# Combination treatment with proteasome inhibitors and antiestrogens has a synergistic effect mediated by p21$^{WAF1}$ in estrogen receptor-positive breast cancer

MARIE MAYNADIER, ILARIA BASILE, AUDREY GALLUD, MAGALI GARY-BOBO and MARCEL GARCIA

Institut des Biomolécules Max Mousseron (IBMM), CNRS, Université Montpellier, ENSCM, Faculté de Montpellier, 34093 Montpellier Cedex 5, France

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**Abstract.** Although antiestrogens significantly improve the survival of patients with ER-positive breast cancer, therapeutic resistance remains a major limitation. The combinatorial use of antiestrogen with other therapies was proposed to increase their efficiency and more importantly, to prevent or delay the resistance phenomenon. In the present study, we addressed their combined effects with proteasome inhibitors (PIs). The effects of antiestrogens (hydroxyl-tamoxifen, raloxifen and fulvestrant) currently used in endocrine therapy were tested in combination with PIs, bortezomib or MG132, on the growth of three ER-positive breast cancer cell lines and in two cellular models of acquired antiestrogen resistance. When compared to single treatments, these combined treatments were significantly more effective in preventing the growth of the cell lines. The regulation of key cell cycle proteins, the cyclin-dependent kinase inhibitors, p21$^{WAF1}$ and p27$^{KIP1}$, were also studied. Bortezomib and MG132 drastically increased p21$^{WAF1}$ expression through elevation of its mRNA concentration. Notably, p27$^{KIP1}$ regulation was quite different from that of p21$^{WAF1}$. Furthermore, the effect of bortezomib in combination with antiestrogen was evaluated on antiestrogen-resistant cell lines. The growth of two antiestrogen-resistant cell lines appeared responsive to proteasome inhibition and was strongly decreased by a combined therapy with an antiestrogen. Collectively, these findings provide new perspectives for the use of PIs in combination with endocrine therapies for breast cancer and possibly to overcome acquired hormonal resistance.

*Correspondence to:* Dr Marcel Garcia or Dr Marie Maynadier, IBMM, Institut des Biomolécules Max Mousseron (IBMM), CNRS, Université Montpellier, ENSCM, Faculté de Montpellier, Avenue Charles Flahault, 34093 Montpellier Cedex 5, France
E-mail: marcel.garcia@inserm.fr
E-mail: marie.maynadier@inserm.fr

*Abbreviations:* Bz, bortezomib; CKI, cyclin-dependent kinase inhibitor; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; E2, 17β-estradiol; ER, estrogen receptor α; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OHT, 4-hydroxytamoxifen; p21, p21$^{WAF1}$; p27, p27$^{KIP1}$; PBS, phosphate-buffered saline; PI, proteasome inhibitor; qRT-PCR, quantitative real-time polymerase chain reaction; Ralox, raloxifen; Tam, tamoxifen

*Key words:* estrogen receptor, CKI, cell cycle, breast, cancer

## Introduction

The control of cell proliferation is a major issue in the treatment of cancer. In breast cancer, endocrine therapies targeting the estrogen receptor α (ER) are major therapeutic tools. Antiestrogens which primarily act by competing with estrogens for binding to the ER prevent the mitogenic effect of estrogens (1-3). They act as pure ER antagonist, such as fulvestrant (Faslodex or $ICI_{182780}$) or selective ER modulators, such as tamoxifen (Nolvadex) or raloxifen (Evista). The major limitation of antiestrogens is due to the acquired resistance of responsive breast tumors after several years of treatment. Thus, the sequential or combinatorial use of antiestrogen with other therapeutics has been proposed to overcome this resistance (4).

Previous studies have supported the importance of cyclin-kinase inhibitor (CKI) proteins, p21$^{WAF1}$ (p21) and p27$^{KIP1}$ (p27), in ER signaling and in the antiestrogen response (5-8). Indeed, antiestrogen treatment was found to induce a $G_0/G_1$ arrest in sensitive ER-positive breast cancer cells due to an upregulation of both p21 and p27 levels (5). This $G_0/G_1$ arrest was abrogated whether p21 and p27 were depleted. Moreover, low levels of these CKIs indicate a poor prognosis of breast cancers (9-14).

Proteasome inhibitors (PIs) have likewise been found to regulate these CKIs (15-20) and recently, we demonstrated that in ER-positive breast cancer, ER was a critical mediator of bortezomib-induced inhibition (21). Thus, PIs could be of potential interest in combination therapeutic strategies with antiestrogens. MG132 is a reversible peptide aldehyde used in experimental studies. Bortezomib (formerly known as PS-341 or Velcade) is a boronic acid, that has been approved by the Food and Drug Administration for the treatment of relapsed and refractory multiple myeloma (22,23). Bortezomib and MG132 both inhibit proteasome chymotrypsin-like activity; however, their effect on ER is different (24). Therefore, comparison of these two PIs combined with antiestrogen provided distinct results.

