Bortezomib (PS-341, Velcade) increases the efficacy of trastuzumab (Herceptin) in HER-2–positive breast cancer cells in a synergistic manner

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Abstract

Background: Preclinical and clinical studies have shown that the proteasome inhibitor bortezomib (PS341, Velcade) is highly effective when combined with chemotherapeutic agents. The value of trastuzumab (Herceptin) in HER-2–positive (3+ score by immunohistochemistry or fluorescence in situ hybridization positive) breast cancer is also known; however, the response rate is <40% for metastatic breast cancer. These two pharmacologic agents prevent nuclear factor-κB (NF-κB) activation and induce nuclear accumulation of the cyclin-dependent kinase inhibitor p27kip1, suggesting that combining bortezomib with trastuzumab could increase trastuzumab efficacy. Methods: Drug cytotoxicity, both individually and together, and drug effects on p27 localization and NF-κB activation were investigated on four breast cancer cell lines: SKBR-3 (HER-2 **+**), MDA-MB-453 (HER-2 **+**), HER-2–transfected MCF-7 (HER-2 **++**), and MCF-7 (HER-2 **+**). Results: Bortezomib induced apoptosis in HER-2–positive and HER-2–negative breast cancer cells in a dose- and time-dependent manner. Together, these drugs induced apoptosis of HER-2 **++/+** cells at low concentrations, which had no effect when used alone, indicating there was a synergistic effect. Sequential treatment (trastuzumab then bortezomib) induced either necrosis or apoptosis, depending on the trastuzumab preincubation time. Susceptibility to bortezomib alone and the drug combination correlated with NF-κB activity and p27 localization. Conclusions: The addition of bortezomib to trastuzumab increases the effect of trastuzumab in HER-2 **++/+** cell lines in a synergistic way. This effect likely results from the ability of these two drugs to target the NF-κB and p27 pathways. The potential clinical application of this drug combination is under current evaluation by our group in a phase 1 clinical trial.

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Introduction

The proteasome is a highly conserved multiprotease complex involved in the degradation of cellular factors, leading to a number of cellular processes, including transcription factor activation, cell cycle progression, angiogenesis, cell adhesion, cytokine production, and apoptosis. Many of these pathways contribute to cancer cell growth and survival, suggesting that proteasome inhibitors could provide a new and promising class of anticancer agents. The dipeptide boronic acid analogue bortezomib (Velcade), formerly known as PS-341, was the first proteasome inhibitor used in a clinical setting (1). This drug has been approved by the Food and Drug Administration for treatment of relapsed and refractory multiple myeloma and is currently in clinical trials for the treatment of a wide variety of malignancies, including non-Hodgkin’s lymphoma and other solid tumors. Although bortezomib is active against breast cancer cell lines, in vivo models and phase 1 clinical trials have not found it effective for breast cancer when used as a single agent. However, both preclinical and clinical data have proven its efficacy in combination with several chemotherapeutic agents, suggesting an additive or synergistic effect and a potential role in overcoming resistance to chemotherapy (2). Particularly relevant for breast cancer, bortezomib has been shown to have an additive/synergistic effect with doxorubicin and docetaxel (3–7).

Trastuzumab (Herceptin), a humanized monoclonal antibody directed against the growth factor receptor
HER-2, has proven efficacy for the treatment of metastatic breast cancer, both alone and combined with chemotherapy (8–10). However, even in a selected subset of patients with high HER-2–positive tumors (3+ score by immunohistochemistry or fluorescence in situ hybridization positive), the response rate to trastuzumab is <40%, with a median duration of response between 9 and 12 months (9). Therefore, both de novo and acquired resistance to trastuzumab is an important problem for which a solution needs to be found.

In nonactivated cells, the nuclear factor-κB (NF-κB) is sequestered in an inactive form in the cytoplasm by inhibitors of the IκB family. Activation of the NF-κB pathway involves the proteasome-mediated degradation of the IκB inhibitors (11–13) and the subsequent translocation of NF-κB to the nucleus resulting in the activation of a variety of genes coding for factors favoring cancer cell growth, including antiapoptotic, prometastatic, and proangiogenic factors. NF-κB is activated in a number of human cancers, including HER-2 breast cancer cells. In these cells, overexpression of HER-2 leads to NF-κB activation via the AKT phosphorylation pathway (14). The ubiquitin-proteasome pathway is also involved in HER-2 turnover (1, 15–18). In addition, activation of the Ras/mitogen-activated protein kinase pathway by HER-2 overexpression is associated with enhanced cell cycle progression mediated by proteasome depletion/inactivation, leading to the increased susceptibility of the cells to apoptosis. Thus, bortezomib-mediated inhibition of the proteasome degradation pathway resulting in the stabilization of the IκB/NF-κB complexes in the cytoplasm and trastuzumab-mediated inhibition of HER-2 degradation as well as the targeting of the NF-κB and cyclin-dependent kinase inhibitor p27kip1 and the induction of cyclin G1 (19). This effect can be abrogated by blocking ErbB kinases and/or by proteasome depletion/inactivation, leading to the increased susceptibility of the cells to apoptosis. Thus, bortezomib-mediated inhibition of the proteasome degradation pathway resulting in the stabilization of the IκB/NF-κB complexes in the cytoplasm and trastuzumab-mediated inhibition of HER-2 degradation as well as the targeting of the NF-κB and cyclin-dependent kinase inhibitor p27kip1 pathways might produce additive/synergistic effects, which would benefit breast cancer treatment. The goal of this study was to determine whether the combination of both bortezomib and trastuzumab would increase the efficacy of the treatment and/or could delay or overcome drug resistance compared with either drug alone.

