Bortezomib Enhances the Efficacy of Fulvestrant by Amplifying the Aggregation of the Estrogen Receptor, Which Leads to a Proapoptotic Unfolded Protein Response

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Abstract

Purpose: Fulvestrant is known to promote the degradation of the estrogen receptor (ER) in the nucleus. However, fulvestrant also promotes the aggregation of the newly synthesized ER in the cytoplasm. Accumulation of protein aggregates leads to cell death but this effect is limited as a result of their elimination by the proteasome. We tested whether combining fulvestrant with the proteasome inhibitor, bortezomib, could enhance the accumulation of ER aggregates and cause apoptotic cell death.

Experimental Design: The rate of aggregation of the ER was monitored in ER⁺ breast cancer cell lines, T47D, ZR-75.1, BT474, MDA-MB-361, MCF-7, fulvestrant resistance MCF-7, and tamoxifen-resistant T47D-cyclin D1 cells. Activation of the unfolded protein response, apoptosis, and metabolic rate were also monitored in these cell lines following treatment with fulvestrant, bortezomib, or bortezomib in combination with fulvestrant.

Results: We found that bortezomib enhances the fulvestrant-mediated aggregation of the ER in the cytoplasm without blocking the degradation of the ER in the nucleus. Further, these aggregates activate a sustained unfolded protein response leading to apoptotic cell death. Further, we show that the combination induced tumor regression in a breast cancer mouse model of tamoxifen resistance.

Conclusions: Adding bortezomib to fulvestrant enhances its efficacy by taking advantage of the unique ability of fulvestrant to promote cytoplasmic aggregates of the ER. As this effect of fulvestrant is independent of the transcriptional activity of the ER, these results suggest that this novel combination may be effective in breast cancers that are ER⁺ but estrogen independent. Clin Cancer Res; 17(8); 2292–300.

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Introduction

As 70% of breast cancers express the estrogen receptor (ER), endocrine therapy targeting the ER represents a major therapeutic tool. In addition to tamoxifen and aromatase inhibitors, fulvestrant is a third drug, which acts by promoting the proteasomal degradation of the ER (1, 2).

However, a second effect of fulvestrant is the aggregation of the newly synthesized ER in the cytoplasm (3–5). Such aggregation is not observed following treatment with tamoxifen and occurs following the disappearance of the ER in the nucleus (5). Despite these findings, the current view of the mode of action of fulvestrant only considers its effect on the degradation of the ER in the nucleus.

Accumulation of protein aggregates is cytotoxic. Elimination of protein aggregates can be achieved by 2 pathways: degradation by the proteasome or by autophagy (6). On one hand, macroautophagy and proteasomal degradation of protein aggregates limit the toxicity of protein aggregates. On the other hand, when protein aggregates become insoluble they can lead to the inhibition of the proteasome and the activation of the unfolded protein response (UPR). The UPR is a complex cellular response that can lead to either cytoprotective or proapoptotic responses (7). The outcome of the activation of the UPR is dependent on the intensity of the stress signal, with a mild stress leading to the activation of cytoprotective responses, whereas intense stress leads to apoptosis (7).

In neurodegenerative diseases, the toxicity of protein aggregates involves the activation of a proapoptotic UPR (8, 9). Therefore, these studies offer a precedent for the notion that cytoplasmic protein aggregates promote apoptosis via the activation of the UPR.

Interestingly, resistance to fulvestrant is associated with the activation of a cytoprotective UPR (10, 11).
observations raise the possibility that the ER aggregates formed following treatment with fulvestrant alone may generate a weak signal that activates such cytoprotective UPR. If so, drugs able to amplify these aggregates may allow the conversion to a proapoptotic UPR. However, this possibility was never tested.

Bortezomib is a Food and Drug Administration–approved proteasome inhibitor (12) shown to activate the UPR (13). Therefore, we initiated this study to test whether the addition of bortezomib to fulvestrant may enhance the efficacy of fulvestrant by amplifying the ER aggregates and inducing a proapoptotic UPR.