In the present study, we addressed for the first time, the growth effects of several PIs in the presence of antiestrogens in three distinct ER-positive breast cancer cell lines. We also explored the underlying mechanisms by focusing on p21 and p27 expression. The effects of PIs associated with antiestrogens were also assessed in two cellular models of acquired antiestrogen resistance.

## Materials and methods

*Cell culture*. MCF7, T47D and ZR 75.1 human breast cancer cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). LCC2 and RTx6 cells are sublines of the MCF-7 human breast cancer cell line respectively selected for their resistance to 4-hydroxytamoxifen (OHT) and tamoxifen (Tam), kindly provided by R. Clarke (25) and F. Bayard (26), respectively.

Stock solutions of $ICI_{182,780}$ (also called fulvestrant), tamoxifen or its active metabolite OHT (a kind gift from A. Wakeling, AstraZeneca, Cheshire, UK), raloxifen (Eli Lilly and Company, France) and 17β-estradiol (E2) (Sigma-Aldrich Chimie, Saint-Quentin Fallavier, France) were prepared in ethanol. MG132 (Sigma-Aldrich Chimie) was diluted in DMSO. Bortezomib (a kind gift from Millennium Pharmaceuticals, Cambridge, MA, USA) was protected from light and prepared in aqueous solution. Treatments were always equilibrated for vehicle concentration.

*Cell growth assay*. Steroids were withdrawn from cells by a 6-day culture in phenol red-free DMEM supplemented with 10% steroid-stripped serum. Then, the cells were harvested and plated on 96-well plates. Adherent cells were treated for 5 days with 1 nM E2 and 0.3 $\mu$M MG132 or 26 nM bortezomib combined or not with 1 $\mu$M OHT, tamoxifen, raloxifen or 0.1 $\mu$M $ICI_{182,730}$. Outgrowth assays were performed with 5,000 cells in 96-well plates as previously described (27). After 4 days, the cells were quantified using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (200 $\mu$g/ml MTT for 4 h). Values are expressed as a percentage of E2-stimulated cells.

*Colony formation assay*. These assays were carried out according to Prud'homme *et al* (28). Briefly, using a 12-well plate, 4,000 cells in 0.3% agar (Sigma-Aldrich Chemie, Lyon, France) were layered on preformed 0.7% agar layer (Costar, Fisher Bioblock Scientific, Illkirch, France). After plating, the cells in agar were incubated in conditioned medium and simultaneously treated with 1 nM E2 and 0.3 $\mu$M MG132 or 26 nM bortezomib combined or not with 1 $\mu$M OHT or 0.1 $\mu$M $ICI_{182,730}$. Colonies were counted under the microscope using a low magnification (x5) after 28 days.

*Immunofluorescence*. Cells grown on coverslips were treated for 24 h as indicated before fixation with 4% PFA for 12 min, cold methanol for 4 min, cold acetone for 2 min, and then saturated overnight at 4˚C with 3% goat serum in phosphate-buffered saline (PBS) containing 4% bovine γ-globulin. For immunostaining, the cells were incubated with the mouse monoclonal $p21^{WAF1}$ (Oncogene Research Products, San Diego, CA, USA) or $p27^{KIP1}$ (Interchim, Montluçon, France) antibody for 2 h. The cells were extensively washed and incubated for 1 h with Alexa Fluor 568 anti-mouse antibody (Fisher Bioblock Scientific), and then washed again and incubated with 4',6-diamidino-2-phenylindole (DAPI) (0.5 $\mu$g/ml; Sigma-Aldrich Chimie) for 15 min for nuclear staining. Negative controls were performed with a purified rabbit IgG1.

*Immunoblotting*. For protein expression analysis of whole cell lysates, the cell lysates were extracted by 3 freeze-thaw cycles in buffer containing 50 mM HEPES, 150 mM NaCl, 0.1% NP-40, 10% glycerol, 2.5 mM EGTA and protease inhibitors (Complete, Roche Diagnostics, Indianapolis, IN, USA), and centrifuged at 10,000 x g for 15 min. Proteins were quantified by the Bradford assay and a constant amount was analyzed by immunoblotting. Afterwards, the samples were mixed with equal amounts of sample buffer (125 mM Tris/HCl, 288 mM β-mercatoethanol, 20% glycerol, 2% SDS, 10 $\mu$g/ml bromphenol blue), boiled for 3 min, and blotted as previously described (29). Immunostaining was performed using mouse monoclonal p21 (Oncogene Research Products) and p27 (Interchim). Polyclonal rabbit antibody against actin was purchased from Sigma-Aldrich Chimie (A2066). Immunoblotting was performed with goat anti-rabbit or sheep anti-mouse secondary antibodies, purchased from Amersham (Piscataway, NJ, USA). The bound immunoglobulins were visualized using the ECL detection system (Amersham). Immunoblotting films were analyzed by densitometry with the PC-Bas 2.0 software (Fuji, Stanford, CT, USA).