Materials and Methods

Cell Culture

The human breast cancer cell lines SKBR-3, MDA-MB-453, and MCF-7 were obtained from the American Type Culture collection (Manassas, VA) and grown in DMEM (Invitrogen, Inc., Carlsbad, CA) supplemented with 10% FCS (denatured at 55°C during 20 min). The MCF-7 cell line overexpressing transfected HER-2, MCF-7-Her-2, was kindly provided by Dr. Kern (Southern Research Institute, Birmingham, AL; ref. 20) and cultivated on fibronectin (4 μg/μL; Sigma-Aldrich, St. Louis, MO) with improved minimal essential medium (Invitrogen) supplemented with 10% CCS (calf serum stripped of steroids by charcoal treatment). Although isolated from a single colony, the MCF-7-Her-2 cell line was a mixture of two populations of high HER-2– and low HER-2–expressing cells. Culture of the mixed populations in estrogen-depleted conditions for a period of 5 weeks resulted in the emergence of high HER-2–expressing cells (20).

Breast Cancer Cell Line Characterization

Her-2 gene and protein status were analyzed by fluorescence in situ hybridization and immunohistochemistry, respectively. As previously described, MCF-7-Her-2 and SKBR-3 cells have a similar level of HER-2 overexpression (3+ by immunohistochemistry; ref. 20). MDA-MB-453 and SKBR-3 cells overexpress the HER-2 receptor as a result of amplification of the corresponding oncogene (Her-2/CEP17 ratio = 3.3 and 4.5, respectively).

Bortezomib (Velcade) and Trastuzumab (Herceptin)

The proteasome inhibitor bortezomib, formerly known as PS-341, was provided free of charge by Millenium Pharmaceuticals (Cambridge, MA) and reconstituted in DMSO at appropriate concentration (concentration of DMSO after dilution in culture medium was <0.1% and does not induce cytotoxicity). Trastuzumab was provided by Roche Pharmaceutical (Basel, Switzerland) and reconstituted in water at appropriate concentration.

Her-2 Gene Status Evaluation

Fluorescence in situ hybridization was done using the Food and Drug Administration–approved dual-color probe Her-2 Spectrum orange-CEP17 Spectrum green from Vyssis (Pathvision kit, Downers Grove, CA) as described previously (21). Fluorescence in situ hybridization was evaluated using an Olympus BX51 epifluorescence microscope equipped as described previously (21). Signals from at least 60 nonoverlapping nuclei from the invasive part of the tumor were counted. Amplification was defined as a ratio between the number of Her-2 signals and CEP17 >2.

HER-2 Protein Status Evaluation

HER-2 was evaluated by immunohistochemistry with the mouse monoclonal antibody CB-11, at a dilution of 1:40 (Novoceastra, Newcastle upon Tyne, United Kingdom). Cells were centrifuged at 800 rpm for 10 min on poly-L-lysine–coated slides. Slides were incubated for 10 min in acetone and then stained with the Ventana Nexes System using standard Nexes reagents and biotin endogen blockers (Ventana Medical Systems, Tucson, AZ). The sections were incubated for 30 min at 37°C with the primary antibody and 10 min at 37°C with the biotinylated secondary antibody before incubation with avidin peroxidase for the same amount of time. The chromogen was 3,3'-diaminobenzidine. All the products needed for these steps were included in the 3,3'-diaminobenzidine detection kit provided by the manufacturer. The slides were then counterstained with Meyer’s hematoxylin, dehydrated, and mounted. Membranous staining was defined as positive. Scoring system was used according to the HercepTest scoring (DAKO, Carpinteria, CA).

