Differential cellular and molecular effects of bortezomib, a proteasome inhibitor, in human breast cancer cells

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Abstract
The cellular and molecular effects of the proteasome inhibitor bortezomib on breast cancer cells are as yet poorly characterized. Here, in a panel of six breast cancer cell lines, bortezomib reduced viability in a concentration-dependent, time-dependent, and cell line–dependent manner. Proteasome activity was relatively high in two of the three more resistant cell lines. No relationship was observed between bortezomib effects on cell viability and expression/phosphorylation of HER-2, epidermal growth factor receptor (EGFR), AKT, or extracellular signal-regulated kinase 1/2 (ERK1/2). Molecular effects of bortezomib were further studied in SK-BR-3 and BT-474 cells because they share expression of EGFR and overexpression of HER-2 while, in contrast, SK-BR-3 cells were 200-fold more sensitive to this agent. Proteasome activity was inhibited to a similar extent in the two cell lines, and known proteasome substrates accumulated similarly. In SK-BR-3 cells, a marked inhibition of EGFR, HER-2, and AKT phosphorylation was observed at a clinically relevant concentration of bortezomib. In contrast, phosphorylation of Raf/mitogen-activated protein kinase kinase 1/2 (MEK 1/2)/ERK1/2 increased by bortezomib. In BT-474 cells, the effects were much less pronounced. Treatment of SK-BR-3 cells with bortezomib combined with pharmacologic inhibitors of EGFR, phosphatidylinositol 3’-kinase, or MEK resulted in modest or no enhancement of the effects on cell viability. Collectively, these results show that bortezomib has differential cellular and molecular effects in human breast cancer cells. The bortezomib-observed effects on signaling transduction molecules might be relevant to help to design mechanistic-based combination treatments. [Mol Cancer Ther 2006;5(3):665–75]

Introduction
The proteasome plays a pivotal role in the cellular housekeeping by eliminating mutant, misfolded, and damaged proteins. Moreover, the proteasome is involved in the targeted elimination of regulatory proteins, such as transcription factors, signaling molecules, and cell cycle inhibitors (1). The inhibition of the proteasome results in the abnormal accumulation of many intracellular proteins, thereby disrupting cellular homeostasis. Thus, cells undergo cell cycle arrest or programmed cell death (2). The observation that tumor cells are more sensitive to proteasome inhibition than normal cells led to propose the proteasome as a novel target for cancer treatment (3). Bortezomib (Velcade, formerly known as PS-341) is a selective and reversible proteasome inhibitor that results in a wide range of molecular sequels, including stabilization of cell cycle regulatory proteins, inhibition of nuclear factor-kB (NF-kB) activation, induction of apoptosis, and override of Bcl-2 resistance and antiangiogenesis (3, 4). More importantly, bortezomib has a wide range of antitumor activity and increases the activity of multiple chemotherapeutic agents (5). Bortezomib inhibits 20S proteasome activity in whole cells with a Ki of ~7 nmol/L, consistent with its mean IC50 (7 nmol/L) in the National Cancer Institute panel of 60 cell lines (4). Preclinical studies suggested that twice-weekly regimens resulting in proteasome inhibition, as measured in blood, approaching but not exceeding 80% would be optimal (6). Early clinical trials of bortezomib incorporated a pharmacodynamic ex vivo assay developed to study the degree and kinetics of proteasome inhibition achieved in patients as a key element to select the dose for patients (7–9).

Bortezomib is approved in many countries for the treatment of chemorefractory multiple myeloma patients (10, 11) and is in further clinical development in multiple tumor types, including breast cancer (12–14). Preliminary reports of trials using bortezomib in breast cancer patients have shown lack of significant clinical activity against this disease (15, 16). The potential clinical development of bortezomib in breast cancer would need to be based on combination therapeutic strategies with agents such as docetaxel or the anti-HER-2 antibody trastuzumab (17–20). To fully explore bortezomib-based combinations, it is of importance to gain further knowledge in the molecular effects of bortezomib in breast cancer. In the present study,
we aimed to characterize the effects of bortezomib in a panel of human breast cancer cell lines on viability, cell cycle, and apoptosis. Considering the important role of the epidermal growth factor receptor (EGFR) and HER-2 and their signaling transduction pathways in breast cancer, the effects of bortezomib on these pathways were also investigated. We also sought to explore the effects of cotreatment with bortezomib and selected pharmacologic agents that target these pathways.

**Materials and Methods**

**Cell Lines and Reagents**

Human breast cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD) and cultured at 37°C with 5% CO₂ in a humidified incubator in DMEM/F12 (MDA-MD-231, MDA-MD-453, MDA-MD-468, BT-474, and SK-BR-3) or DMEM (MCF-7) supplemented with 2 mmol/L L-glutamine and 10% fetal bovine serum. For BT-474, insulin (0.01 μg/mL) was added. Media and supplements were obtained from Life Technologies (Gaithersburg, MD). Recombinant human tumor necrosis factor-α (TNF-α) and EGFR were purchased from R&D Systems (Minneapolis, MN). Chemical inhibitors for the EGFR tyrosine kinase (AG1478) and mitogen-activated protein kinase [MEK (PD-098059)] and phosphatidylinositol 3'-kinase [PI3K (LY294002)] were from Calbiochem (San Diego, CA). All of them were dissolved as recommended by the manufacturers, aliquoted, and stored at −20°C. Bortezomib was kindly provided by Millennium Pharmaceuticals (Cambridge, MA). MG-132 was purchased from Calbiochem. In both cases, 10 mmol/L aliquots of drug in DMSO were stored dissolved as recommended by the manufacturers, aliquoted, and stored at −20°C. Bortezomib was kindly provided by Millennium Pharmaceuticals (Cambridge, MA).