*Quantitative real-time (qRT)-PCR*. Total RNA was isolated from the cells using the RNeasy Mini kit from Qiagen (Courtaboeuf, France). For analyzing the transcription of p21 and p27, 1 $\mu$g of total RNA was reverse-transcribed in 20 $\mu$l reaction mix using the SuperScript II First-Strand Synthesis System (Invitrogen).

qRT-PCR was carried out in Roche LightCycler using DNA double-strand-specific SYBR-Green I dye for detection (Roche). P21 primer sequences were: 5'-CTG GTG ACT CTC AGG GTC GAA-3' (sense primer) and 5'-GGA TTA GGG CTT CCT CTT GGA-3' (antisense primer). Primer sequences for p27 were: 5'-AGA CGG GGT TAG CGG AGC-3' (sense primer) and 5'-GAA CCG TCT GAA ACA TTT TCT TCT GT-3' (antisense primer). The relative mRNA levels in cells were calculated using the ΔΔCt method with endogenous RS9 mRNA as a control (21).

## Results

In the present study, we investigated the synergism between antiestrogens and antiproteasome treatments in cancer cell lines. At first, we studied the effects of 3 antiestrogens with partial agonist/antagonist or pure antagonist activities in combination with PIs at $IC_{50}$ concentrations [as previously described (21)] of 0.3 $\mu$M MG132 and 26 nM bortezomib. Combinations appeared significantly more effective at reducing the growth than the individual treatments (Fig. 1A and B) in the MCF7 cancer cells. As shown in Fig. 1A, a 4-day treatment with OH-tamoxifen and MG132 resulted in a 62% inhibition of growth, whereas the separate treatments inhibited 43% of