Immunofluorescence and Confocal Microscopy

Cells were centrifuged at 800 rpm for 5 min on poly-L-lysine–coated slides with a cytospin centrifuge (Shandon, Astmoo, United Kingdom) and permeabilized.
by immersion in 100% methanol for 6 min at −20°C. Cells were preincubated for 30 min at room temperature in the saturation solution: PBS containing 0.5% gelatin (Bio-Rad, Richmond, CA) and 0.25% bovine serum albumin (Life Technologies, Gaithersburg, MD). The specimens were incubated for 1 h at room temperature with the primary antibodies: a monoclonal antibody directed against p27 (isotype IgG2a, clone 1B4; Novoceastra) and a monoclonal antibody directed against HER-2 (isotype IgG1, clone CB11; Novoceastra). The primary antibodies were diluted in the saturation solution at 1:20 and 1:100, respectively. Cells were rinsed thrice for 10 min with PBS containing 0.2% gelatin and incubated for 1 h at room temperature with the secondary antibodies: a goat anti-mouse IgG2a conjugated to Fluor Alexa 488 (Molecular Probes, Eugene, OR) diluted 1:100 and a goat anti-mouse IgG1 coupled to biotin diluted 1:200 in the saturation solution. After washing thrice for 10 min with PBS containing 0.2% gelatin, the specimens were incubated for 30 min with streptavidin-CY5, washed again thrice, and then mounted using DABCO-based substrate detection system (Roche Pharmaceutical).

Nuclear Extracts and Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from 2 × 10^7 cells according to the method described by Osborn et al. (22) with minor modifications. All buffers contained the protease inhibitor cocktail complete from Roche Pharmaceutical, pepstatin (1 µg/ml), and DTT 0.5 mmol/L. Briefly, cells were washed with ice-cold PBS and twice with cold buffer A [10 mmol/L HEPES buffer (pH 7.9), 1.5 mmol/L MgCl_2, 10 mmol/L KCl]. The pellets were resuspended and incubated twice for 10 min with ice-cold lysis buffer A containing 0.2% NP40. The pellet (nuclear fraction) was incubated for 20 min with ice-cold extraction buffer C [20 mmol/L HEPES buffer (pH 7.9), 25% glycerol, 1.5 mmol/L MgCl_2, 420 mmol/L NaCl, 0.2 mmol/L EDTA] and then centrifuged (20,800 × g) for 10 min at 4°C. The nuclear supernatants were diluted with 150 µL of buffer D [20 mmol/L HEPES buffer (pH 7.9), 20% glycerol, 50 mmol/L KCl, 0.2 mmol/L EDTA] and kept frozen at −80°C. Protein concentrations were determined by the Bradford method (23). Electrophoretic mobility shift assays (EMSA) were done as previously described (24). Single-stranded oligonucleotides were 5’ end-labeled with [γ-32P]ATP (>5,000 Ci/mm); Amersham, Arlington Heights, IL) using T4-polynucleotide kinase, annealed to their complementary strand, purified by gel electrophoresis, and recovered from polycrylamide using the QIAEX II kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instruction before their use in EMSAs. The nucleotide sequence of the coding strand of the double-stranded oligonucleotides used for this study are 5’-TGAGGGGACCTTTCTACGC-3’ (NF-κB binding site; Promega, Madison, WI) and 5’-TGTCGAATG-3’ (Oct-1 binding site; Santa Cruz Biotechnology, Santa Cruz, CA).

Nuclear extracts (10 µg of protein) were preincubated for 10 min (without the probe) in the presence of a reaction mixture containing 10 µg of bovine serum albumin (Pharmacia, Piscataway, NJ), 1.5 µg of the nonspecific competitor DNA poly(deoxyinosinic-deoxycytidylic acid) (Pharmacia), 50 µmol/L ZnCl_2, 1 mmol/L DTT, 20 mmol/L Tris-HCl (pH 7.5), 60 mmol/L KCl, 1 mmol/L MgCl_2, 0.1 mmol/L EDTA, and 10% (v/v) glycerol.

Supershift Assay

Antibodies directed to the NF-κB family proteins p50 (SC-1190), p65 (SC-109), and c-Rel (SC-6955); all from Santa Cruz (SC-1190) were incubated with the nuclear extract at a 1:100 dilution in a buffer containing 10% glycerol, 0.5 mmol/L MgCl_2, and 10 mmol/L Tris (pH 7.5) for 1 h at room temperature. The nuclear extracts were then processed for EMSA as described above.

Supershift conditions were determined by titration. After the nuclear extracts were incubated with mAbs for 1 h, the reaction was stopped by the addition of EMSA loading buffer and loaded onto a 4% native polyacrylamide gel. The gel was run in Tris-glycine buffer (pH 8.4), and the DNA was visualized with ethidium bromide staining. The NF-κB supershifts were identified by the addition of antibodies to the bands with altered mobility.

Western Blotting

Samples containing 15 µg of nuclear protein extract, 25% lithium dodecyl sulfate, and 0.05 mol/L DTT were heated at 100°C for 20 min and loaded on a NuPage 4% to 12% Bis-Tris gel (Invitrogen) in MOPS [3-(N-morpholino)-propane sulfonic acid] buffer and transferred onto a nitrocellulose membrane. The membrane was washed for 30 min with 4% milk in TBST buffer (50 mmol/L Tris, 0.15 mol/L NaCl, 0.1% Tween 20), incubated overnight with a 100-fold dilution of mouse monoclonal anti-p27 IgG2a (clone 1B4; Novoceastra) in TBST/2% milk, and washed thrice in TBST. The membrane was then incubated with an anti-mouse IgG conjugated to horseradish peroxidase for 1 h in TBST/2% milk and washed thrice in TBST. Proteins were visualized using the Lumi-light Western blotting substrate detection system (Roche Pharmaceutical).