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**3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay**

The effects of bortezomib on the proliferation and viability of breast cancer cells were studied by use of the colorimetric method provided by the MTS-CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay is a modification from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and depends on two solutions: a tetrazolium compound (MTS) and an electron coupling reagent (phenazine methosulfate). MTS is bioreduced by cells into a formazan product. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in cells that are metabolically active. The quantity of formazan product as measured by the amount of 490-nm absorbance is directly proportional to the number of living cells in culture (21). We did the MTS assay in 96-well, flat-bottomed plates (Nunc, Naperville, IL). Approximately 1 × 10⁴ cells were seeded in 100 μL drug-free media and incubated for 24 hours before drug treatment; 100 μL of various 2× drug concentration (1× final concentration) were added for times from 24 to 72 hours. Forty microliters of MTS/phenazine methosulfate solution were added to the wells, and the cells were further incubated for 2 to 4 hours. The amount of soluble formazan produced, by cellular reduction of the MTS, was measured by the absorbance on a microplate spectrophotometer ( Molecular Dynamics, Sunnyvale, CA) at 490 nm (test wavelength) and 690 nm (reference wavelength). The percentage of surviving cells was estimated by dividing the A₄₉₀ nm − A₆₉₀ nm of treated cells by the A₄₉₀ nm − A₆₉₀ nm of control cells. Approximate IC₅₀ values were determined from the dose-response curve. Data were derived from at least three independent experiments (in triplicate).

**Flow Cytometry**

Cells (2 × 10⁶) were seeded in 100-mm plates, allowed to attach overnight, and then treated as indicated for times up to 72 hours. At each time point, cells (floating and adherent) were collected, counted, and washed twice with cold PBS. For cell cycle analysis based on DNA content, cells (1 × 10⁶/mL) were fixed with 70% ethanol in PBS at −20°C for 2 to 3 days, centrifuged, and stained with propidium iodide (Sigma; 20 μg/mL in PBS with 0.1% Triton X-100) in the presence of RNase A (0.2 mg/mL; Sigma) at room temperature for 30 minutes in the dark. The cells were then analyzed with FACScan and Cell-Quest software (Becton Dickinson, Mountain View, CA). For apoptosis analysis, phosphatidylserine externalization was measured using the Annexin V/FITC Apoptosis Detection kit (Roche, Indianapolis, IN). Cells were labeled with Annexin V/FITC and counterstained with propidium iodide according to the manufacturer’s protocol. Dual-variable flow cytometric analysis was done to determine the percentage of apoptotic cells (Annexin V alone–positive cells), necrotic cells (propidium iodide–positive cells), or viable cells (staining negative for Annexin V and propidium iodide). All assays were done on two separate occasions.

**Proteasome Activity Assay**

Cell lysates were prepared, and the fluorogenic peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity), was used according to the procedures described by the 20S Proteasome Activity Assay kit (Chemicon, Temecula, CA). In brief, control or drug-treated cells were broken in a lysis buffer [150 mmol/L NaCl, 20 mmol/L Tris (pH 7.2), 1% Triton X-100, 1 mmol/L DTT] without protease inhibitors. Total cell lysate (50 μg) was incubated with 20 μmol/L of fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC at 45°C in 100 μL of assay buffer [25 mmol/L HEPES (pH 7.5), 0.5 mmol/L of 0.05% NP40, and 0.001% SDS]. Free AMC liberated by the substrate hydrolysis was quantified for 90 minutes at 1-minute intervals on a microtiter plate fluorometer (FLUOstar Optima; BMG Labtech, Durham, NC; excitation, 355 nm; emission, 460 nm). Preliminary experiments with control cells indicated that reaction rates were linear for at least 2 hours. A fluorescence standard curve with known dilutions of Suc-Leu-Leu-Val-Tyr-AMC was generated. The data were plotted as arbitrary fluorescence units.

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versus time, and we obtained the slope of a line fit to the data using an appropriate linear regression program. Proteasome activity values (% control) were derived by dividing the slope obtained in the presence of bortezomib by the slope obtained in its absence × 100. For specific activity calculations, we used the formula: activity (pmol/min) = slope (arbitrary fluorescence units/min) × conversion factor (µg AMC/arbitrary fluorescence unit) × assay volume (µL).

