Bortezomib enhances the efficacy of fulvestrant by amplifying the aggregation of the estrogen receptor, which leads to a pro-apoptotic unfolded protein response.

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**Translational relevance:** Endocrine therapy is the most frequent treatment of estrogen receptor (ER) positive breast cancers. Endocrine therapy acts by repressing the transcriptional activity of the ER. One major difficulty with this approach is that it allows for the selection of mutations that results in the growth of cells that have adapted to low estrogen conditions and causes recurrence. Results presented here suggest that the efficacy of endocrine therapy can be enhanced using the unique ability of fulvestrant to cause the aggregation of the ER. We describe that by combining fulvestrant to the proteasome inhibitor, bortezomib, the aggregation of the ER results in apoptosis and tumor regression. Fulvestrant is currently approved in post-menopausal women with breast cancers that have recurred on other endocrine therapies. The result presented in this study served as the basis for a phase II randomized trial comparing fulvestrant alone or in combination with bortezomib in this patient population.
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 estrogen receptor positive breast cancer cells lines, T47D, ZR-75.1, BT474, MDA-MB361, MCF7, 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. Since 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 positive but estrogen independent.
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 proteosomal degradation of the ER (Buzdar & Robertson, 2006; Stenoien et al, 2001).

However, a second effect of fulvestrant is the aggregation of the newly synthesized ER in the cytoplasm (Dauvois et al, 1993; Htun et al, 1999; Pick et al, 2007). Such aggregation is not observed following treatment with tamoxifen and occurs following the disappearance of the ER in the nucleus (Pick et al, 2007). 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 two pathways; degradation by the proteasome or by autophagy (Ding & Yin, 2008). 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 pro-apoptotic responses (Schroder, 2008). 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 a cytoprotective responses, while intense stress leads to apoptosis (Schroder, 2008).

In neurodegenerative diseases, the toxicity of protein aggregates involves the activation of a pro-apoptotic UPR (Nishitoh et al, 2002; Nishitoh et al, 1998). 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 (Davies et al, 2008; Gu et al, 2002). These 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 amplified these aggregates may allow the conversion to a pro-apoptotic UPR. However, this possibility was never tested.

Bortezomib is an FDA approved proteasome inhibitor (Bross et al, 2004) shown to activate the UPR (Landowski et al, 2005). 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 pro-apoptotic UPR.
Materials and Methods

Cell culture, reagent and transfection. T47D and ZR75.1 cells were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS), insulin (5 mg/ml), and antibiotics (Life Technologies, Inc.). MCF7, MCF7-F40, BT474, MDA-MB-361, MDA-MB-453 and MDA-MB 157 cells were grown in DMEM medium supplemented with 10% FBS and antibiotics. For depletion of estrogen, cells were cultured in phenol-red-free RPMI 1640 or DMEM medium 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 mM as a stock solution (Millennium). Fulvestrant (Tocris) was dissolved in 100% ethanol. Transient transfections for GFP-ER were performed using the Amaxa nucleofection technology according to manufacture’s instruction.

MTT assay. Methylthiazoletetrazolium (MTT, Sigma) assays were performed as described previously (Ishii et al, 2008) using the indicated drugs.

Western blot analysis. Western analysis were performed as described previously (Ishii et al, 2008). Using the following antibodies: rabbit anti-ER antibody (G-20Santa 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 using Amaxa nucleofection. Staining was performed using the rabbit anti-ubiquitin antibody (Sigma Aldrich) and anti-mouse Alexa Fluor 594 (Molecular probes) as described previously (Ishii et al, 2008).

