens influence ER level in a same way as these ligands [22]. Such released compounds may act by themselves or in synergy with minute, undetectable amounts of residual ligands. Thus, ER expression could be controlled, at least in part, by an autocrine/paracrine regulatory mechanism, as described for the control of cell proliferation. This concept may explain why growth factors [23,24] and various inhibitors/activators of kinases involved in signal transduction [25] modulate ER level.

The ability of ER to bind ligands depends on an additional regulatory process involving the association of the neosynthesized receptor with chaperones such as heat shock proteins ($hsp_s$) [26,27]. Ligands normally provoke the dissociation of hsp-ER oligomers leading to a decrease of estrogen binding ability [27–29] concomitant with ER anchorage to the nuclear matrix. This step precedes receptor down regulation, explaining why tamoxifen and analogues [14], which fail to induce efficient down regulation of ER, favor the accumulation of a pool of receptors unable to bind estradiol [30]. This property suppresses the sensitivity of ER to estrogenic stimuli while it would sustain its ligand-independent transactivation activity.

The impact of these regulatory processes on ER transcriptional activity is far from being definitely established. Some authors claim that the proteasomal degradation of the receptor is required for the expression of ER-targeted genes [31–33], while others support the opposite concept ([34] and ref. therein). Thus, proteasomal inhibitors (i.e. MG-132, lactacystin, LLnL, etc.) were reported in some studies to abrogate the ability of $E_2$ to transcribe reporter genes while, in other reports, they appeared stimulatory. To some extent, differences in experimental conditions and cells may explain this disturbing discrepancy, even though the promoter context of these reporter genes may also be advocated [34]. Obviously, studies are required to clarify the situation. In particular, it would be of interest to know whether or not the ubiquitin-proteasome pathway governs the elimination of the whole ER population or only a given receptor subpopulation (newly synthesized, mature, activated, senescent receptors), in view of our recent finding of distinct degradation pathways for ligand-independent and agonist-mediated receptor elimination [35].

The aim of the present study was to further characterize the role of the proteasome in the regulation of ER steady state, estrogen binding capacity and transcriptional activity. Special emphasis was devoted to the influence of estrogens and antiestrogens on the turnover rate of the receptor. To this end, MCF-7 cells pre-exposed to [$^{35}$S]methionine were treated with a given ligand ($E_2$, the partial antiestrogen OH-Tam or the pure antiestrogen RU 58,668) in order to evaluate the ability of the latter to change the degradation rate of the receptor. Examination of cells exposed to this radioactive precursor either in the absence or presence of the same ligand enabled us to assess the potential effect of the latter on ER synthesis. [$^3$H]$E_2$ binding capacity as well as ERE-dependent transcription of a reporter gene (luciferase) were assessed in parallel to establish relationships between receptor level and function. The outcome of such experiments in presence of the proteasome inhibitor MG-132 led us to delineate the paramount importance of the ubiquitin-proteasome pathway in receptor turnover and function.

## 2. Materials and methods

### 2.1. Ligands and reagents

L-[$^{35}$S]methionine (>100 mCi/mmol) was purchased from Amersham Biosciences (Buckinghamshire, UK). Estradiol ($E_2$) and 4-hydroxytamoxifen (OH-Tam) were obtained from Sigma (St. Louis, MO). RU 58,668 was a gift from Roussel-Uclaf (Romainville, France). MG-132 came from Calbiochem (San Diego, Ca), PMSF (phenylmethylsulfonyl fluoride) from Sigma (St. Louis, Mo) and TPCK (tosyl-L-phenylalaninechloromethylketone) from Roche Diagnostics (Mannheim, Germany).

### 2.2. Cell culture conditions

During the whole period of experiments, MCF-7 cells (received from the Michigan Cancer Foundation in 1977) were maintained in culture at 37 °C (95% air and 5% $CO_2$) in Phenol Red-containing MEM supplemented with 10% heat-inactivated (56 °C, 1 h) fetal calf bovine serum (FCS), L-glutamine, penicillin–streptomycin at the usual concentrations (Gibco BRL – Life Technologies, Ghent, Belgium). All experiments were conducted in Phenol Red-free MEM containing 10% dextran-coated charcoal (DCC) treated FCS.

### 2.3. Effect of ligands on ER turnover rate

#### 2.3.1. $^{35}$S-ER labeling

MCF-7 cells were plated in 60 cm$^2$ Petri dishes ($4.5 \times 10^5$ cells per dish). After 4 days of culture, they were fed with MEM devoid of L-methionine (Gibco BRL) and kept in that medium for 2 h before exposure to 10 nM [$^{35}$S]methionine under appropriate conditions for assessing of the influence of ligands upon ER synthesis and degradation (treatment up to 4 h). Thus, in order to examine changes in ER synthesis, investigated ligands were added to the medium at the time of cell labeling with [$^{35}$S]methionine; ligand-induced ER degradation was performed in methionine-containing MEM (regular medium) on cells pre-exposed for 2 h to [$^{35}$S]methionine. At the end of each incubation, cells were processed for ER extraction as described below.

#### 2.3.2. ER immunoprecipitation

After medium removal, cell monolayers were rinsed twice with TBS (50 mM Tris–HCl pH 7.5 with 150 mM NaCl) at room temperature. All further steps were run at 4 °C. Cells were lyzed, using 1 ml of lysis solution (TBS pH 7.5, 0.5% deoxycholic acid, 1% Nonidet P-40, 0.1% SDS, 50 mM NaF, 1 mM $Na_3VO_4$ and 5 mM EDTA) containing freshly added proteolysis inhibitors PMSF (0.6 mM) and TPCK (0.3 mM); they were maintained in contact with this solution during 15 min and then scraped. Lysates were harvested in microtubes and passed four times through a 1 ml syringe fitted with 0.4 mm × 19 mm needle followed by additional 15 min incubation and finally clarified by centrifugation at 17,530 × *g* for 30 min. Protein concentration of such supernatants was measured in an aliquot using the BCA protein assay from Pierce (Rockford, Ill) using BSA as standard.

Clarified supernatants containing equivalent amounts of proteins were distributed in microtubes (900 μl), incubated with 45 μl of anti-rabbit IgG agarose for 2 h under agitation and centrifuged to remove proteins that may non-specifically cross react at time of immunoprecipitation. Supernatants were incubated overnight with 2 μg of HC-20 anti-ER polyclonal antibody (Santa Cruz Biotechnology). ER-antibody complexes were precipitated with 45 μl of anti-rabbit IgG agarose (2 h) and agarose-complexes were collected by centrifugation and washed four times with 900 μl TBS containing detergents (0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS). Pellets were suspended in 60 μl electrophoresis buffer (100 mM Tris–HCl, pH 6.8, 17% glycerol, 8.5% β-mercaptoethanol, 5% SDS, 0.2 mg/ml bromophenol blue) and boiled for 5 min to liberate ER. Such denatured ER samples were stored at −80 °C for assessment of their ER labeling and/or content (Western blot).