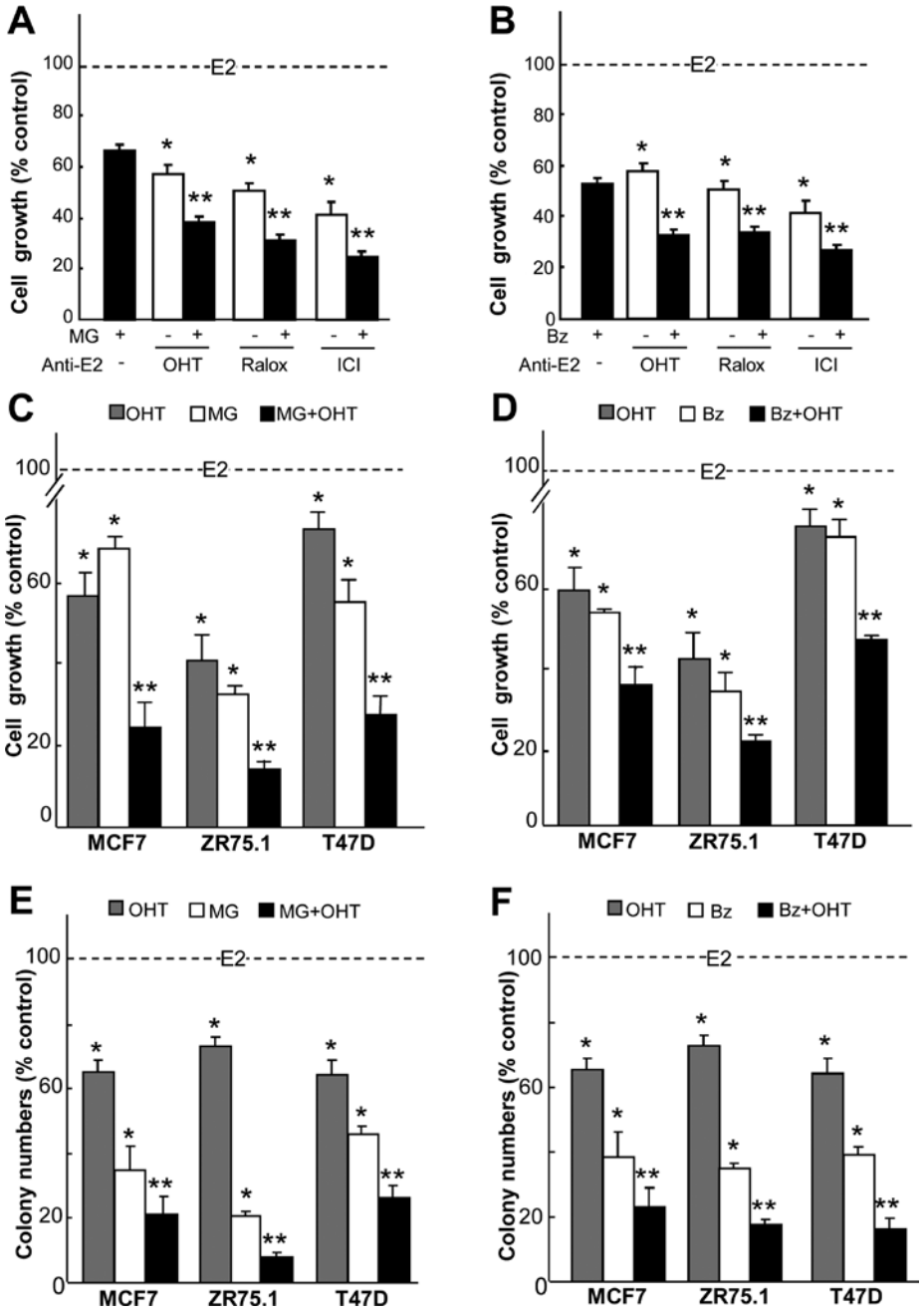


Figure 1. Cell viability in the presence of antiestrogens and PIs. Monitoring of MCF7 cell growth in the presence of antiestrogens and (A) MG132 (MG) or (B) bortezomib (Bz). Cell growth was evaluated by MTT assay after a 4-day treatment with 1 nM estradiol (E2) alone or combined with 1 $\mu$M OH-tamoxifen (OHT), 1 $\mu$M raloxifen (Ralox), 0.1 $\mu$M fulvestrant (ICI), 0.3 $\mu$M MG132 (MG) or 26 nM bortezomib (Bz). Values are expressed as a percentage of E2-treated cells. Values represent the mean ± SD of 3 experiments. Effects of (C) MG132 (MG) and (D) bortezomib (Bz) on cell outgrowth were evaluated on several ER-positive cell lines. The experiment was performed with 1 nM E2, 1 $\mu$M OH-tamoxifen (OHT) and 0.3 $\mu$M MG132 (MG) or 26 nM bortezomib (Bz). Values represent the mean ± SD of 3 experiments with E2 as control. (E and F) Colony forming assays in soft agar. Colony numbers were evaluated after OH-tamoxifen (OHT) (1 $\mu$M), MG132 (MG) (0.3 $\mu$M) or bortezomib (Bz) (26 nM) treatments. Values are expressed as percentage of colonies in the E2-treated cells. Mean ± SD of 2 independent experiments in duplicate. *P<0.01 from Student's t-test. PIs, proteasome inhibitors.

the growth. OH-tamoxifen also showed a synergistic effect with bortezomib (Fig. 1B) where growth inhibition reached 70%. Ralox and $ICI_{182,780}$ combination also obtained an additive antiproliferative activity with MG132 and bortezomib. However, in the case of $ICI_{182,780}$, the benefit attributed to the combination was limited by the strong effect of $ICI_{182,780}$ alone. Therefore, we focused on OH-tamoxifen to test the combinatorial treatments on several ER-positive breast cancer cell lines. Cell viability assays of MCF7, ZR75.1 and T47D cells were then carried out with PIs and OH-tamoxifen. For all cell lines, the antiproliferative activity of the combined agents was significantly higher than that of the single treatments (Fig. 1C and D). Next, we compared the effects of the combination on colony formation in soft agar. Treatments with MG132 (Fig. 1E) or bortezomib (Fig. 1F) in combination with OH-tamoxifen were found to drastically decreased cell colony formation as compared to single treatment. This confirmed, in an anchorage-independent condition, the synergistic activity