Proliferation Analysis

Cellular growth was assessed by cell counting using the trypan blue dye exclusion protocol after 24, 48, 72, and 96 h of incubation. Results are the mean of duplicate wells.

Morphologic Studies

To assess morphologic changes after drug treatment, the cells were centrifuged, air-dried, stained with May Grünwald Giemsa, and analyzed by light microscopy.

Annexin V Binding Assay

Flow cytometric analysis of Annexin V-FITC and propidium iodide–stained cells was done using the kit purchased from BioSource International (Camarillo, CA) as recommended by the manufacturer. Data are presented as dot plots showing the changes in mean fluorescence intensity of Annexin V-FITC/propidium iodide. The data shown correspond to the mean value from three independent experiments. The mean SD between these values is 3%.

Cell Cycle Analysis

Cells were washed with PBS, permeabilized, and incubated with a solution containing propidium iodide and RNase (Coulter DNA-prep Reagent). The tubes were placed at 4°C in the dark overnight before analysis by flow cytometry to identify DNA content. Cell cycle distribution was analyzed using Multicycle software (Phoenix Flow Systems, San Diego, CA). Cells with a hypodiploid DNA content were counted as apoptotic cells.

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MCF7 (ER+ HER-2) were preincubated with nuclear extracts for 1 h on ice before the addition of the radio-labeled probe for the supershift assay.

Oligonucleotides encoding wild-type and deleted NF-κB binding sites are NF-κBwt, 5′-TTGAGGGGACTTTC-CAGGC-3′ and NF-κBmut, 5′-TTGAGCTCACTTTCC-CAGGC-3′.

Analysis of Combined Treatment of Trastuzumab with Bortezomib: Combination Index

Combination index calculations were done as described by Chou and Talalay (25) with the use of CalcuSyn software (Biosoft, Cambridge, United Kingdom). According to this method, a combination index can be calculated from dose-response curves obtained following exposure to bortezomib and/or trastuzumab as single agent and in combination. Combination index values ~1.0 indicate additive interactions between the two agents; combination index values <1 indicate synergy; and, conversely, combination index values >1 indicate antagonism. With the use of CalcuSyn software, synergy is further refined as synergism (combination index values >1.0 indicate antagonism). With the use of CalcuSyn software, synergy is further refined as synergism (combination index values <1 indicate synergy; and, conversely, combination index values >1 indicate antagonism). With the use of CalcuSyn software, synergy is further refined as synergism (combination index values >1 indicate antagonism). With the use of CalcuSyn software, synergy is further refined as synergism (combination index values >1 indicate antagonism).

Statistical Analysis

The correlation between the percentages of apoptosis induced by drug treatment and the level of NF-κB complex binding and nuclear p27 levels assessed as continuous variables was measured by calculating nonparametric Spearman’s rank correlation coefficients.

Results

Induction of Apoptosis in HER-2 Positive and HER-2 Negative Cell Lines after Bortezomib Treatment

To evaluate the ability of bortezomib alone to induce apoptosis in HER-2–positive and HER-2–negative breast cancer cell lines, the apoptotic effect of bortezomib was quantified in an Annexin binding assay (Table 1). MCF-7 (HER-2) and MDA-MB-453 (HER-2) cell lines were similarly susceptible to bortezomib (PS-341) with 4% to 9% apoptosis elicited by a treatment for 48 h at 10^{-3} μmol/L and 20% to 30% apoptosis at 10^{-2} μmol/L. In contrast, the HER-2++ SKBR-3 cells were extremely susceptible to bortezomib with 70% apoptosis attained at 10^{-3} μmol/L. Alternatively, MCF-7-Her-2 cells (HER-2++) were extremely resistant to treatment with no apoptosis observed after 48 h of treatment and drug concentration up to 1 μmol/L and only 22% apoptosis elicited after 72 h and 0.1 μmol/L bortezomib. When MCF-7-Her-2 cells were cultured in estrogen-containing media for 6 months, which induces HER-2 down-regulation (20), drug susceptibility increased with 22% apoptosis observed after a 48 h of incubation with 10^{-3} μmol/L bortezomib. These results were confirmed by visual observation of the apoptotic morphology of the cells, and there was not correlation between susceptibility to bortezomib and cell proliferation (data not shown). Thus, susceptibility to bortezomib clearly depended on the genetic background of the cells and, when analyzed in the same background, a reduced expression of HER-2 increased susceptibility to the drug.

In contrast to bortezomib, trastuzumab activity was previously shown to be very dependent on the presence of HER-2 receptors (26, 27). We also confirmed that Her-2 transgene expression was necessary and sufficient to render MCF-7 cells susceptible to trastuzumab (data not shown).