Materials and Methods

Cell culture, reagent, and transfection
T47D and ZR-75.1 cells were grown in RPMI medium supplemented with 10% FBS, insulin (5 mg/mL), and antibiotics (Life Technologies, Inc.). MCF-7, MCF-7-F40, BT474, MDA-MB-361, MDA-MB-453, and MDA-MB-157 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and antibiotics. For depletion of estrogen, cells were cultured in phenol red–free RPMI 1640 or DMEM supplemented with 10% charcoal/dextran-treated FBS (HyClone), insulin (5 mg/mL), and antibiotics before the treatments. Bortezomib was dissolved in saline solution at a dose of 2.6 mmol/L as a stock solution (Millennium). Fulvestrant (Tocris) was dissolved in 100% ethanol. Transient transfections for green fluorescent protein (GFP)-ER were conducted by the Amaxa nucleofection technology according to the manufacturer’s instructions.

Western blot analysis
Western blot analyses were carried out as described previously (14). Using the following antibodies: rabbit anti-ER antibody (G-20; Santa Cruz), mouse anti-BiP (DB Biosciences), rabbit anti-LC3 antibody (PM036; MBL International), rabbit anti-cleaved caspase 3 antibody (Cell Signaling), rabbit anti-cleaved caspase 9 antibody (Cell Signaling), or mouse anti-tubulin antibody (University of Iowa).

Immunofluorescence staining
T47D cells were transfected with GFP-ER and ubiquitin by Amaxa nucleofection. Staining was done by using the rabbit anti-ubiquitin antibody (Sigma Aldrich) and antimouse Alexa Fluor 594 (Molecular probes) as described previously (14).

Proteasomal activity assay
10 μg of T47D cell extracts treated with or without fulvestrant and/or bortezomib were incubated for 2.5 hours at 37°C in 100 μL assay buffer (50 mmol/L Tris-HCl, pH 7.5) with 10 mmol/L fluorogenic substrate Suc-LLVY-AMC (CALBIOCHEM). Release of free hydrolyzed 7-amino-4-methylcoumarin (AMC) groups was measured by using an ISS Counter with an excitation filter of 380 nm and an emission filter of 460 nm (PerkinElmer).

Xenograft Implantation and Measurement of Tumor Size
Xenografts were established as described previously (14). Bortezomib (0.05 mg/kg body weight) was given by tail vein injection twice weekly. Fulvestrant (5 mg) dissolved in 100% ethanol and diluted in peanut oil was injected subcutaneously, weekly. Our protocol was approved by the animal ethic committee at Mount Sinai School of Medicine.

Results

Bortezomib does not block the fulvestrant-mediated degradation of the ER but enhances the cytoplasmic aggregation of the ER by fulvestrant

Fulvestrant promotes the proteasome-dependent degradation of the ER in the nucleus (2, 15, 16). Therefore, as expected, we found that treatment with fulvestrant alone led to a reduction in the ER level in the nucleus (Fig. 1A and B) and these effects of fulvestrant were inhibited when cells were incubated in the presence of the proteasome inhibitors, LLnL and MG132 (Fig. 1A and B). In contrast, incubation in the presence of the proteasome inhibitor bortezomib did not rescue the level of the ER to the level of untreated cells by Western blot analysis (Fig. 1A) and did not prevent the elimination of the ER from the nucleus by treatment with fulvestrant (Fig. 1B). As bortezomib inhibits only 1 catalytic activity of the proteasome, whereas LLnL and MG132 inhibit all 3 catalytic activities, this result
C, T47D cells were pretreated with or without 15 nmol/L of bortezomib for 30 minutes, then treated with or without 0.1 mM fulvestrant (Ful) and/or bortezomib (Bor), LLnL, or MG132 for 15 hours. 

Figure 1. The fulvestrant-mediated cytoplasmic ER aggregates are amplified by bortezomib. A, T47D cells were pretreated with or without 15 nmol/L of bortezomib, LLnL, or MG132 for 30 minutes, then treated with or without 0.1 mM of fulvestrant for 2 hours. ER levels were determined by Western blotting. B, immunofluorescence of GFP-ER in T47D cells treated with or without fulvestrant (Ful) and/or bortezomib (Bor), LLnL, or MG132 for 15 hours. C, T47D cells were pretreated with or without 15 nmol/L of bortezomib for 30 minutes, then treated with or without 0.1 mM of fulvestrant for additional 2 hours. The levels of ER in the soluble and insoluble fractions were determined by Western blotting. D, T47D cells were pretreated with or without 15 nmol/L of bortezomib for 30 minutes, then treated with or without 0.1 mM of fulvestrant for 1, 2, 3, or 4 hours. The levels of endogenous ER in the insoluble fractions were determined by Western blotting, whereas the localization of endogenous ER was determined by immunofluorescence (IF) at the 4 hours treatment time point. DAPI, 4',6-diamidino-2-phenylindole.