### 2.4. Assessment of $^{35}$S-ER labeling

#### 2.4.1. Radioactivity measurement

Aliquots (10 μl) of denatured ER samples were evaluated for radioactivity in a β-scintillation counter; non-specific binding was assessed on extracts processed in parallel with no primary antibody (ER labeling value = labeling after immunoprecipitation − non-specific labeling). ER labeling was normalized according to the concentration of the protein measured in the corresponding clarified supernatants. For comparison of the data, labeling values were expressed in percentage of a control corresponding to a cell culture unexposed to any ligand (for ER degradation, at the time of exposure of the $^{35}$S pre-labeled cells to the investigated ligand; for ER synthesis, after 1–3 h of $^{35}$S labeling).

#### 2.4.2. Autofluorography of electrophoresis gels

Denatured ER samples were submitted to electrophoresis on 10% polyacrylamide gels (50 mA for two gels). Gels were then successively fixed (acetic acid 10%, methanol 40%, distilled water 50%), washed with distilled water, incubated in an ethanolic solution of salicylic acid (to increase the radioactive signals) and finally dried. Radiolabeled ER bands (67 kDa) onto the gels were identified by autofluorography (3 days at −80 °C with hyperfilm MP, Amersham Biosciences).

### 2.5. Assessment of total ER level (western blot)

Proteins were separated on the polyacrylamide gels and electrotransferred onto Hybond ECL nitrocellulose membranes (Amersham, UK) using a semi-dried blotting apparatus (Bio-Rad, Ca) and subsequently proceeded for immunodetection using the D-12 anti-ER antibody (dilution 1:2000; Santa Cruz Biotechnology) according to a procedure already described [14]. Intensity of each ER band was estimated by a computer-assisted gel scanning densitometer (GS-710 Calibrated Imaging Densitometer and Quantity One Software, Bio-Rad, CA). Data were expressed in percentage of corresponding controls (see above). Loading controls performed with an anti-actin antibody (Sigma) failed to show any influence of investigated ligands or MG-132 on actin level indicating the specificity of variation of ER level induced by these compounds.

### 2.6. ER immunofluorescence staining

Trypsinized MCF-7 cells were suspended in phenol-red-free medium containing 10% DCC-treated FCS and plated at densities of 0.5–1 × $10^4$ cells/cm$^2$ on sterile round glass coverslips in 12-well dishes. Two days after seeding, cells were fed fresh medium containing $E_2$, RU 58,668, MG-132 and/or cycloheximide at concentrations specified in Section 3. After drug exposure, cell monolayers were rinsed with Dulbecco's PBS (DPBS) and fixed at 4 °C with phosphate-buffered 4% paraformaldehyde (PAF) or Carnoy's mixture. Following fixation, PAF or Carnoy's mixture were changed for DPBS or 70% ethanol, respectively, where cell cultures were stored at 4 °C until immunostaining, which was performed within the next 20 h.

Demonstration of ER by immunofluorescence was achieved as described previously [36]. In short, cells monolayers were rinsed several times with phosphate buffered saline (PBS, 40 mM $Na_2HPO_4$, 10 mM $KH_2PO_4$, 120 mM NaCl, pH 7.2). In the case of PAF-fixed cells, 0.1% Triton X-100 was included in buffer used for all incubation and rinsing steps, in order to ensure cell permeabilization. Before exposure to the primary antibody, cells were preincubated for 20 min in PBS containing 5% normal goat serum (PBS-NGS) and 50 mM $NH_4Cl$ to prevent non-specific adsorption of immunoglobulins. Cells were exposed for 60 min to HC-20 antibody diluted 1:50 in PBS-NGS. Thereafter, the cell preparations were incubated for 30 min in the presence of a dextran polymer conjugated with both peroxidase and antibodies raised against rabbit immunoglobulins (EnVision™, Dakopatts, Glostrup, Denmark). The next step consisted in a 30 min incubation with rabbit antiserum raised against horseradish peroxidase (Laboratory of Hormonology, Marloie, Belgium), followed by a 30 min incubation in presence of biotinylated swine anti-rabbit immunoglobulins antibodies (from Dakopatts). Texas Red labeling was completed by exposing cells for 30 min to Texas Red-conjugated streptavidin (Vector Laboratories, Burlingame, CA). After thorough rinses in PBS, the coverslips were mounted on glass slides using commercial antifading medium (Vectashield®, Vector Laboratories). Negative controls were produced by omitting the primary antibody. This modification resulted in a virtual disappearance of the signal.

The cell preparations were examined on a Leitz Orthoplan microscope equipped with a Ploem system for epi-illumination. Texas Red fluorescence was examined at an excitation wavelength of 596 nm and an emission wavelength of 615 nm. The appearance of immunostained cell preparation was documented by using a PC-driven digital camera (Leica DC 300F, Leica Microsystems AG, Heerbrugg, Switzerland). Microscopic fields were digitalized thanks to 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).

### 2.7. Assessment of [$^3$H]$E_2$ binding parameters (ER whole cell assay [37])

MCF-7 cells were plated in 24-well plastic dishes (30,000 cells/well) and cultured for 3 days. Medium was then replaced by a serum-free medium containing $E_2$, RU 58,668 or OH-Tam either in the presence or absence of MG-132; control cells were cultured in parallel with or without MG-132. After 3 h of incubation, medium was removed and the cells exposed to [$^3$H]$E_2$ increasing concentrations (from 0.05 to 1 nM); additional wells were filled with the same concentrations of [$^3$H]$E_2$ and 500-fold excess of unlabeled $E_2$ for non-specific binding measurements. After 1 h of incubation, specifically incorporated [$^3$H]$E_2$ (bound to ER) was measured and the [$^3$H]$E_2$ binding parameters ($K_d$, $n$) established according to Scatchard plot analysis. Binding capacities ($n$) were normalized to protein content measured in parallel on cell extracts.

### 2.8. ERE-dependent transcriptional activity

Assays were run on MVLN cells [38] i.e. MCF-7 cells stably transfected with a pVit-tk-Luc reporter plasmid; control experiments run in our laboratory demonstrated that this transfection failed to affect the ligand-induced mechanisms regulating ER level. In practice, MVLN cells were cultured for 3–4 days in 35 mm Ø Falcon dishes (80,000 cells per dish) in MEM containing 10% DCC-treated serum. Subsequently, they were exposed for 4 h to either $E_2$ or RU 58,668 at various concentrations in serum-free medium in the presence or absence of MG-132 at 1 μM; control cells were maintained in culture without any ligand both with or without MG-132. Extracted luciferase was measured by luminometry according to a protocol previously described [22].

## 3. Results

### 3.1. Ligands affect ER degradation and production

Short-term culture (4 h) in serum-containing medium of MCF-7 cells preincubated with [$^{35}$S]methionine (chase period) led to a gradual decrease of $^{35}$S-labeled ER, which progressively accelerated: clearance was relatively slow within the two first hours of the chase (∼20%) and subsequently led to a rapid reduction of ER labeling (∼80%) (Fig. 1, upper panel, left). This biphasic clearance was not due to a persistence of [$^{35}$S]methionine incorporation after the withdrawal of the precursor, since a delay between [$^{35}$S]methionine withdrawal and the measurement of degradation kinetics failed to suppress the slow phase of labeled ER elimination. It was thus ascribed to a higher stability of neosynthesized ER molecules. Incidentally, the measurement of total ER level in the same samples did not reveal any significant change during the chase period (western blots, Fig. 1, lower panel, left), indicating that our experimental conditions maintained ER homeostasis (loss was totally compensated by synthesis).