Induction of Apoptosis in HER-2 Positive Cell Lines after Treatment with the Trastuzumab-Bortezomib Combination

To evaluate the potential of trastuzumab combined with bortezomib to induce apoptosis in HER-2–positive breast cancer cell lines, we exposed cancer cells to both drugs at

Table 1. Apoptosis induction by bortezomib

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>No bortezomib, %</th>
<th>Bortezomib (10^{-5} μmol/L), %</th>
<th>Bortezomib (10^{-4} μmol/L), %</th>
<th>Bortezomib (10^{-3} μmol/L), %</th>
<th>Bortezomib (10^{-2} μmol/L), %</th>
<th>Bortezomib (10^{-1} μmol/L), %</th>
<th>Bortezomib (1 μmol/L), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7 (ER+ HER-2−)</td>
<td>48</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>4</td>
<td>30</td>
<td>ND</td>
</tr>
<tr>
<td>MDA-MB-453 (ER− HER-2++)</td>
<td>48</td>
<td>0</td>
<td>ND</td>
<td>3</td>
<td>9</td>
<td>17</td>
<td>22</td>
</tr>
<tr>
<td>SKBR3 (ER− HER-2++)</td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>25.5</td>
<td>70</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MCF7-Her-2 (ER− HER-2++)</td>
<td>48</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MCF7-Her-2 (ER− HER-2++)</td>
<td>72</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>9</td>
<td>22.2</td>
</tr>
<tr>
<td>MCF7-Her-2 reverted (ER− HER-2−)</td>
<td>48</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>22</td>
<td>70</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE: Percentages of apoptosis measured with 10^{-5} to 1 μmol/L bortezomib after 48 h of incubation for MCF7, MDA-MB-453, SKBR3, and the reverted MCF7-Her-2 (down-regulation of HER-2 receptor) and after 48 and 72 h for MCF7-Her-2. The percentage of apoptosis was normalized according to the control without drug. Data are the mean values of three independent experiments. Mean SD is of 3%. Abbreviations: ER, estrogen receptor; ND, not done.
different doses and sequences. Two drug delivery protocols were tested with the drugs either added simultaneously or with trastuzumab added before bortezomib (Fig. 1). The susceptibility to the two drugs was very different for each cell line. In SKBR3 cells, the addition of 2 μg/mL trastuzumab with 10 μmol/L bortezomib elicited 60% apoptosis, whereas, individually, the drugs did not induce apoptosis (Fig. 1A). Although less susceptible than SKBR3, MCF-7-Her-2 cells displayed 26% apoptosis when 20 μg/mL trastuzumab was added to 10 μmol/L bortezomib compared with 0% and 9% with either drug separately (Fig. 1B). In MDA-MB-453, addition of 2 μg/mL trastuzumab with 10 μmol/L bortezomib elicited 15% apoptosis, whereas the individual drugs induced very low or undetectable levels of apoptosis (Fig. 1C).

A sequential treatment for 24 h with trastuzumab followed by a 48-h treatment with bortezomib resulted in apoptosis level similar to that observed with a 48-h treatment with both drugs simultaneously (Fig. 1D). Increasing the incubation time with trastuzumab to 48 h in this regimen resulted in necrosis. We hypothesized that the alterations in the cell cycle induced by trastuzumab at 48 h directed the cells to necrosis rather than apoptosis upon the addition of bortezomib.

Cytomorphologic analysis of MDA-MB-453 cells confirmed that the treatment with the two drugs induced apoptosis at concentrations eliciting no or few effects with drugs individually. Trastuzumab concentration had no effect on the apoptosis rate. Sequential incubation of trastuzumab for 24 h and bortezomib for 48 h induced apoptosis, whereas sequential incubation of both drugs for 48 h each induced necrosis (data not shown). Mean of three independent experiments. Mean SD is of 3%.

**Figure 1.** Simultaneous and sequential incubation with trastuzumab and bortezomib. Simultaneous incubation with both drugs induced apoptosis at concentration eliciting no or few effects with drugs individually. Trastuzumab concentration had no effect on the apoptosis rate. Sequential incubation of trastuzumab for 24 h and bortezomib for 48 h induced apoptosis, whereas sequential incubation of both drugs for 48 h each induced necrosis (data not shown). Mean of three independent experiments. Mean SD is of 3%.