**Proteasomal activity assay.** 10ug 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 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 (Ishii et al, 2008). Bortezomib (0.05 mg/kg body weight) was given by tail vein injection twice weekly. 5mg of fulvestrant 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 (Callige et al., 2005; Callige & Richard-Foy, 2006; Stenoien et al., 2001). Therefore, as expected, we found that treatment with fulvestrant alone led to a reduction in the ER level in the nucleus (Fig. 1A, B) and these effects of fulvestrant were inhibited when cells were incubated in presence of the proteasome inhibitors LLnL and MG132 (Fig. 1A, 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). Since bortezomib inhibits only one catalytic activity of the proteasome, while LLnL and MG132 inhibit all three catalytic activities, this result 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 using 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 blot, while 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 (Ding & Yin, 2008). 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 while 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. Since 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. Since the proteasome is required for the continual turn-over 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 using a fluorogenic peptide. As expected, we found that bortezomib alone was very efficient (80% inhibition of cleavage) at blocking cleavage of this peptide, since 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 poly-ubiquitinated 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 higher molecular weight poly-ubiquitinated 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 poly-ubiquitinated proteins (Fig. 2C). These results indicate that combining the specific inhibition of the chymotrypsin-like activity and the inhibition of translocation of poly-ubiquitinated 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 unfolded protein response (UPR), which is characterized by elevated level of the chaperone BiP (Schroder, 2008). Therefore, we analyzed the level of BiP by Western blot in T47D cells. We found that as expected bortezomib led to an elevation in the level of BiP (Fig. 3A) and that 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 pro-caspase8, nor did it led to the cleavage and activation of caspase 3 and 9 compared to the untreated cells (Fig. 3B), suggesting that fulvestrant alone generates a mild stress, which activates a cyto-protective outcome of the UPR. Treatment with bortezomib alone led to a reduction in bid, bcl-2 and pro-caspase 8, as well as the detection of cleaved caspase 3 and 9 (Fig. 3B), however, these effects were drastically more severe upon 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 pro-apoptotic UPR.

To determine the effect of the combination, we next performed an MTT assay on T47D and ZR-75.1 cells treated with bortezomib alone or in combination with fulvestrant. We found that while 12.5 nM bortezomib led to a 30% (Fig. 3C) and 50% (Fig. 3D) reduction in metabolic rate respectively, addition of fulvestrant reduced the metabolic rate by 60% in T47D cells (Fig. 3C) and 73% reduction 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 nM of bortezomib for p values of 0.0005 and 0.01 respectively) and ZR-75.1 cells (10 and 12.5 nM of bortezomib for p values of 0.001 and 0.03 respectively).

The sensitivity to the fulvestrant-bortezomib was further tested in two additional ER positive cell lines, BT474 and MDA-MB-361. We found that BT474 cells were insensitive to both 3μM fulvestrant alone and 10, 30 and 50nM bortezomib alone (Fig. 3E). However, when 3μM fulvestrant was combined to 30 and 50nM 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 lower in BT-474 and MDA-MB-361 cells compared to 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 negative cell lines should be resistant to the fulvestrant-bortezomib combination. To test this possibility the ER negative cell lines, MDA-MB453 and MDA-MD 157 were treated the combination. We found that in both cases, the combination had no significant effect compared to 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 pro-apoptotic UPR.
Autophagy confers resistance to the fulvestrant-bortezomib combination.

The sensitivity of the ER+ breast cancer cell line MCF-7 was also tested. While 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 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 panel), while there was a minimal elevation in LC3-II levels in T47D cells (Fig. 4B, lower panel). Induction of autophagy was confirmed by the detection of arc shape membranes and late autophagosomes by electron microscopy (Fig. 4C). Since autophagy represents a mechanism of resistance to proteasome inhibition (Milani et al, 2009), 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 (Coser et al, 2009) was obtained and tested for its sensitivity. We found that these cells were resistant to both 3μM fulvestrant alone or increasing doses of bortezomib (Fig. 4D). However, treatment with 3μM fulvestrant in combination with 30 and 50nM 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 using T47D-cyclin D1 cells, which overexpress cyclin D1 (Ishii et al, 2008). 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 nM of bortezomib respectively).