Addition of $E_2$ to the chase medium abolished the slow phase preceding the rapid elimination of the $^{35}$S-labeled ER pool (60% loss within the two first hours), suggesting that the hormone may sensitize the newly synthesized receptor, making it as vulnerable to degradation as the mature form of ER (Fig. 1, upper panel, right). Apparent lack of compensatory ER synthesis under hormone treatment (decline of total ER level as shown in Fig. 1, lower panel, right) supported this view. However, a blockade of receptor synthesis [4–7] occurring in parallel could not be excluded.

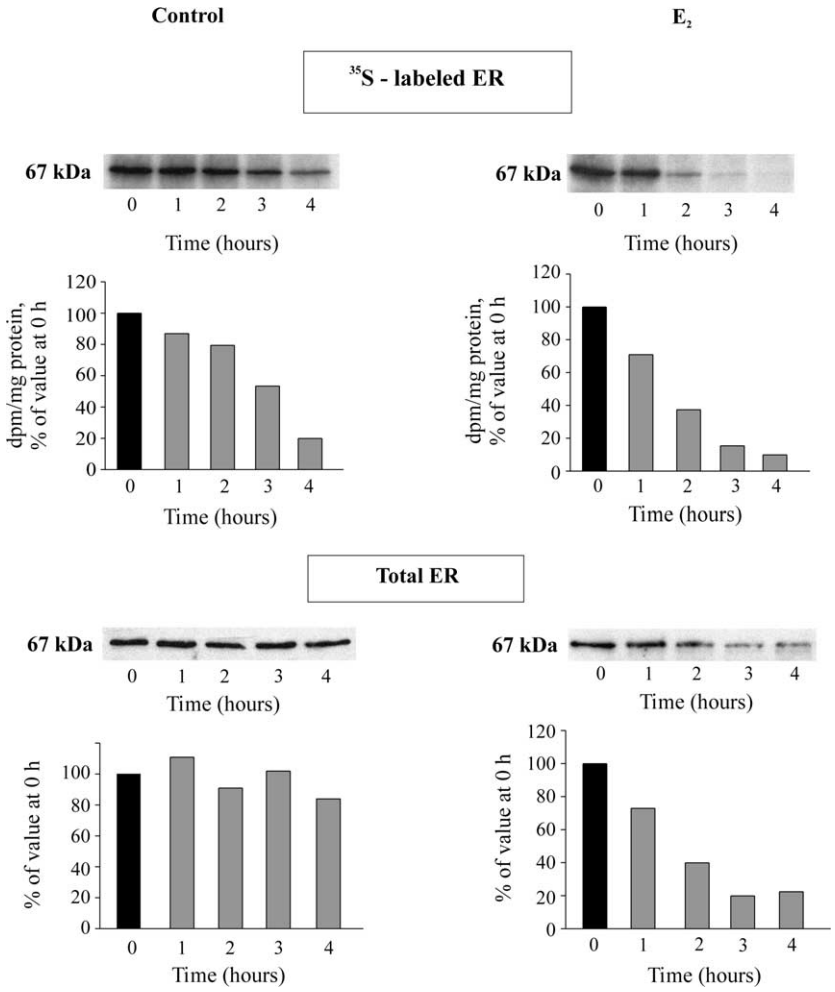


Fig. 1. Effect of $E_2$ on kinetics of ER degradation. MCF-7 cells were labeled with 10 nM [$^{35}$S]methionine for 2 h. After removal of the medium, cells were maintained in culture without (control, left) or with 10 nM $E_2$ (right) for 1–4 h. Immunoprecipitated ER from cell extracts were then submitted to SDS-PAGE and identified by autofluorography (top) or western blotting (bottom). Quantitative data: level of $^{35}$S-labeled ER was measured by scintillation counting (top); total ER level was estimated from scanning of the western blots (data refer to the mean value of two independent experiments, which gave similar results; variation around the mean: 0–9% (left) and 0–5% (right)).

Comparison of residual ER level ($^{35}$S-labeled and total) after 3 h of treatment with $E_2$, RU 58,668 or OH-Tam at various concentrations confirmed and extended our observations on the effect of these ligands on both stability and synthesis of the receptor (Fig. 2). The elimination of $^{35}$S-labeled ER and the decrease of ER steady-state level were induced by similar concentrations of $E_2$ or RU 58,668, suggesting that these ligands modify ER homeostasis by promoting the degradation of newly synthesized receptor. On the other hand, OH-Tam, which blocks receptor degradation [14], failed to produce any decrease of $^{35}$S-labeled ER and increased the original ER pool, as could be expected from previous studies [15–17].

CHX has been shown to block $E_2$-induced ER down regulation [25,35,39]. In this study, we observed that it also exerts an inhibitory effect on the decline of the $^{35}$S-labeled receptor pool in response to $E_2$ (Fig. 3). Since CHX failed to block the degradation of the unliganded native ER (elimination almost identical in cells exposed and unexposed for 3 h to CHX, see controls), it seems that a rapidly turning over protein is involved in the molecular events triggered by the hormone and required for the down regulation of the receptor. Interestingly, such a stabilizing effect of CHX did not affect $^{35}$S-labeled ER elimination provoked by RU 58,668, confirming that this antiestrogen differs from $E_2$ with regard to ER down regulation process [12,17,29]. As already shown [12], the hypothesis of a non-proteasomal pathway for ER degradation in RU 58,668-treated cells must nevertheless be rejected since the antiproteasome MG-132 inhibited the ability of this pure antiestrogen to eliminate $^{35}$S-labeled ER (see below).

These conclusions were confirmed by ER immunofluorescence staining in PAF-fixed cells (Fig. 4). Control cells exhibited a nuclear signal, which underwent no modification after treatment with CHX or MG-132 (Fig. 4a–c). $E_2$ or RU 58,668 produced a drastic reduction of immunofluorescence signal (Fig. 4a, d and g). In both cases, the intensity of the signal was restored by MG-132 (Fig. 4f and i) (note that the proteasome inhibitor was less active on RU 58,668-induced

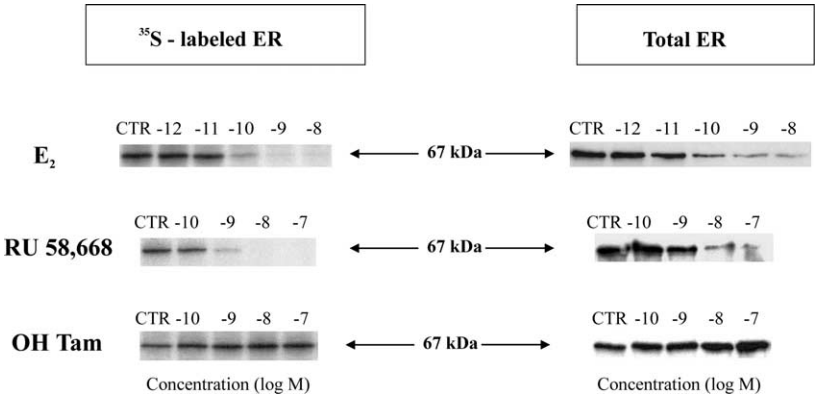


Fig. 2. Effects of $E_2$, RU 58,668 or OH-Tam on ER degradation. MCF-7 cells were labeled with 10 nM [$^{35}$S]methionine for 2 h. After removal of the medium, cells were exposed to increasing amounts of $E_2$, RU 58,668 or OH-Tam for 3 h; control cells were maintained in culture without any ligand (CTR). ER immunoprecipitated from cell extracts were then submitted to SDS-PAGE and revealed by autofluorography (left) or western blotting (right).