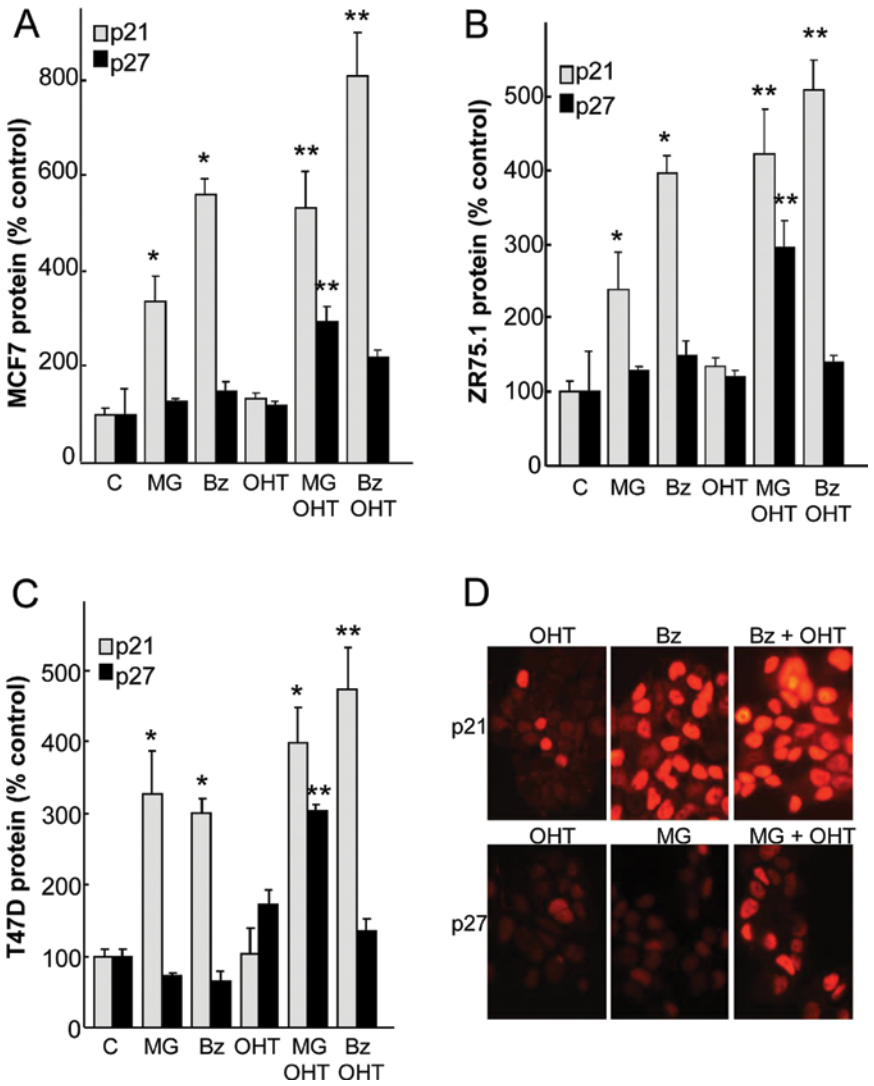


Figure 2. Control of p21 and p27 protein levels by PIs and antiestrogens after a 24 h treatment. Quantification of p21 and p27 expression by western blot analysis in (A) MCF7, (B) ZR75.1 and (C) T47D cell lysates. Cells were treated for 24 h with 1 nM estradiol (E2), 1 $\mu$M OH-tamoxifen (OHT), 0.3 $\mu$M MG132 (MG) and 26 nM bortezomib (Bz). Analysis was carried out for 50 $\mu$g protein and normalized with actin detection in the same samples. Values represent the mean ± SD of 3 experiments with E2 as control. *P<0.01 from Student's t-test. (D) Immunofluorescence with p21 or p27 monoclonal antibody detected by Alexa Fluor 568 dye in the MCF7 cells after a 24-h treatment. Images are representative of 2 distinct experiments. PIs, proteasome inhibitors.

of PIs and antiestrogens as shown by cell viability assays. One can also note that the importance of the combination also lies in using minimized concentrations of active compounds to obtain a maximal inhibition.

As PIs and OH-tamoxifen are known to regulate cell growth by controling cell cycle proteins, we investigated their effects on p27 and p21 expression in three ER-positive cell lines (Fig. 2A-C). A 24-h treatment with 0.3 $\mu$M MG132 or 30 nM bortezomib significantly increased p21 levels in all cell lines in a range of 2.4- to 5.5-fold. Combination with OH-tamoxifen with each PI significantly raised p21 levels over the values of the single treatments. These effects correspond to a synergistic action of OH-tamoxifen and PI. In contrast to p21, the regulation of p27 appeared different, since the expression of this CKI was not significantly affected by treatments except for OH-tamoxifen plus MG132, which significantly increased p27 in all cell lines. Taken together, these data suggest a synergism in the action of antiestrogens and PIs on p21 and p27 expression.

As previous data indicate that CKI overexpression could be associated with cellular delocalization (30), we verified the nuclear localization of these two CKIs by immunofluorescence (Fig. 2D). After treatments with PI and/or OH-tamoxifen, the overexpression of p21 and p27 was found to be mainly located in the nucleus in the MCF7 cells.