Figure 1.

indicates that the remaining catalytic activities are sufficient to promote the degradation of the ER in presence of bortezomib and fulvestrant.

We confirmed that fulvestrant leads to the accumulation of the newly synthesized GFP-ER in the cytoplasm (Fig. 1B) and found that in the presence of bortezomib and fulvestrant, the staining became localized into foci (Fig. 1B). Accumulation of ER aggregates in the cytoplasm was confirmed by immunofluorescence of endogenous ER (Fig. 1D). Further, the cytoplasmic accumulation of endogenous ER corresponded to the appearance of the ER in the insoluble fraction (Fig. 1C). However, detection of the ER in the insoluble fraction was abolished after 3 hours (Fig. 1D) by Western blotting, whereas the ER aggregates remained detectable after 4 hours by immunofluorescence (Fig. 1D) suggesting that they may become further modified.

These results revealed that bortezomib does not inhibit the elimination of the ER in the nucleus but enhances the accumulation of ER aggregates in the cytoplasm.

The cytoplasmic aggregates of the ER are ubiquitinated and inhibit the activity of the proteasome

Aggregation of proteins results from their misfolding. Misfolded proteins are eliminated by ubiquitin–proteasome pathway (6). We therefore tested whether the ER aggregates observed following fulvestrant and bortezomib treatment are ubiquitinated. T47D cells were transfected with both GFP-ER and ubiquitin and then treated with fulvestrant and bortezomib. We found that although ubiquitin localized throughout the cells, as expected, the intensity of the ubiquitin staining was stronger in the area where the ER aggregates were detected (Fig. 2A), suggesting that the aggregates are ubiquitinated. As ubiquitination results in a shift in molecular weight chains, this finding explains the disappearance of ER by Western blot analysis at a time point where the staining persists by immunofluorescence (Fig. 1D).

We next addressed the effect of endogenous ER aggregates on the activity of the proteasome. The lid of the proteasome contains ubiquitin receptor and unfoldases, which allow translocation of substrates into the catalytic core. We reasoned that ER aggregates may exceed the unfolding capacity of the lid and as a result prevent their translocation. As the proteasome is required for the continual turnover of proteins, the binding to the lid in absence of translocation may block the entrance of the catalytic core and therefore indirectly inhibit the proteasome. To test this possibility, we first measured the chymotrypsin activity of the proteasome by using a fluorogenic...
peptide. As expected, we found that bortezomib alone was very efficient (80% inhibition of cleavage) at blocking cleavage of this peptide, because bortezomib is an inhibitor of the chymotrypsin-like activity (Fig. 2B). However, we also found that fulvestrant alone inhibits the chymotrypsin-like activity by 20% (Fig. 2B) suggesting that the accumulation of endogenous ER aggregates following fulvestrant are sufficient to partially limit the access of the peptide to the enzyme. Combining fulvestrant and bortezomib led to an additive inhibition of the chymotrypsin-like activity (Fig. 2B).

Second, we assessed the ability of fulvestrant, bortezomib, or both to inhibit the elimination of polyubiquitinated proteins in general. We found that the baseline level of ubiquitination was not significantly affected by bortezomib alone (Fig. 2C). Treatment with fulvestrant alone led to the mild accumulation of high molecular weight polyubiquitinated proteins, suggesting that unlike bortezomib, the inhibition of the proteasome as a result of the formation of ER aggregates is not specific to any catalytic activity but acts by blocking the entrance of the catalytic core. However, treatment with both fulvestrant and bortezomib led to a drastic accumulation of high molecular weight polyubiquitinated proteins (Fig. 2C). These results indicate that combining the specific inhibition of the chymotrypsin-like activity and the inhibition of translocation of polyubiquitinated proteins represent a double lock on the activity of the proteasome (Fig. 2D).