ER down regulation). By contrast, CHX only suppressed the $E_2$-induced extinction of ER immunofluorescence (Fig. 4e) and had no visible effect on RU 58,668-induced ER down regulation (Fig. 4h).

As suggested by data illustrated here, the protective effect of CHX and MG-132 against ligand-induced ER down regulation might be relevant to the ability of these drugs to stabilize ER within the cell nucleus (a property already described for OH-Tam [14]). In recent work, we have shown that ER stabilization caused by OH-Tam can be demonstrated morphologically by ER immunofluorescence staining after fixation with an alcohol-based mixture (more specifically, Carnoy's fixative [14,35]). As illustrated by Fig. 5a and b, exposure to OH-Tam indeed causes a clear-cut increase of ER immunofluorescence signal in nuclei of Carnoy-fixed cell (note that, in untreated cells, Carnoy fixation results in a more or less extensive loss of ER immunofluorescence, as revealed by comparison with PAF-fixed cells, Figs. 4a and 5a). Interestingly enough, cell treatment with CHX or MG-132 provokes an augmentation of nuclear ER immunofluorescence signal, similar to that induced by OH-Tam (Fig. 5c and d).

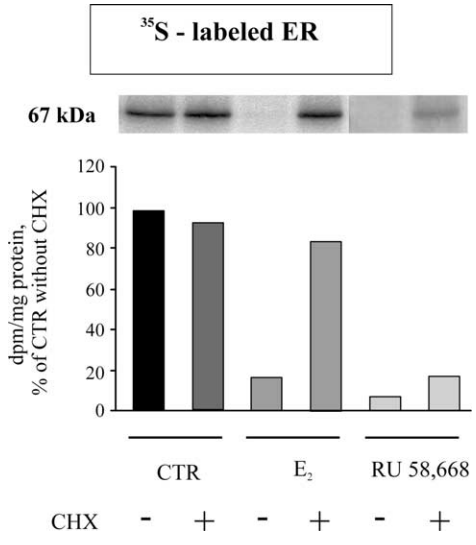


Fig. 3. Effect of cycloheximide on ligand-induced ER degradation. MCF-7 cells were labeled with 10 nM [$^{35}$S]methionine for 2 h. After removal of the medium, cells were exposed to 50 μM CHX alone (CTR) or in combination with either 10 nM $E_2$ or 0.1 μM RU 58,668 for 3 h. Immunoprecipatated ER from cell extracts were then submitted to SDS-PAGE and identified by autofluorography. For quantitative data, level of $^{35}$S-labeled ER was measured by scintillation counting (data refer to the mean value of two independent experiments, which gave similar results; variation around the mean: 0–2%).

### 3.2. *Influence of ligands on ER turnover rate*

Insofar as $^{35}$S-ER labeling is routinely performed in serum-free medium, ER turnover rate was examined in the absence of DCC-treated serum. Preliminary experiments revealed that this condition slightly slowed down the rate of $^{35}$S-labeled ER degradation (40% loss after 4 h) without affecting the potency of $E_2$, RU 58,668 and OH-Tam to modulate this elimination process (see below). Lack of growth factors and/or minute amounts of residual estrogens ($E_2$ is already active at 0.1 nM, Fig. 2) potentially present in DCC-treated serum may explain this difference. A putative persistence of ER-labeling after [$^{35}$S]methionine withdrawal would constitute another explanation. The observation of a weak increase of the $^{35}$S-labeled ER pool (~3%) during the chase period in OH-Tam-treated cells indeed revealed the existence of such a persistent labeling, in contrast with the experiments performed in the presence DCC-treated serum where it was not recorded [14].

As shown in Fig. 6, in the absence of any ligand-induced stimulation, the loss of $^{35}$S-labeled ER (ER elimination, upper panel) was totally compensated by $^{35}$S-ER labeling (ER synthesis, lower panel), indicating a maintenance of ER homeostasis in serum-free condition. The addition of MG-132 to the medium totally blocked the elimination of $^{35}$S-labeled ER, and also increased ER labeling most probably because of the absence of degradation. Thus, the newly synthesized receptor was prone to proteasomal degradation like the mature receptor. In this context, it should be stressed that the $^{35}$S-labeled ER pool slightly increased in the presence of MG-132, a finding that confirms a continuation of ER la-