The sensitivity of T47D-cyclin D1 cells was further tested using a xenograft model. Tumors were allowed to growth for 3 weeks and mice were then randomized to one of 4 groups; 1) control, 2) fulvestrant alone, 3) bortezomib alone, or 4) fulvestrant-bortezomib combination. We found that both fulvestrant and bortezomib led to a reduction in the growth rate of the tumors compared to tumors in the untreated group (Fig. 5B). Repeated Measures ANOVA statistical analysis revealed p values of 0.031 for control group versus bortezomib group and of 0.043 for the control versus fulvestrant group. However, tumor volumes were found to regress in the fulvestrant-bortezomib group. Repeated Measures ANOVA statistical analysis revealed p values of 0.0019 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 (Herynk & Fuqua, 2004). However, since 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 while 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 pro-apoptotic 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. While the ability of fulvestrant to promote the aggregation of the ER in the cytoplasm has been described by several groups (Dauvois et al, 1993; Htun et al, 1999; Pick et al, 2007), whether these aggregates play a role in the mode of action of fulvestrant had never been
investigated previously. Our data shows 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 NFκB, two cytoprotective pathways downstream of the activation of the UPR have been identified as mechanisms of resistance to fulvestrant (Davies et al, 2008; Gomez et al, 2007; Gu et al, 2002; Pratt et al, 2003; Riggins et al, 2005). These results imply that the efficacy of fulvestrant may be limited by its ability to activate these cytoprotective responses.

However, our data also suggests that by adding bortezomib, the activation of a cytoprotective UPR by fulvestrant is converted into a pro-apoptotic UPR. Since bortezomib is well known to stabilize the inhibitor of NFκB and therefore block the activity of NFκB (McConkey & Zhu, 2008) and that bortezomib was also reported to block the splicing of XBP-1 (Lee et al, 2003), these observations suggest that the switch from a cytoprotective UPR with fulvestrant alone to a pro-apoptotic UPR with the fulvestrant-bortezomib combination.

Therefore, we propose that bortezomib enhances the efficacy of fulvestrant by acting at two distinct points; 1) the enhancement of the fulvestrant-mediated aggregation of the ER and 2) the inhibition of NFκB activity and XBP-1 splicing. This unique mode of action represents a strong scientific rational 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 shows that while MCF-7 cells were the most sensitive to fulvestrant alone, addition of bortezomib did not significantly increased 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 on one hand, the observation that autophagy contributes to the efficacy of fulvestrant in these cells (Clarke et al, 2009) and on the other hand, that autophagy induces resistance to bortezomib (Milani et al, 2009; Zhu et al). 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 non-overexpressing cells is likely due to the fact that the threshold of induction of apoptosis by bortezomib is lower in these cells (Ishii et al, 2008). Further, our data raises 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-7F40 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 anti-estrogen resistance does not arise from acquired mutation but rather from selection of a small pre-existing population of cells that are intrinsically resistant (Coser et al, 2009). Since 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 pro-apoptotic UPR. Alternatively, high doses of bortezomib were recently shown to cause the complete repression of the transcription of the ER (Powers et al). 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 where 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 (30-50nM) were required. Considering that the serum levels of bortezomib reaches 509uM (Bross et al, 2004) 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 two different mechanisms makes this combination even more attractive.
References


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Legends to figures:

Figure 1: The fulvestrant-mediated cytoplasmic ER aggregates are amplified by bortezomib. A) T47D cells were pretreated with or without 15 nM of bortezomib, LLnL or MG132 for 30 minutes, then treated with or without 0.1 μM of fulvestrant for two hours. ER levels were determined by western blotting. (B) Immunofluorescence of GFP-ER in T47D cells treated with or without fulvestrant and/or bortezomib, LLnL or MG132 for 15 hours. (C) T47D cells were pretreated with or without 15 nM of bortezomib for 30 minutes, then treated with or without 0.1 μM of fulvestrant for additional two 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 nM of bortezomib for 30 minutes, then treated with or without 0.1 μM of fulvestrant for 1, 2, 3 or 4 hours. The levels of endogenous ER in the insoluble fractions were determined by western blotting, while the localization of endogenous ER was determined by immunofluorescence at the 4 hours treatment time point.