The fulvestrant–bortezomib combination induces the unfolded protein response and apoptosis

Inhibition of the proteasome activates the UPR, which is characterized by elevated level of the chaperone BiP (7). Therefore, we analyzed the level of BiP by Western blotting in T47D cells. We found that, as expected, bortezomib led to an elevation in the level of BiP (Fig. 3A) and, further, fulvestrant alone also led to an elevation in the levels of BiP, a result that is consistent with the ability of fulvestrant alone to partially inhibit the proteasome (Fig. 2B, D). However, BiP levels were drastically more elevated following treatment with both drugs (Fig. 3A).

To determine the outcome of the activation of the UPR by fulvestrant, bortezomib, or both, we next analyzed a panel of apoptotic markers in T47D cells. We found that fulvestrant alone did not affect the levels of bid, bcl-2, or procaspase 8, nor did it lead to the cleavage and activation of caspases 3 and 9 compared with the untreated cells (Fig. 3B); suggesting that fulvestrant alone generates a mild stress, which activates a cytoprotective outcome of the UPR. Treatment with bortezomib alone led to a reduction in bid, bcl-2, and procaspase 8, as well as the detection of cleaved caspases 3 and 9 (Fig. 3B); however, these effects were drastically more severe on treatment with both fulvestrant and bortezomib (Fig. 3B). These results suggest that the addition of bortezomib to fulvestrant results in a strong stress signal that activates a proapoptotic UPR.

To determine the effect of the combination, we next carried out an MTT assay on T47D and ZR-75.1 cells treated with bortezomib alone or in combination with fulvestrant. We found that although 12.5 nmol/L bortezomib led to a moderate reduction in metabolic rate, respectively, addition of fulvestrant reduced the metabolic rates by 60% in T47D cells (Fig. 3C) and 73% in ZR-75.1 cells (Fig. 3D). One-way ANOVA test also revealed that there is statistically significant difference when fulvestrant is combined with higher concentration of bortezomib.
in both T47D cells (10 and 12.5 nmol/L of bortezomib for P values of 0.0005 and 0.01, respectively) and ZR-75.1 cells (10 and 12.5 nmol/L of bortezomib for P values of 0.001 and 0.03, respectively).

The sensitivity to the fulvestrant–bortezomib was further tested in 2 additional ER⁺ cell lines, BT474 and MDA-MB-361. We found that BT474 cells were insensitive to both 3 μmol/L fulvestrant alone and 10, 30, and 50 nmol/L bortezomib alone (Fig. 3E). However, when 3 μmol/L fulvestrant was combined to 30 and 50 nmol/L bortezomib, a reduction in metabolic rate was observed and was statistically significant with P values of 0.001 and 0.0001 (Fig. 3E). However, MDA-MB-361 cells were not affected by either drug alone or in combination (data not shown). Considering that the levels of the ER is drastically low in BT474 and MDA-MB-361 cells compared with T47D and ZR-75.1 cells (Fig. 3F), the requirement for increased dose of bortezomib is in fact not surprising. Further, the observation that the level of the ER is even lower in MDA-MB-361 than in BT474 cells, also offers an explanation for the insensitivity of MDA-MB-361 cells.
These results and the fact that fulvestrant acts on the ER predicts that ER\(^+\) cell lines should be resistant to the fulvestrant–bortezomib combination. To test this possibility the ER\(^+\) cell lines, MDA-MB-453 and MDA-MB-157, were treated with the combination. We found that in both cases, the combination had no significant effect compared with bortezomib alone (Supplementary Data) indicating that the efficacy of the combination is dependent on the expression of the ER.

Collectively, these results suggest that the inhibition of the proteasome that results from treatment with the combination leads to the activation of a proapoptotic UPR.

**Autophagy confers resistance to the fulvestrant–bortezomib combination**

The sensitivity of the ER\(^+\) breast cancer cell line MCF-7 was also tested. Although MCF-7 cells were found to be sensitive to fulvestrant alone, the addition of bortezomib did not significantly increase the sensitivity (Fig. 4A). Further, we found that unlike T47D cells (Fig. 3A) BiP was unaffected (Fig. 4B), indicating that the UPR is not activated in MCF-7 cells.