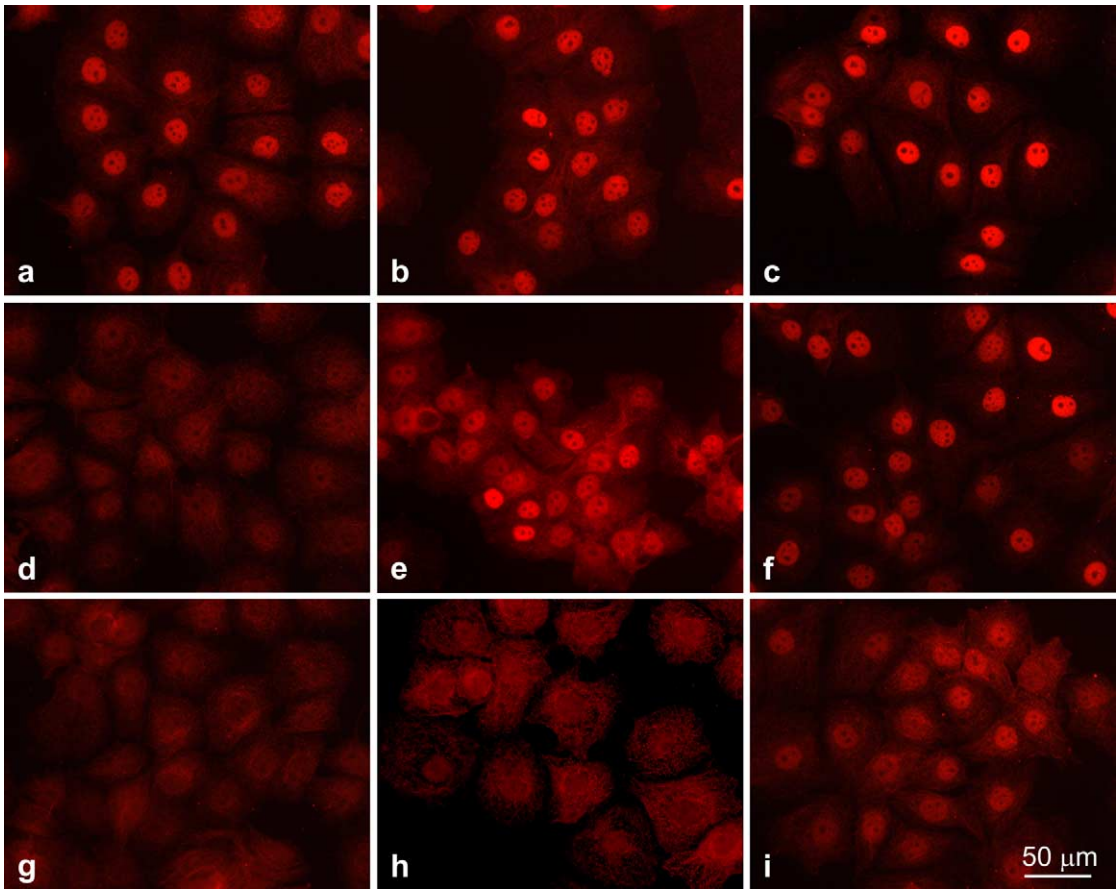


Fig. 4. Effect of cycloheximide and MG-132 on ER down regulation induced by $E_2$ or RU 58,668. The receptor was demonstrated in PAF-fixed cell by immunofluorescence staining, using HC-20 antibody. a: untreated cells; b: cells treated for 6 h with 50 μM CHX; c: cells treated for 6 h with 10 μM MG-132.; d–f: cells exposed for 5 h to 1 nM $E_2$ alone (d), in combination with CHX (e) or in combination with MG-132 (f). g–i: cells exposed for 5 h to 100 nM RU 58,668 alone (g), in combination with CHX (h), or in combination with MG-132 (i). Treatment with CHX or MG-132 was initiated 1 h before addition of $E_2$ or RU 58,668. Texas Red labeling.

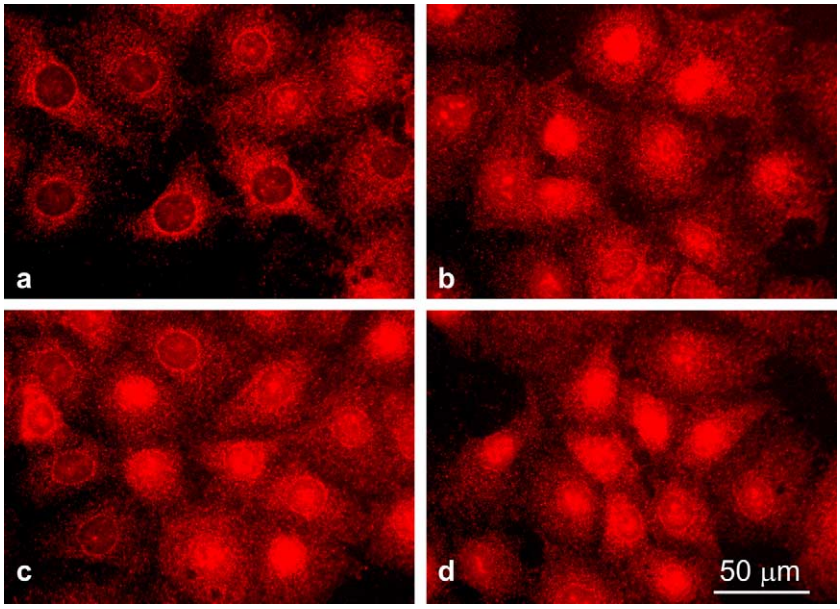


Fig. 5. Morphological demonstration of ER stabilization/nuclear anchoring induced by OH-Tam, cycloheximide and MG-132. The receptor was demonstrated in Carnoy-fixed cells by immunofluorescence staining, using HC-20 antibody. a: untreated cells; b: cells treated for 5 h with 100 nM OH-Tam; c: cells treated for 6 h with 50 μM CHX; d: cells treated for 6 h with 10 μM MG-132. Texas Red labeling.

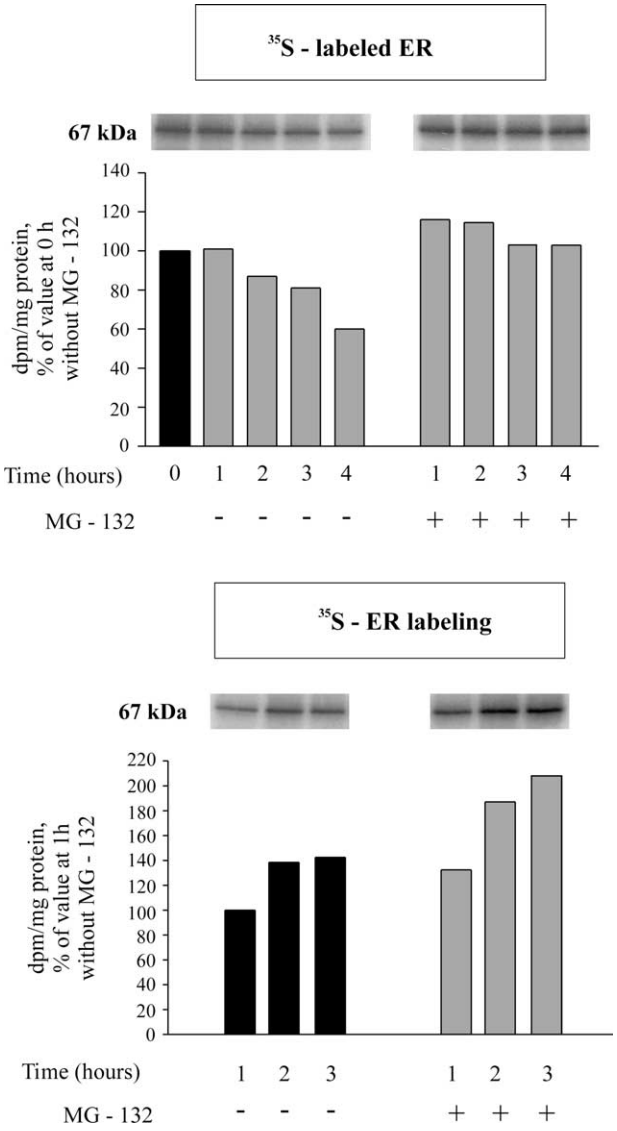


Fig. 6. Influence of MG-132 on basal rates of ER elimination and synthesis. To assess the kinetics of ER elimination, MCF-7 cells were labeled with 10 nM [$^{35}$S]methionine for 2 h. After removal of medium, cells were maintained in serum-free medium with or without 1 μM MG-132 for 1–4 h (top). To assess ER synthesis, additional cells were incubated with 10 nM $^{35}$S-methionine in the absence or presence of 1 μM MG-132 for 1–3 h (bottom). Immunoprecipitated ER from cell extracts were then submitted to SDS-PAGE and revealed by autofluorography. The quantitative data gave the level of $^{35}$S-labeled ER measured by scintillation counting (data refer to the mean value of two independent experiments, which gave similar results; variation around the mean: 1–12% (top) and 3–10% (bottom)).

beling after the withdrawal of [$^{35}$S]methionine under such experimental condition.