In order to explore the potential mechanism that increases CKIs, we semi-quantified mRNA levels of p21 and p27 using qRT-PCR assays after a 24-h treatment of PIs combined or not with antiestrogens. The two PIs increased p21 mRNA levels in all cell lines, from 3- to 4-fold in the T47D cells, 8- to 10-fold in the MCF7 cells and 12- to 13-fold in the ZR75.1 cells (Fig. 3). OH-tamoxifen treatment was not significantly effective alone on p21 mRNA and did not exhibit synergism when combined with PI. Considering p27, the results showed that PI alone or PI in combination with OH-tamoxifen downregulated p27 mRNA levels. These results indicate that p27 overexpression of CKIs is independent of its mRNA expression, whereas p21 expression can be regulated at the mRNA level by PIs.

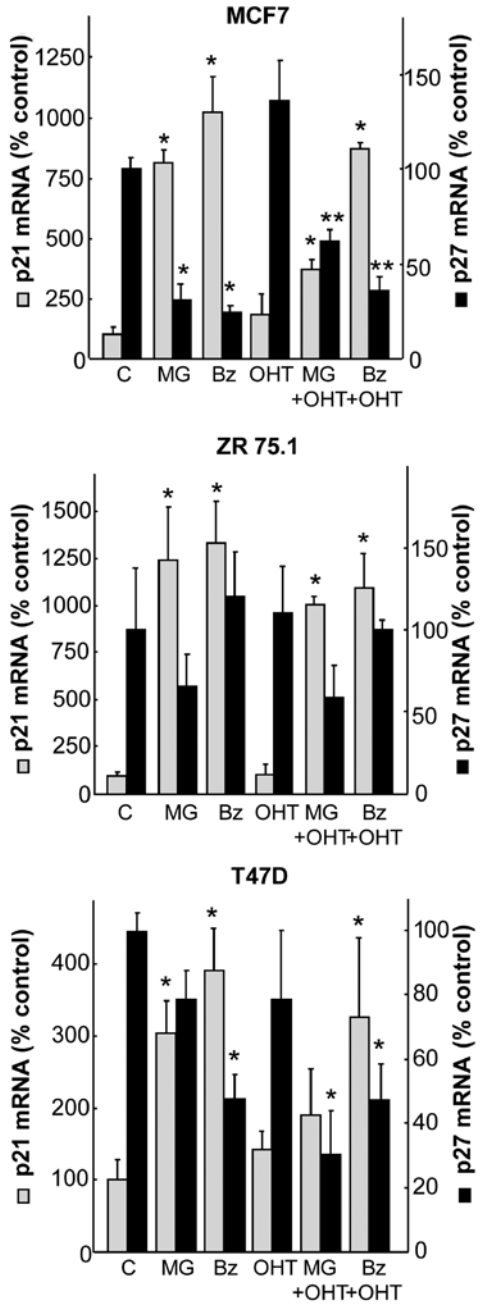


Figure 3. Expression levels of p21 and p27 mRNA by PIs and antiestrogens after a 24-h treatment. The concentrations of p21 and p27 mRNA were evaluated by qRT-PCR in extracts of MCF7, ZR75.1 and T47D cells treated with 1 nM estradiol (E2), 1 $\mu$M OH-tamoxifen (OHT), 0.3 $\mu$M MG132 (MG) and 26 nM bortezomib (Bz). E2-treated mRNA level was considered as an internal control and taken as 100%. Values represent the mean ± SD of 3 experiments. *P<0.01 from Student's t-test. PIs, proteasome inhibitors.