We next tested whether autophagy is activated by using the elevation of LC3-II levels as a marker. We found that bortezomib, but not fulvestrant, leads to the elevation in LC3-II in MCF-7 cells (Fig. 4B, middle), whereas there was a minimal elevation in LC3-II levels in T47D cells (Fig. 4B, bottom). Induction of autophagy was confirmed by the detection of arc shape membranes and late autophagosomes by electron microscopy (Fig. 4C). As autophagy represents a mechanism of resistance to proteasome inhibition (17), we concluded that macroautophagy causes resistance to the combination in MCF-7 cells.

We next aimed at defining the sensitivity of fulvestrant-resistant cells to the combination. The MCF-7-F40 line (18) was obtained and tested for its sensitivity. We found that these cells were resistant to both 3 \(\mu\)mol/L fulvestrant alone or increasing doses of bortezomib (Fig. 4D). However, treatment with 3 \(\mu\)mol/L fulvestrant in combination with 30 and 50nmol/L bortezomib reduced their metabolic rate significantly with \(P\) values of 0.0008 and 0.0001. Therefore, this result suggests that the fulvestrant–bortezomib combination can restore sensitivity in otherwise fulvestrant-resistant cells.
The bortezomib–fulvestrant combination promotes regression of tamoxifen-resistant breast cancer xenografts

Overexpression of cyclin D1 is associated with resistance to tamoxifen and we confirmed this observation by using T47D-cyclin D1 cells, which overexpress cyclin D1 (14). We tested whether T47D-cyclin D1 cells are sensitive to the fulvestrant–bortezomib combination and found that these cells were the most sensitive, with a 90% decrease in metabolic rate (Fig. 5A). One-way ANOVA test also revealed that there is statistically significant difference when fulvestrant is combined with bortezomib (P values of 0.004, 0.0005, and 0.00045 for 7.5, 10, and 12.5 nmol/L of bortezomib, respectively).

The sensitivity of T47D-cyclin D1 cells was further tested by using a xenograft model. Tumors were allowed to growth for 3 weeks and mice were then randomized to 1 of following 4 groups: (i) control, (ii) fulvestrant alone, (iii) bortezomib alone, and (iv) fulvestrant–bortezomib combination. We found that both fulvestrant and bortezomib led to a reduction in the growth rate of the tumors compared with tumors in the untreated group (Fig. 5B). Repeated measures ANOVA statistical analysis revealed P values of 0.0045 for control group versus fulvestrant + bortezomib group. Therefore, these results indicate that the fulvestrant–bortezomib combination is effective in this model of tamoxifen resistance.

Activating mutations in the ER have been identified in ER+ but tamoxifen-resistant breast cancer patients (19). However, because the fulvestrant–bortezomib approach relies on the ability to promote toxic aggregates of the ER, we reasoned that mutants ER might be more prone to aggregation as they are misfolded. To test this possibility, we created 2 mutants, Y537N and Y537S, by site directed mutagenesis, transfected these plasmids in T47D cells and incubated the cells in the presence of fulvestrant and bortezomib for 1 hour. We found that although no aggregation of the ER is observed at this time point in cells expressing wild-type ER, aggregation in the cytoplasm is readily detectable in cells expressing mutants ER (Fig. 5C). Therefore, these results support the notion that ER+ but estrogen-independent breast cancers, due to mutation in the ER, maybe sensitive to the fulvestrant–bortezomib combination.

Discussion

Figure 5D shows a model that summarizes the effect of fulvestrant and bortezomib. First, fulvestrant promotes the degradation of the ER in the nucleus, blocking its ability to promote transcription. Second, fulvestrant causes the
aggregation of newly synthesized ER in the cytoplasm, which causes inhibition of the proteasome by clogging the lid. Third, bortezomib inhibits the proteasome and increases the ER aggregates formed by fulvestrant. The excessive accumulation of ER aggregates then causes activation of a proapoptotic UPR.