Cell treatment with $E_2$ or RU 58,668 enhanced ER elimination (Fig. 7, upper panel, left) and reduced its labeling (Fig. 7, lower panel). The impact of these ligands was virtually suppressed by MG-132, indicating that their action on ER turnover focuses essentially on the degradation of the receptor (a pretreatment with the proteasome inhibitor slightly enhanced its effect, upper panel, right).

Table 1
Effect of ligands on [$^3$H]$E_2$ binding capacity of MCF-7 cells in the absence or presence of the antiproteasome MG-132 (two independent experiments, a and b)

| | | | MG-132 (1 μM) | |
|---|---|---|---|---|
| | $K_d$[a] | $n$[a] | $K_d$ | $n$ |
| CTR | | | | |
| a | 0.37 | 508 [100][b] | 0.45 | 740 [100][b] |
| b | 0.19 | 424 [100] | 0.28 | 695 [100] |
| $E_2$ (0.001 nM) | | | | |
| a | 0.36 | 517 [102] | 0.48 | 742 [100] |
| b | 0.24 | 513 [121] | 0.36 | 699 [101] |
| $E_2$ (0.01 nM) | | | | |
| a | 0.43 | 439 [86] | 0.39 | 601 [81] |
| b | 0.25 | 278 [66] | 0.29 | 486 [70] |
| $E_2$ (0.1 nM) | | | | |
| a | 1.60 | 200 [39] | 0.49 | 247 [33] |
| b | 0.56 | 111 [26] | 0.47 | 223 [32] |
| CTR | | | | |
| a | 0.44 | 594 [100] | 0.41 | 737 [100] |
| b | 0.13 | 578 [100] | 0.19 | 842 [100] |
| OH-Tam (0.1 nM) | | | | |
| a | 0.34 | 499 [84] | 0.49 | 771 [105] |
| b | 0.11 | 530 [92] | 0.19 | 825 [98] |
| OH-Tam (1 nM) | | | | |
| a | 0.73 | 368 [62] | 0.49 | 490 [66] |
| b | 0.13 | 285 [49] | 0.24 | 574 [68] |
| CTR | | | | |
| a | 0.29 | 583 [100] | 0.33 | 994 [100] |
| b | 0.29 | 554 [100] | 0.38 | 843 [100] |
| RU 58,668 (0.1 nM) | | | | |
| a | 0.28 | 559 [82] | 0.37 | 942 [95] |
| b | 0.25 | 467 [84] | 0.35 | 797 [95] |
| RU 58,668 (1 nM) | | | | |
| a | 0.43 | 229 [34] | 0.46 | 412 [52] |
| b | 0.39 | 231 [42] | 0.35 | 343 [41] |

[a] Values expressed: $K_d$ in nM, $n$ in fmol/mg protein.
[b] Percentage.

Concerning the effect of OH-Tam on ER turnover rate, we recorded a slight increase of $^{35}$S-ER labeling after 3 h of treatment in addition to the expected blockade of degradation of $^{35}$S-labeled ER, exactly as found in cells exposed to MG-132. When tested together, OH-Tam and MG-132 did not produce any additive effect (data not shown).

### 3.3. *Effect of ligands on [$^3$H]$E_2$ binding capacity*

Under serum-free culture conditions used to assess ER turnover rate, MG-132 markedly increased the capacity of the cells to incorporate [$^3$H]$E_2$ (~40% increase after 3 h of treatment; Table 1, see controls). This effect may result from an inhibition of ER degradation associated with unaffected ER synthesis.

Exposure of MCF-7 cells to a ligand is known to produce a dramatic loss of their capacity to accumulate [$^3$H]$E_2$ [20,40], a phenomenon which persists for an extended pe-

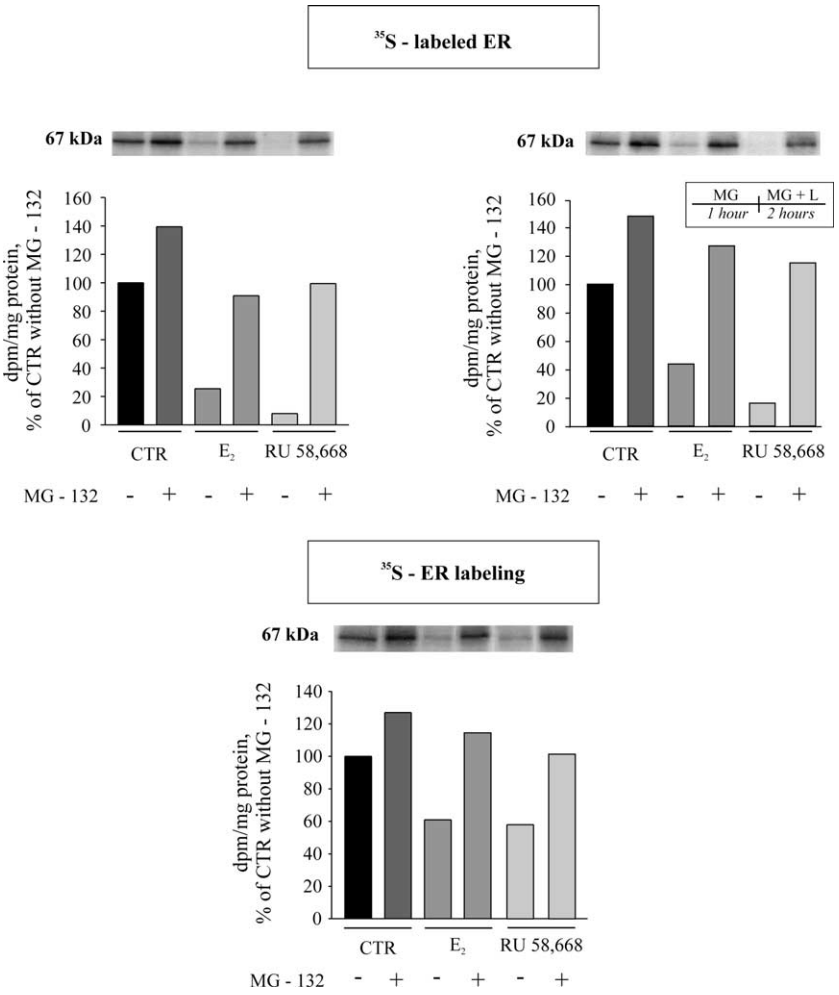


Fig. 7. Influence of MG-132 on $E_2$- and RU 58,668-induced ER elimination and synthesis. To assess ER elimination (top), MCF-7 cells were incubated with 10 nM [$^{35}$S]methionine. After removal of the medium, cells were exposed in serum-free medium to vehicle (CTR), 10 nM $E_2$ or 100 nM RU 58,668 alone or in combination with 1 μM MG-132 for 3 h (**left**); additional cells were pretreated for 1 h with MG-132 before exposure to these ligands for 2 h (**right**). To assess ER synthesis (**bottom**), MCF-7 cells were incubated with 10 nM [$^{35}$S]methionine in the presence of 10 nM $E_2$ or 100 nM RU 58,668 alone or in combination with 1 μM MG-132 for 3 h. In both conditions, immunoprecipitated ER from cell extracts were submitted to SDS-PAGE and revealed by autofluorography. The quantitative data revealed the level of $^{35}$S-labeled ER determined by scintillation counting (data refer to the mean value of two independent experiments, which gave similar results; variation around the mean: 0–9% (**top**) and 4–6% (**bottom**)).