Figure 2. Insoluble ER aggregates co-localize with ubiquitin. (A) Cells transiently transfected with GFP-ER were treated with fulvestrant for 12 hours. Immunofluorescence was performed using anti-ubiquitin antibody. The representative pictures are shown. (B) The chymotrypsin-like activity of the proteasome was measured using the fluorogenic substrate Suc-LLVY-AMC as described in the material and methods. C) T47D cells were transiently transfected with Myc-ubiquitin for 24 hours and subsequently treated with either 15nM bortezomib, 3 μM fulvestrant or both for 2 days. After 2 days, total amount of poly-ubiquitinated proteins in the extracts were determined by Western blot analysis. (D) Diagram of the lid and catalytic core of the proteasome.
Figure 3. Bortezomib-fulvestrant combination activates the UPR and apoptosis. (A) T47D cells were treated with 15nM bortezomib or 3μM fulvestrant or both for 2 days and the level of BiP determined by western analysis. Tubulin was used as the loading control. (B) T47D cells were treated with 15nM bortezomib or 3μM fulvestrant or both for 2 days and the levels of bid, bcl-2, pro-caspase 8, cleaved caspase 9 and cleaved caspase 3 were determined by western analysis. C) T47D cells were treated with increasing concentration of fulvestrant and/or bortezomib for two days. The percentage of inhibition in metabolic rate after treatment was determined by MTT assay. Results are presented as the mean from one independent experiment performed in triplicate. D) ZR75.1 cells were treated with increasing concentration of fulvestrant and/or bortezomib for two days. The percentage of inhibition in metabolic rate after treatment was determined by MTT assay. Results are presented as the mean from one independent experiment performed in triplicate. E) BT474 cells were treated with 3μM fulvestrant and increasing concentrations of bortezomib alone or with fulvestrant for two days. The percentage of inhibition in metabolic rate after treatment was determined by MTT assay. Results are presented as the mean from one independent experiment performed in triplicate. F) Western blot of the ER in T47D, ZR75.1, BT474 and MDA-MB-361 cells.

Figure 4. Resistance to the fulvestrant-bortezomib in ER positive cells correlates with the induction of autophagy. (A) The ER positive cell line MCF-7 was treated with increasing doses of bortezomib in absence of fulvestrant or in presence of 0.3 μM and 3 μM fulvestrant for 2 days. Metabolic rate was determined by MTT assay. (B) MCF-7 cells were treated with 15nM of bortezomib, 3 μM fulvestrant or both for 2 days and the level
of BiP and the autophagy marker LC3-II determined by western analysis. Tubulin was used as a loading control. T47D cells were treated the same as MCF-7 cells and the activation of autophagy determined by monitoring the level of LC3-II by western analysis. (C) MCF-7 cells were treated with 15nM of bortezomib and 3 μM fulvestrant for 2 days and the cells fixed for electron microscopy. D) MCF-7F40 cells were treated with 3 μM fulvestrant alone, increasing doses of bortezomib alone or in combination with fulvestrant and their metabolic rate determined by MTT assay 2 days after treatment.

Figure 5. Tamoxifen resistant cells are sensitive to the fulvestrant-bortezomib combination. (A) The ER positive cell lines T47D-cyclin D1 were treated with increasing doses of bortezomib in absence of fulvestrant or in presence of 0.3 μM and 3 μM fulvestrant for 2 days. Metabolic rate was determined by MTT assay. B) Four groups of eight nude mice were injected with 1x10^7 T47D-cyclin D1 cells and tumors allowed to grow for 3 weeks. At week 3, mice were treated with vehicle (control), bortezomib alone, fulvestrant alone or bortezomib plus fulvestrant. Tumor growth was determined over a period of 7 weeks. The average tumor volume in each group was adjusted to 100mm^3 to allow for a direct comparison of the four groups. Statistical difference in tumor volumes between groups was determined using Repeated measures ANOVA. (C) T47D cells were transfected with the indicated plasmids for 24 hours and the incubated the cells in the presence of 3 μM fulvestrant and 12 nM bortezomib for 1 hour. (D) Model of the effect of the fulvestrant-bortezomib combination.
Figure 1

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<td>MG132</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
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</tbody>
</table>