The ability of fulvestrant to promote the degradation of the ER is currently considered the only mechanism by which fulvestrant prevents the growth of ER$^+$ breast cancer cells. Although the ability of fulvestrant to promote the aggregation of the ER in the cytoplasm has been described by several groups (3–5), whether these aggregates play a role in the mode of action of fulvestrant had never been investigated previously. Our data show that these cytoplasmic aggregates of the ER lead to a mild inhibition of the proteasome and the induction of the UPR without the activation of apoptosis. Therefore, fulvestrant activates a cytoprotective UPR, which suggests that the aggregates do not contribute to the mode of action of fulvestrant to prevent the growth of ER$^+$ breast cancer cells. In fact, these observations raise the possibility that the induction of a cytoprotective UPR by fulvestrant may contribute to resistance to fulvestrant. In agreement with this possibility, splicing of XBP1 and activation of NF-kB, 2 cytoprotective pathways downstream of the activation of the UPR have been identified as mechanisms of resistance to fulvestrant (10, 11, 20–22). These results imply that the efficacy of fulvestrant may be limited by its ability to activate these cytoprotective responses.

However, our data also suggest that by adding bortezomib, the activation of a cytoprotective UPR by fulvestrant is converted into a proapoptotic UPR. As bortezomib is known to stabilize the inhibitor of NF-kB and therefore block the activity of NF-kB (23) and that bortezomib was also reported to block the splicing of XBP-1 (24), these observations suggest the switch from a cytoprotective UPR with fulvestrant alone to a proapoptotic UPR with the fulvestrant–bortezomib combination.

Therefore, we propose that bortezomib enhances the efficacy of fulvestrant by acting at 2 distinct points: (i) the enhancement of the fulvestrant-mediated aggregation of the ER and (ii) the inhibition of NF-kB activity and XBP-1 splicing. This unique mode of action represents a strong scientific rationale for the design of a clinical trial testing the superiority of the fulvestrant–bortezomib combination over fulvestrant alone in ER$^+$ breast cancer patients that have recurred on prior endocrine therapy.

However, our data also show that although MCF-7 cells were the most sensitive to fulvestrant alone, addition of bortezomib did not significantly increase the efficacy of fulvestrant in these cells. This observation is in agreement with the induction of autophagy rather than the UPR in these cells. This result is consistent with the observations that autophagy contributes to the efficacy of fulvestrant in these cells (25) and induces resistance to bortezomib (17, 26). Therefore, autophagy may be a mechanism by which bortezomib does not enhance the efficacy of fulvestrant. Our data suggest that the fulvestrant–bortezomib combination works best in tamoxifen-resistant models. The observation that cyclin D1-overexpressing cancer cells are more sensitive than nonoverexpressing cells is likely because of the fact that the threshold of induction of apoptosis by bortezomib is lower in these cells (14). Further, our data raise the possibility that mutations affecting the ER may serve to sensitize cells further to this novel combination by exploiting their altered conformation. If so, ER$^+$ but estrogen-independent cells that are resistant to all form of classical endocrine therapy approach are predicted to be sensitive to the fulvestrant–bortezomib combination.

As for the fulvestrant-resistant MCF-7-F40 cells, we found that they are also resistant to bortezomib suggesting that these cells are very different from MCF-7 cells. This possibility supports the recent hypothesis that antiestrogen resistance does not arise from acquired mutation but rather from selection of a small preexisting population of cells that are intrinsically resistant (18). As we found that addition of higher dose of bortezomib in combination with fulvestrant was able to reverse resistance in these cells, we propose that the fulvestrant-resistant cells may simply have an increased capacity of induction of cytoprotective UPR and that in this context higher dose of bortezomib is required to convert this response to a proapoptotic UPR. Alternatively, high doses of bortezomib were recently shown to cause the complete repression of the transcription of the ER (27). Therefore, by promoting the degradation of the ER by fulvestrant and the inhibition of its transcription by bortezomib, the combination may completely abolish ER signaling in these cells.

In addition, we found that even in cells in which the expression of the ER is very low such as that observed in BT474 cells, the fulvestrant–bortezomib combination was effective although higher doses of bortezomib (30–50 nmol/L) were required. Considering that the serum levels of bortezomib reaches 509 μmol/L (12) after injection and gradually decreases, these higher doses are easily achievable clinically.

In summary, the ability of bortezomib to enhance the efficacy of fulvestrant by at least 2 different mechanisms makes this combination even more attractive.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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