Effects Observed with Trastuzumab Plus Bortezomib Are Associated with Altered NF-κB Complex Formation

Trastuzumab and bortezomib both block NF-κB activation and increase p27 nuclear concentrations. Therefore, we...
investigated both their individual and combined effects on these biological end points. The effect of trastuzumab plus bortezomib on NF-κB complexes and nuclear p27 location was assessed at drug concentrations where little or no effect was observed with either drug individually. Drug-induced alterations in the DNA binding of nuclear NF-κB complexes was assessed using both EMSA and EMSA supershifts to show specific changes in the DNA-binding activity of RelA (p65) and p50 containing complexes. Parallel EMSA experiments were done using an Oct-1 probe to control for equivalent protein binding in all lanes (Fig. 3). Analysis of the SKBR3 cells by EMSA revealed two complexes that were supershifted by the anti-p50 antibody but not by the anti-cRel antibody, whereas the anti-p65 antibody only supershifted the upper complex (Fig. 3A). These results indicate that the SKBR3 cell line contains abundant p50/p50 and p50/p65 complexes specifically bound to a NF-κB consensus sequence.

p50/p50 homodimers were found in nuclear extracts from all four cell lines (Fig. 3B), with significantly greater amounts detected in the SKBR-3 cell line. p50/p65 heterodimers were detected in nuclear extracts from all cell lines except MCF-7, indicating that constitutive NF-κB activation was prominent in HER-2–positive cells. MCF-7-Her-2 and MDA-MB-453 had moderate and SKBR3 had sizable amounts of DNA-binding p65, reflecting their respective degree of constitutive NF-κB activation. Thus, a positive correlation was observed between the level of HER-2 receptor expression and constitutive NF-κB activation in all four cell lines. Basal NF-κB activation also significantly correlated with cell line susceptibility to bortezomib, except for the MCF-7-Her-2 cell line that was very resistant to treatment but displayed a moderate level of NF-κB activation (P = 0.01).

The effect of the two drugs on NF-κB activation was assessed using nuclear extracts from SKBR-3 (Fig. 3C). p65-containing nuclear complexes were reduced by individual treatment with 20 μg/mL trastuzumab or 10⁻⁶ μmol/L bortezomib (Fig. 3C) but not at bortezomib concentration of 10⁻⁶ μmol/L (data not shown). Interestingly, NF-κB complexes were barely detected when these cells were treated with both 20 μg/mL trastuzumab and 10⁻⁶ μmol/L bortezomib simultaneously. Similarly, the drug treatments reduced nuclear p50/p50 homodimers in SKBR-3 cells. The treatment also reduced NF-κB complexes in the other cell lines, although the basal level of these complexes was too low to enable accurate quantification.

**Effects Observed with Trastuzumab plus Bortezomib Are Associated with Altered p27 Nuclear Localization**

We then analyzed the intracellular localization of p27 in cell treated or not with the two drugs. To this end, the cells were analyzed by immunofluorescence staining followed by confocal microscopy (Fig. 4), and nuclear extracts were submitted to Western blotting (Fig. 5). First, the four cell lines and SKBR3 cells treated with trastuzumab and/or bortezomib were analyzed by dual immunofluorescence staining with anti-p27 and anti-HER-2 antibodies. p27 was present predominantly in the nuclei of the HER-2⁺⁺⁺ MCF-7 (Fig. 4A) and HER-2⁺⁺⁺ MCF-7-Her-2 (Fig. 4B) cells and in both the nuclei and the cytoplasm in HER-2⁺⁺ MDA-MB-453 cells (Fig. 4C). In HER-2⁺⁺⁺ SKBR3, untreated (Fig. 4D) or treated with trastuzumab (Fig. 4E), p27 was distributed essentially in the cytoplasm. In contrast, treatment of these cells with bortezomib at 10⁻⁴ μmol/L (Fig. 4F) and combined treatment with 20 μg/mL trastuzumab and 10⁻⁶ μmol/L bortezomib (Fig. 4G) resulted in the presence

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**Table 2. Analysis of combined treatment of trastuzumab with bortezomib: combination index calculation through the Chou and Talalay method**

<table>
<thead>
<tr>
<th>Bortezomib (μmol/L)</th>
<th>Trastuzumab (μg/mL)</th>
<th>Combination index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKBR-3 (48-h treatment)</td>
<td>10⁻⁸</td>
<td>2</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>2</td>
<td>0.659 (+++)</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>2</td>
<td>0.000 (++++)</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>2</td>
<td>0.000 (++++)</td>
</tr>
<tr>
<td>MDA-MB-453 (48-h treatment)</td>
<td>10⁻⁴</td>
<td>2</td>
</tr>
<tr>
<td>10⁻³</td>
<td>2</td>
<td>0.005 (++++)</td>
</tr>
<tr>
<td>10⁻²</td>
<td>2</td>
<td>0.005 (++++)</td>
</tr>
<tr>
<td>MCF-7-Her-2 (72-h treatment)</td>
<td>10⁻⁴</td>
<td>2</td>
</tr>
<tr>
<td>10⁻³</td>
<td>2</td>
<td>0.018 (++++)</td>
</tr>
<tr>
<td>10⁻²</td>
<td>2</td>
<td>1.670 (−)</td>
</tr>
</tbody>
</table>

*+++++, very strong synergism; ++++, strong synergism; ++, synergism, ±, nearly additive; −, moderate antagonism; −−, antagonism.
of p27 in the nuclei in addition to its presence in the cytoplasm and led to the accumulation of the HER-2 receptor inside the cells. The nuclear envelope delimiting the cytoplasm and the nuclei is clearly pictured on the differential interference contrast.