riod of time after ligand removal and which can be best observed when the latter does not induce any ER down regulation (i.e. OH-Tam and related compounds [30]). As shown in Table 1, this phenomenon also occurred when ER degradation was abolished by MG-132. Thus, after treatment with $E_2$, RU 58,668 or OH-Tam, the amounts of [$^3$H]$E_2$ binding sites (*n* values) decreased to the same extent in the presence and absence of the proteasome inhibitor, indicating that these ligands modify the binding capacity of ER and its steady-state level by distinct mechanisms. Consequently, it seems that impairment of ER degradation would not significantly hinder the ability of a ligand to modify the conformation of the receptor. In this regard, it is noteworthy that the binding affinity of residual receptor sites ($K_d$ values) appeared virtually unaffected, rejecting a possible interference of minute amounts of ligands in our assays, as already reported [41].

### *3.4. Influence of blockade of ER proteasomal degradation on ERE-dependent transcriptional activity*

Addition of MG-132 (1 μM) to the serum-free medium used to assess ER turnover rate led to a decrease of luciferase activity after 4 h of treatment (Fig. 8). The extent of this decrease varied among experiments with a mean value of 50% ($n = 3$), roughly corresponding to the augmentation of ER protein measured in the presence of the proteasomal inhibitor (see above). Hence, blockade of ligand-independent ER degradation was associated with a loss of basal ERE-dependent transcriptional activity, supporting the concept that a prolongation of ER half-life may decrease its ability to enhance gene expression [32,33].

The loss of basal luciferase activity induced by MG-132 was reflected by the efficiency of $E_2$ and RU 58,668 to modulate the expression of this reporter gene. $E_2$, while still en-

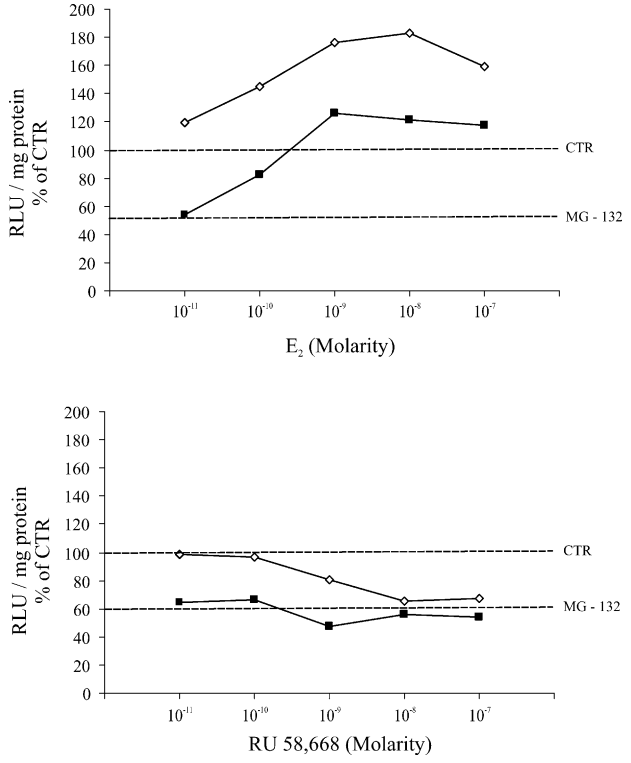


Fig. 8. Influence of MG-132 on ERE-dependent transcription in presence of $E_2$ and RU 58,668. MVLN cells were incubated for 30 min with 1 μM MG-132 and subsequently exposed to increasing amounts of $E_2$ or RU 58,668 in the presence of the proteasome inhibitor for additional 3 h 30 (closed symbols). Additional cells were maintained along the whole experiment either in the absence of MG-132 (open symbols). Cells were then submitted to luciferase assay. Dashed lines represent values in control (CTR) and MG-132-treated cells.

hancing luciferase, was unable to restore the level measured without the proteasome inhibitor. The stimulatory effect of the hormone varied among experiments, but mean relative values were almost the same as those established in control cells, suggesting that MG-132 failed to interfere in the activation of the receptor. Hence, a loss of basal ER transactivation ability provoked by proteasome inhibition may partly mask the stimulatory effect of estrogens, a fact which may explain the antagonistic activity of proteasome inhibitors reported by some authors [31–33]. On the other hand, RU 58,668 did not enhance the MG-132-induced loss of basal luciferase, disproving the possibility of a cooperative effect between these compounds. A similar lack of drug interaction was also recorded with OH-Tam at 100 nM in a complementary experiment (% inhibition: 29 and 31 without and with MG-132 respectively).

## 4. Discussion

The rate at which ER is synthesized and degraded (turnover) is a major factor regulating cell responsiveness to estrogens and antiestrogens. According to current opinions expressed in the literature, the turnover rate might not solely control ER steady state level but also its transcriptional activity, even though the precise modalities of this regulation remain controversial [31–34]. Ubiquitination [10] and neddylation [42] of the receptor, which govern its breakdown in the proteasome compartment, have been identified as key regulatory factors in ER turnover. Ligands strongly influence these metabolic events, leading to either accelerated (estrogens, pure antiestrogens) or reduced (partial antiestrogens) ER elimination. The issue that we addressed here concerned the nature of the receptor molecules affected by this clearance process: would ligands enhance the elimination of the whole ER population or only a subset of this population? Our data highly suggest that the action of ligands essentially targets newly synthesized receptor molecules, which appear more refractory than the mature receptors to proteasomal degradation. Thus, $E_2$ and RU 58,668 provoke a quasi immediate proteolysis of nascent receptors (at the time of synthesis), while OH-Tam, as well as the proteasome inhibitor MG-132 favor their nuclear stabilization.

The concept of a higher stability of neosynthesized receptors was deduced from the biphasic character of $^{35}$S-labeled ER clearance (slow followed by rapid ER elimination), which was only observed in untreated cells. While a residual $^{35}$S labeling of ER after removal of labeled methionine may partly contribute to this elimination profile, this does not invalidate the hypothesis of neosynthesized receptor being preferentially affected by ligands. Actually, ligands seem to favor the elimination of neosynthesized ER which otherwise would accumulate to maintain receptor homeostasis.

Enhanced proteasomal ER degradation is not the sole mechanism leading to its down regulation. Estrogens have indeed been shown to repress the transcription of ER gene [4–7] as well as to decrease the half-life of its mRNA [43]. While such regulatory processes may affect ER level in a context of $E_2$-induced stimulation, they cannot explain ER elimination and stabilization in the presence of RU 58,668 and OH-Tam, respectively. Indeed, these two compounds as well as other antagonists do not modulate ER mRNA level [5,8,9]. Moreover, effects of proteasome inhibition on ER mRNA level have never been reported. Hence, it is reasonable to assume that most, if not all data recorded here pertain to mechanisms regulating the stability of ER protein. However, the possibility of a marginal influence of $E_2$ on receptor production (blockade of the compensatory synthesis required to maintain ER homeostasis in untreated cells) cannot be totally excluded.