IB: ER

IB: Tubulin

IB: ER

IB: Tubulin

B

<table>
<thead>
<tr>
<th>Condition</th>
<th>Nuc</th>
<th>Cyto</th>
<th>Aggr</th>
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<tbody>
<tr>
<td>Control</td>
<td>82%</td>
<td>18%</td>
<td>0%</td>
</tr>
<tr>
<td>Bor</td>
<td>84%</td>
<td>16%</td>
<td>0%</td>
</tr>
<tr>
<td>Ful</td>
<td>33%</td>
<td>53%</td>
<td>14%</td>
</tr>
<tr>
<td>Ful+Bor</td>
<td>28%</td>
<td>41%</td>
<td>31%</td>
</tr>
<tr>
<td>Ful+LnLL</td>
<td>80%</td>
<td>20%</td>
<td>0%</td>
</tr>
<tr>
<td>Ful+MG132</td>
<td>82%</td>
<td>18%</td>
<td>0%</td>
</tr>
</tbody>
</table>

GFP-ER

Dapi

Merged

IB: ER

IB: Tubulin

C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
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<th>+</th>
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<tbody>
<tr>
<td>Fulvestrant</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bortezomib</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ER/tubulin ratio

IB: ER

Soluble

Insoluble

IB: ER

IB: Tubulin

D

<table>
<thead>
<tr>
<th>Condition</th>
<th>Soluble</th>
<th>Insoluble</th>
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<tbody>
<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
<td></td>
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<td>3</td>
<td></td>
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<tr>
<td>4</td>
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</table>

IB: ER

IB: Tubulin

IF: endogenous ER/Dapi

4hrs Ful+Bor
Figure 2

A

ER  Ubiquitin  Merged

C

Myc-Ub

IB: anti-Myc

1  3  0.9  26

High MW Poly-Ub proteins

D

Fulvestrant-mediated

ER aggregates

Bortezomib

Lid

Cataytic core

Trypsin

Caspase

B

% Average of Non-UB-AM

(Chymotrypsin-specific substrate)

p = 0.04

p = 0.05

C  B  F  F+B

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Figure 3

A

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>F</th>
<th>F+B</th>
</tr>
</thead>
<tbody>
<tr>
<td>BiP</td>
<td>1.5</td>
<td>3.7</td>
<td>10.6</td>
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<tr>
<td>Tubulin</td>
<td>1</td>
<td>5.5</td>
<td>3.7</td>
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</tbody>
</table>

B

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>F</th>
<th>F+B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bid</td>
<td>1</td>
<td>0.16</td>
<td>0.05</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>1</td>
<td>0.45</td>
<td>0.17</td>
</tr>
<tr>
<td>Pro-caspase 8</td>
<td>1</td>
<td>0.19</td>
<td>1.2</td>
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<tr>
<td>Cleaved caspase 9</td>
<td>11.7</td>
<td>1</td>
<td>42.8</td>
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<tr>
<td>Cleaved caspase 3</td>
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<td>7.9</td>
<td>2.0</td>
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<tr>
<td>Tubulin</td>
<td>1</td>
<td>55.1</td>
<td></td>
</tr>
</tbody>
</table>

C

D

ZR 75.1 (ER+)

% Remaining metabolic activity

- Ful (μM)
  - 0
  - 0.3
  - 3.0

E

BT474 (ER+/−)

% Remaining metabolic activity

- Ful (μM)
  - 0
  - 3.0

F

T47D

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>F</th>
<th>F+B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubulin</td>
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<td>5.5</td>
<td>3.7</td>
</tr>
</tbody>
</table>

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Figure 4

A

% Remaining metabolic activity

B

MCF-7 (ER+)

Ful (μM)

- 0
- 0.3
- 3.0

Bortezomib (nM)

0 7.5 10 12

B

MCF-7

C

F+L

BiP

Tubulin

1 1.3 0.9 1.2

T47D

LC3-II

1 6.7 1.0 5.0

LC3-II

1 2.7 1.3 2.6

Tubulin

D

% Remaining metabolic activity

MCF-7 F40 (ER+)

(Fulvestrant resistant)

Ful (μM)

- 0
- 3.0

Bortezomib (nM)

0 10 30 50
Clinical Cancer Research

Bortezomib enhances the efficacy of fulvestrant by amplifying the aggregation of the estrogen receptor, which leads to a pro-apoptotic unfolded protein response.

Yuki Ishii, Luena Papa, Urvashi Bahadur, et al.

Clin Cancer Res  Published OnlineFirst February 3, 2011.

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