Finally, Western blot analysis of nuclear extracts confirmed the presence of p27 in the nuclei of MCF-7, MCF-7-Her-2, and MDA-MB-453 but not in the nuclei of SKBR3 untreated or treated with trastuzumab. In addition, quantification of the intensities of the p27 species indicated that the treatments with bortezomib alone or in combination with trastuzumab resulted in a dramatic increase (nearly 10 times) of the p27 pool in the nuclei of SKBR3 cells treated for 24 h with each drug. Relative activity was normalized according to the Oct-1 factor (Bio-Profil Bio-1D V99-03).

In conclusion, the level of apoptosis induced by the treatments of SKBR3 statistically correlated with a reduced level of NF-κB activation and an increased pool of nuclear p27 \( (P = 0.01) \). In addition, these end points were obtained at concentrations of the combined drugs that had no or few effects when used alone. Finally, cell lines with a low level of activated NF-κB and a high nuclear pool of p27 were poorly susceptible to the treatment.

**Discussion**

Inhibition of the proteasome has been shown to stabilize a multitude of cellular proteins, initially inducing arrest at...
Protein loading may be appreciated with Ponceau red coloration. 24 h were immunoblotted with anti-p27 specific antibody. Amount of lane) from breast cancer cells treated with trastuzumab and bortezomib for level of p27 assessed by Western blot. Protein nuclear extracts (15 μg per lane) from breast cancer cells treated with trastuzumab and bortezomib for 24 h were immunoblotted with anti-p27 specific antibody. Amount of protein loading may be appreciated with Ponceau red coloration.

Figure 5. Effect of bortezomib and trastuzumab on the nuclear protein level of p27 assessed by Western blot. Protein nuclear extracts (15 μg per lane) from breast cancer cells treated with trastuzumab and bortezomib for 24 h were immunoblotted with anti-p27 specific antibody. Amount of protein loading may be appreciated with Ponceau red coloration.

the G2-M transition and ultimately leading to programmed cell death. As previously reported by Teicher et al. (28), our results also indicate that in contrast to trastuzumab, HER-2–negative cell lines (i.e., MCF-7) are susceptible to bortezomib, although the threshold is higher than in HER-2+++ cell lines. Moreover, the reduction of HER-2 expression in HER-2–transfected MCF-7 cells increased the susceptibility to bortezomib, suggesting that HER-2 receptor expression in and of itself does not increase susceptibility to bortezomib and may even reduce their responsiveness.

Tumor regression induced by Trastuzumab is most likely provoked by several mechanisms. One possibility is ubiquitin conjugation of HER-2 after binding with trastuzumab (29), which likely induces HER-2 degradation by the proteasome. A recent study (30) has shown that HER-2 can migrate to the nucleus where it functions as a transcription factor. The data presented in the present study show that bortezomib treatment seems to lead to accumulation of HER-2 in the cytoplasm. It is not known whether this HER-2 accumulated in the cytoplasm is inactive or active (i.e., activates genes involved in the apoptotic pathway) nor its characteristics or the specific mechanism that leads to its accumulation. This certainly warrants further investigation.

Some HER-2–positive tumors are resistant to trastuzumab, and HER-2 receptor expression can induce pathways resulting in loss of bortezomib susceptibility, which lead us to question whether bortezomib could increase trastuzumab efficacy. We found that the combination of both drugs induce apoptosis in HER-2+++ and HER-2++ cell lines at concentrations that had no observable effect with the individual drugs, suggesting a synergistic combined action that was confirmed using the Chou and Talalay method (25). Importantly, depending on the incubation time with trastuzumab, the sequential addition of trastuzumab then bortezomib induced cellular necrosis rather than apoptosis, an effect previously observed only at high concentrations or with prolonged exposure times to bortezomib alone (15, 31). Cell cycle alterations induced by trastuzumab at 48 h seem to predispose cells to bortezomib-induced necrosis. We found that trastuzumab-treated MDA-MB-453 cells, with trastuzumab taken before detectable cell cycle alterations occurred (24 h), were apoptotic rather than necrotic. This effect is potentially mediated by p27, a protein affected by both drugs that is incapable of inducing apoptosis in G1 blocked cells (32). Our data suggest that in the clinical setting, bortezomib might increase the efficacy of trastuzumab in HER-2+++ tumors and might favor trastuzumab activity in HER-2++ tumors.