Exposure of MCF-7 cells to ER ligands provokes a loss of the cell capacity to incorporate [$^3$H]$E_2$ [20,40]. This property may be partly ascribed to the irreversible locking of these ligands within the receptor's hormone-binding domain, because of a drastic conformational change of the latter. Under such circumstances, a significant proportion of ER molecules takes a conformation preventing exchange of bound ligands [44]. Alteration of the hormone-binding domain in ER–ligand complexes still capable of dissociation may also contribute to the loss of binding capacity [40]. As shown here, this loss

of capacity to incorporate [$^3$H]$E_2$ persisted when the cells were exposed to MG-132, indicating that the ligand-induced remodeling of ER surface topology precedes the events leading to receptor degradation (or stabilization). We assume that the fixed conformation imposed by each ligand might act as a specific signature favoring (or hindering) ubiquitination, neddylation or other processes required for changing ER steady state level. Hence, ligand-induced degradation of newly synthesized ER is more likely to result from a conformational change of the receptor rather than from the induction of a particular proteolytic activity. In other words, a mechanism switching ER from a stabilized to a labile form may govern its half-life. Such a mechanism most probably also holds for the native, unliganded receptor even if the conversion of the latter is slower. In this case, one might conceive that the stable to labile switch of ER is governed by cross-talk with other signal transduction pathways [25].

The ability of estrogens and pure antiestrogens to down regulate ER molecules where the hormone-binding domain is covalently blocked by tamoxifen aziridine [21] seems in contradiction with the concept outlined above. However, we have shown that the degradation of such blocked receptors is actually related to the presence of a pool of neosynthesized, unliganded ER [21]. One may therefore consider that newly produced ER molecules, once transformed by cognate ligands (i.e. estrogens or pure antiestrogens), may dimerize with tamoxifen aziridine-bound ER, conferring to the latter the appropriate conformation for proteolysis. Estrogen or antiestrogen-induced displacement of tamoxifen aziridine-bound ER from a compartment where it is stabilized would be another explanation. This reasoning also applies to unliganded ER under subsaturating ligand concentration, since only 10% of the hormone-binding sites need to be saturated in order to deplete the whole receptor population [20]. However, as stated above, factors operating as autocrine/paracrine regulators may also contribute to this amplification process.

The biphasic kinetics of $^{35}$S-labeled ER elimination observed with untreated cells is consistent with the concept that ER evolves through different stages of maturity, this evolution culminating with ER degradation. This process would most probably be the combined result of posttranslational modifications and ER association with co-regulators including chaperone proteins. Thus, neosynthesized ER enters what is viewed as a protein assembly line involving the sequential recruitment and dissociation of chaperones and cochaperones [26]. Obviously, the primary role of this process is to stabilize ER and prime it for ligand binding [27,28]. Little is known so far concerning the involvement of chaperones in the early steps committing ER to degradation, except that some cochaperones are closely connected with the ubiquitin-proteasomal pathway [45]. Besides, it has been shown recently that compounds like geldanamycin and radicicol, which disrupt the activity of the chaperone Hsp90, precipitate ER degradation [35,46,47]. Overall, the biphasic kinetics of ER elimination is consistent with a population of relatively stable receptors which must be converted into a more labile form before undergoing breakdown. The finding supports a likely involvement of chaperones in the slow phase of ER disposal insofar as this slow phase is abolished by $E_2$ which binds the receptor and dissociates it from chaperone proteins.

Exposure of cells to MG-132 in absence of any hormonal stimulation increased their capacity to incorporate [$^3$H]$E_2$. Such an expansion of binding receptor pool would have been expected to result in an augmentation of global transactivation ([34] and ref. therein). Surprisingly, this was not the case since ER-mediated ERE-dependent transactivation (luciferase expression) was reduced by ∼50%, suggesting that a sizeable part of the accumulated receptors was in inactive form. Thus, it seems that, after its production and maturation (i.e. association with chaperones), ER evolves toward a state of non-functionality, which becomes apparent when its elimination is abrogated. An alternative interpretation would be that MG-132 by itself interferes with receptor-mediated transactivation, as suggested by other authors [48]. Anyway, MG-132 modified the cell response to $E_2$ and RU 58,668 in terms of gene transactivation, shifting to higher values the dose–response curve to $E_2$ and suppressing the inhibitory effect of RU 58,668. Hence, accumulated inactive ERs might hamper the function of active receptors. Of note, our results explain why in previous work proteasome inhibition had been claimed to hinder ER-mediated transactivation [31].

MG-132 provokes a nuclear stabilization of ER, as already reported for OH-Tam. As shown previously (14, 35), this nuclear stabilization can be demonstrated morphologically by ER immunofluorescence staining in cells fixed with an alcohol-based mixture (Carnoy's fixative). One may anticipate that both OH-Tam and MG-132 anchor the receptor in a nuclear subcompartment where it is less accessible to the transcriptional machinery, explaining thereby their inhibitory effect against basal ER-mediated expression of luciferase. CHX, which also stabilized ER in the nucleus, may act similarly.

As revealed by ER immunofluorescence staining after PAF fixation, MG-132 totally abrogated ER downregulation induced by $E_2$ while it was less effective against that caused by RU 58,668. The difference between $E_2$- and RU 58,668-induced ER downregulation was still more apparent in cells exposed to CHX, since the latter inhibitor totally failed to modify ER downregulation provoked by RU 58,668. This could be related to differences in the ER conformation imposed by distinct ligands. One is tempted to assume that $E_2$-induced ER down regulation depends on regulatory proteins expressed as a result of ER-mediated gene transactivation. So far the nature of these proteins remains elusive, but they may be involved in ER transport to a compartment where it is degraded. Other attractive candidates are elements of the ubiquitin pathway committing ER to degradation, some ER coactivators like AIB1 [49], or protein kinases targeting ER such as ERK7 [50]. The latter kinase is thought to prime ER for ubiquitination. As revealed by recent work, a dominant-negative ERK7 in human breast cells markedly decreases the

rate of ER degradation following estrogenic stimulation. Furthermore, MCF-7 cells exhibit ERK7 expression, albeit at a low level, and in these cells the protein turns over rapidly. What remains to be investigated, however, is whether ERK7 expression is modulated by estrogen agonists.

Overall, our findings reveal that, in breast carcinoma cells, ER steady-state level, ligand binding capacity and transactivation potency fit together in a complex regulatory scheme involving distinct mechanisms, which may dissociate from each other under various biochemical/hormonal treatments. While our work is essentially fundamental in nature, it is not devoid of potential clinical interest. Indeed, hormonotherapy based on estrogen antagonists is known to improve the survival of patients with breast cancer. Maintenance of a high level of functional ER appears therefore of prime importance. Hence, a detailed knowledge of ER regulation in breast cancer cells would allow one to better appreciate the effectiveness and limitations of antiestrogens and contribute to a rational use of these drugs in breast cancer management.

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