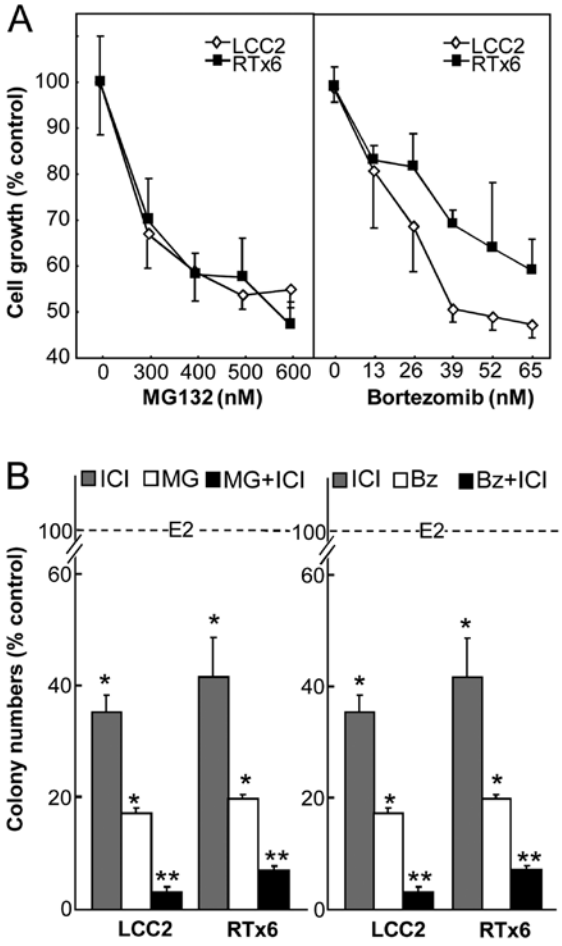


Figure 4. Outgrowth assay of antiestrogen-resistant cells treated with PIs. (A) Cell growth was evaluated in OH-tamoxifen-resistant cells (LCC2) and tamoxifen-resistant cells (RTx6). Both cell lines were selected from MCF7 cells by continuous exposure with antiestrogens. After a 4-day treatment with MG132 (MG) or bortezomib (Bz), cell growth was evaluated by MTT assay. Values are expressed as the percentage of E2-treated cells. Values represent the mean ± SD of 3 experiments. (B) Colony forming assays in soft agar. Colony number following treatment with 0.3 $\mu$M MG132 (MG) or 26 nM bortezomib (Bz) combined or not with 0.1 $\mu$M $ICI_{182780}$. Values are expressed as the percentage of E2-treated cells. Mean ± SD of 2 independent experiments in duplicate. *P<0.01 from Student's t-test. PIs, proteasome inhibitors.

To gain further insight into the study of PIs in combination with antiestrogens, we investigated the effects of PIs on tamoxifen- and OH-tamoxifen resistant cells. For this purpose, the MCF7 sublines LCC2 and RTx6 respectively selected for their resistance to OH-tamoxifen and tamoxifen were analyzed. At first, we tested their sensitivity to MG132 and bortezomib by cell growth assays (Fig. 4A). The dose-dependent growth inhibition of these cell lines appeared in the same range after a 4-day treatment. The $IC_{50}$ value was 0.35 $\mu$M for MG132 and ~40 nM for bortezomib. When compared under the same experimental conditions, the inhibition obtained by 65 nM bortezomib was 51 and 40%, respectively, for LCC2 and RTx6 cells whereas the wild-type MCF7 had a 73% inhibition of growth (21). Therefore, these cell sublines were less sensitive to bortezomib than the original cell line. We then analyzed the effects on colony formation in soft agar with 0.1 $\mu$M $ICI_{182,780}$ combined with PIs (0.3 $\mu$M MG132 or 26 nM bortezomib) (Fig. 4B). Treatments were found to synergistically decrease cell colony formation as compared to single agent treatment. This indicates the interest of a PI and an antiestrogen combination even in resistant cell lines. Importantly, we were surprised to find that the combination restored sensitivity in the resistant cell lines close to that of the MCF7 parental cell line.

To gain further insight into the ability of bortezomib to reverse resistance, p21 and p27 expression levels were analyzed after a 24-h treatment (Fig. 5A and B). In the resistant cell lines, the PI-induced overexpression of p21 was also