The rationale for combining trastuzumab and bortezomib is based on their reported mutual targeting of NF-κB and p27. NF-κB activity is regulated by proteasome-mediated degradation of IκB, an inhibitory protein that functions to sequester NF-κB in the cytoplasm (11–13, 33). Bortezomib, by stabilizing IκB, inhibits NF-κB activation, ultimately leading to apoptosis (15, 34, 35). In addition, HER-2 overexpression induces NF-κB activation via the phosphatidylinositol 3-kinase/AKT pathway and degradation of IκB (14). Our demonstration that a correlation exists between constitutive NF-κB activation and HER-2 overexpression further supports this idea. SKBR-3 cells contain the highest levels of active NF-κB, which supports the observation that NF-κB activity is elevated in estrogen receptor–negative human breast cancer (36). Correlation analyses revealed that susceptibility to bortezomib is positively associated with the basal level of NF-κB activation except in cells transfected with the Her-2 gene for which elevated NF-κB activation is not linked to increased susceptibility. Bortezomib or trastuzumab treatment reduced RelA-containing complexes in all of the HER-2–positive cell lines, with the most dramatic effect observed in SKBR-3 cells. NF-κB inhibition seems to occur early because the trastuzumab concentration we used did not detectably alter the cell cycle. Additionally, the effect of these drugs is not specific for RelA-containing complexes only because p50/p50 homodimers were also reduced. NF-κB inhibition seems to be a major therapeutic mechanism for bortezomib, particularly in estrogen receptor–negative tumors for which the level of NF-κB activation seems to be positively correlated with drug susceptibility.

HER-2 overexpression also induces Ras/mitogen-activated protein kinase signaling, which is known to enhance cell cycle progression by destabilizing and promoting proteasome-mediated p27 degradation (15, 37–39). We observed decreased p27 nuclear immunoreactivity in association with HER-2 overexpression, which has previously been shown for lymph node–negative breast cancer (40). Cytoplasmic p27 was detected in SKBR-3 cells, whereas significant levels of nuclear p27 were observed in MCF-7 and MDA-MB-453 cells, although the majority of p27 was cytoplasmic in the latter. Our data agree with previous studies showing that low nuclear p27 is correlated with estrogen receptor–negative status (41, 42). Nuclear p27 was detected in the highly sensitive HER-2+++ SKBR3 cells after treatment with bortezomib. Accumulation of nuclear p27 has been previously reported (19, 39, 43, 44), and p27 phosphorylation was widely recognized as the major...
posttranslational mechanism regulating its stability and thereby nuclear accumulation (39). Nuclear p27 induction could also result from decreased mitogen-activated protein kinase phosphorylation as shown previously (45). An inverse correlation was observed between the susceptibility to bortezomib and the basal level of nuclear p27. This correlation is also valid for MCF-7-Her-2, where nuclear p27 levels are similar to the parental MCF-7 cells despite HER-2 overexpression and mitogen-activated protein kinase activation. This is perhaps explained by the inability of the transgene to completely activate the Ras/mitogen-activated protein kinase signaling pathway leading to p27 degradation. This result may partially explain the differences in bortezomib susceptibility between MCF-7-Her-2 and SKBR3 and is in agreement with the inverse correlation observed between p27 nuclear induction and apoptosis in SKBR3. Therefore, differences in p27 nuclear accumulation may determine the potency of bortezomib as previously shown for trastuzumab (37). SKBR3 cells have been shown to proliferate at low rates in the presence of trastuzumab (37), which supports our observation that nuclear p27 levels did not increase in SKBR-3 cells treated with trastuzumab for 24 h, the time required for maximum association between cyclin-dependent kinase 2 and p27 in the HER-2++ BT474 cells (37). Our data concur with recently published findings (46) showing that a trastuzumab-resistant clone of SKBR3 has decreased p27 levels in association with increased cyclin-dependent kinase 2 activity and heightened susceptibility to proteasome inhibition. Exogenous addition of p27 increased trastuzumab susceptibility leading these authors to speculate that trastuzumab resistance could be associated with decreased p27; therefore, inducing p27 expression with proteasome inhibitors would increase drug susceptibility (37). Our results showing that apoptosis induced by bortezomib or bortezomib in combination with trastuzumab were negatively correlated with p27 nuclear induction and positively correlated with NF-kB inhibition support this hypothesis. We further found that this drug combination induced apoptosis and NF-kB inhibition and p27 nuclear induction at concentrations eliciting no or very low effect with the individual drugs. Therefore, the synergistic apoptotic effect induced by the combination of both drugs could be related to the activity of both drugs on NF-kB and p27.

The susceptibility of HER-2−positive and HER-2−negative breast cancer cell lines to bortezomib is linked to NF-kB activity and p27 localization. In this study, we show that a strong correlation exists between HER-2 overexpression and constitutive NF-kB activation as well as p27 nuclear scarcity. In HER-2++/+ cells, the trastuzumab-bortezomib combination has a strong effect on apoptosis or cytotoxicity in vitro, depending on the drug delivery protocol. This effect is synergistic and could result from both drugs targeting NF-kB and p27; two proteins whose function and localization are closely linked with HER-2 overexpression. The potential clinical applications of this drug combination are currently under evaluation in a phase 1 clinical trial by our group.

References


Molecular Cancer Therapeutics

Bortezomib (PS-341, Velcade) increases the efficacy of trastuzumab (Herceptin) in HER-2-positive breast cancer cells in a synergistic manner

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