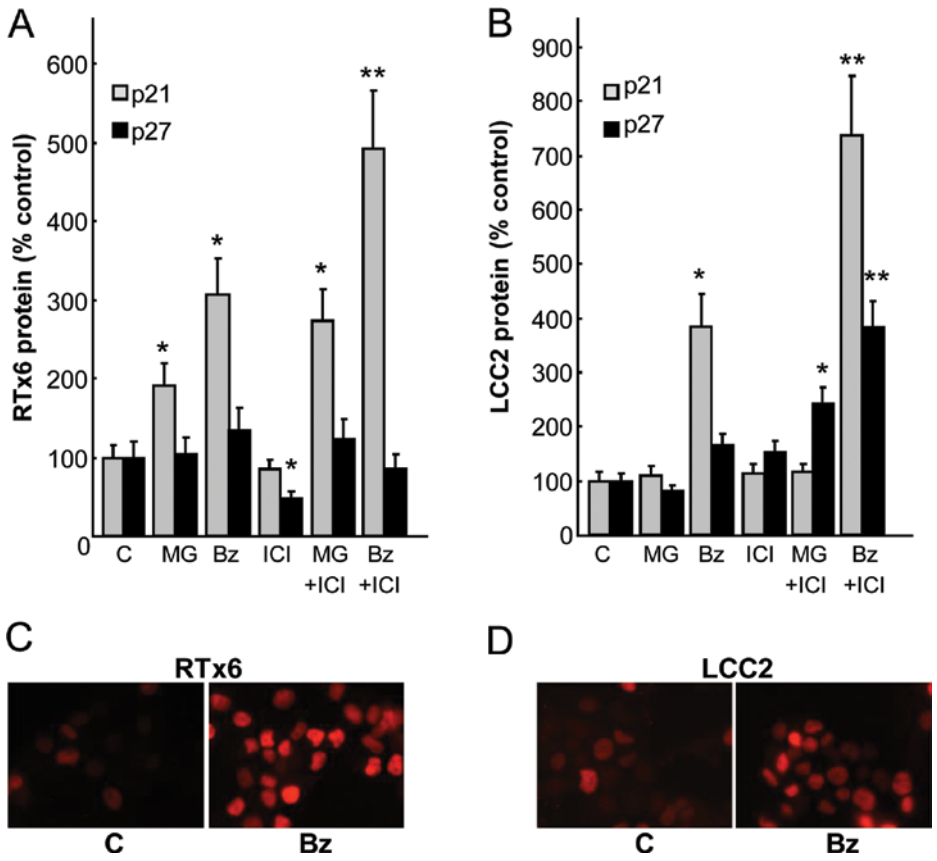


Figure 5. p21 and p27 protein levels in antiestrogen-resistant cells treated with PIs and antiestrogens for 24 h. Quantification of p21 and p27 expression by western blot analysis in (A) RTx6 and (B) LCC2 cell lysates. Cells were treated for 24 h with 1 nM estradiol (E2) alone or in combination with 0.1 $\mu$M $ICI_{182780}$ (ICI), 0.3 $\mu$M MG132 (MG) or 26 nM bortezomib (Bz). Analysis was carried out on 50 $\mu$g protein and normalized with actin detection in the same samples. Values represent the mean ± SD of 3 experiments with E2 as control. *P<0.01 from Student's t-test. (C and D) Immunofluorescence with the p21 monoclonal antibody detected by Alexa Fluor 568 dye in RTx6 and LCC2 cells after a 24-h treatment with 1 nM estradiol (E2) alone or in combination with 26 nM bortezomib (Bz). Images are representative of 2 distinct experiments. PIs, proteasome inhibitors.

noted, although the increase was inferior to that observed in the parental MCF7 cells. Notably, the addition of antiestrogens that are poorly or not effective alone, synergized with bortezomib as in the parental MCF7 cells. Concerning p27 expression, a synergistic effect was observed with $ICI_{182,780}$ but only in the RTx6 cell lysates. We also confirmed that overexpression of p21 occurs in the nucleus of these cells by immunofluorescence (Fig. 5C and D). The present study also confirmed that combined therapy could also be utilized on targeting resistant breast cancers.

## Discussion

Since some PIs have been approved by the US Food and Drug Administration (FDA) for primary treatment of multiple myeloma, they have emerged as an important therapeutic strategy in hematologic malignancies and solid tumors. To date, primary treatment with bortezomib alone in patients with advanced metastatic breast cancers have shown no positive responses (31-33). However, bortezomib may be successful for the treatment of breast cancer patients in combination with other therapies. Combination effects of bortezomib with chemotherapeutic agents, such as taxanes, anthracyclins or antibodies have already demonstrated significant positive responses in phase II trials (34-36).

In the present study, the combined treatments of antiestrogens and PIs were found to synergistically inhibit the growth of three ER-positive breast cancer cell lines. Furthermore, these combinations were also active on the growth of two antiestrogen-resistant cell lines. This later result is in agreement with data from Periyasamy-Thandavan *et al*, who demonstrated that bortezomib prevented cell survival of an OHT-resistant MCF7 cell line (37).

Notably, we found that, depending on the PI used, the CKI recruitment was different. MG132 combination was associated with a p27 accumulation in breast cancer cells while bortezomib action was preferentially correlated with p21 overexpression. Such difference in recruitment of CKIs has been reported for ubiquitylation and proteasome-mediated degradation (38).

Furthermore, as an antiestrogen, bortezomib also requires an intact ER expression. Indeed, although the specific mechanisms of PI-induced growth arrest remain to be elucidated, we showed that bortezomib efficacy on ER-positive cells relies on a functional ER (21). Notably, cancers with endocrine acquired resistance or estrogen-independency are often associated with the expression of a functional ER (5,39-42). Therefore, our data suggest that ER-positive cells resistant to classical endocrine therapies could be sensitive to PI action.

Collectively, PI and antiestrogen combination offers a new approach to treat ER-positive breast cancer. As antiestrogen

efficacy in breast cancer treatment is limited by the frequent development of acquired resistance, the current data provide a rationale for further insight into the clinical evaluation of PI-combined therapies.

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