**Immunofluorescence Assay**

Briefly, cells were seeded in 35-mm tissue culture plates on sterile glass coverslips until subconfluent. After appropriate treatments, cells were washed with PBS and fixed with methanol for 1 hour at −20°C. After blocking with 1% (w/v) bovine serum albumin in PBS for 30 minutes at 37°C, cells were incubated with anti-NF-κB p65 antibody (1:180 dilution in PBS plus 1% bovine serum albumin) for 2 hours at 37°C. Controls included buffer alone or nonspecific purified rabbit immunoglobulin G. Then cells were washed with PBS and incubated with the secondary antibody Alexa 546–coupled goat anti-rabbit IgG (1:1,000 dilution in PBS plus 1% bovine serum albumin) for an additional hour. The slides were further washed with PBS and then mounted in Mowiol (Calbiochem) for fluorescent microscopic examination. Fluorescence confocal and phase images were acquired using a Leica TCS SL laser scanning confocal spectral microscope (Leica Microsystems, Heidelberg GmbH, Mannheim, Germany). Image assembly and treatment were done using the Image Processing Leica Confocal Software.

**Western Blot Analysis**

For Western blot assays, cells were cultured in six-well plates and left untreated or treated as indicated in each experiment. Cells were lysed in ice-cold NP40 buffer [1% NP40, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA containing 5 mmol/L NaF, 2 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, 5 µg/ml leupeptine, and 5 µg/ml aprotinin]. After incubating for 30 minutes at 4°C, the samples were centrifuged, and the supernatant was kept as the NP40-soluble fraction. Unless otherwise specified, Western blot assays were done using the abovementioned conditions. In selected experiments, the pellet resuspended in SDS buffer [2% SDS, 80 mmol/L Tris (pH 6.8), 100 mmol/L DTT, and 10% glycerol] and sonicated constituted the NP40-insoluble fraction, as reported by others (22). Cell extracts (10 µg/lane) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Western blotting was carried out according to a standard procedure using horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The following primary antibodies were used: antibodies to phospho-ERK (p-ERK; Thr202/Tyr204), ERK, p-MEK1/2 (Ser217/Ser221), MEK1/2, phospho-c-Jun NH2-terminal kinase (p-JNK; Thr183/Tyr185), JNK, p-Raf (Ser259), p-AKT (Ser473), AKT, p-EGFR (Tyr1068), EGFR, PI3K (p85), PTEN, and p27 were obtained from Cell Signaling Technology (Beverly, MA). Antibodies to p21 (sc-6246) and MKP-1 (sc-370) were obtained from Santa Cruz Biotechnology. Rabbit polyclonal anti-p-HER-2 (Y1248) was bought from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal anti-HER-2 was from BioGenex (San Ramon, CA). Immunoblotting with β-tubulin mouse monoclonal antibody (Sigma) was done to confirm equal protein loading. Target proteins were visualized after enhanced chemiluminescence treatment (Amersham, Piscataway, NJ) of membranes and subsequent exposure to X-OMAT X-ray film (Sigma).

**Statistical Analysis**

Statistical analysis was carried out with SPSS version 11.0 (SPSS, Inc, Chicago, IL). To analyze correlations, the unpaired t test was used to determine the statistical differences. Statistical tests were conducted at the two-sided 0.05 level of significance.

**Results**

**Differential Effects of Bortezomib on Viability in Breast Carcinoma Cell Lines**

The antiproliferative effects of bortezomib were assessed by MTT in a panel of six human breast cancer cell lines cultured with increasing concentrations of bortezomib for periods of 24, 48, or 72 hours. Bortezomib caused a time-dependent and dose-dependent reduction in cell viability in all tested cell lines. An evident inhibition in cell viability was observed as early as 24 hours in some cell lines (data not shown), and an IC50 was achieved after 72 hours of incubation in all of them (Fig. 1A). Bortezomib IC50 ± SD varied widely among cell lines: 5 ± 1.4 nmol/L for SK-BR-3, 5 ± 2.4 nmol/L for MDA-MB-468, 7 ± 2.7 nmol/L for MDA-MB-231, 100 ± 8.4 nmol/L for MCF-7, 100 ± 12.1 nmol/L for MDA-MB-453, and 1,000 ± 14.4 nmol/L for BT-474 cells. This distinction may be of clinical importance because concentrations of 10 nmol/L are consistent with the one that results in proteasome inhibition at therapeutic doses of bortezomib (4).

Searching for potential molecular correlates of the heterogeneous antiproliferative response to bortezomib, we assayed the basal proteasome activity of each cell line. Protein extracts were prepared under similar culture conditions used for the MTT assays. Proteasome activity was consistently higher in BT-474 and MCF-7 cells compared with the other four cell lines. To assess specific proteasome activity, we did a standard calibration curve with AMC substrate, and proteasome activity in cell viability was observed as early as 24 hours in some cell lines (data not shown), and an IC50 was achieved after 72 hours of incubation in all of them (Fig. 1A). Bortezomib IC50 ± SD varied widely among cell lines: 5 ± 1.4 nmol/L for SK-BR-3, 5 ± 2.4 nmol/L for MDA-MB-468, 7 ± 2.7 nmol/L for MDA-MB-231, 100 ± 8.4 nmol/L for MCF-7, 100 ± 12.1 nmol/L for MDA-MB-453, and 1,000 ± 14.4 nmol/L for BT-474 cells. This distinction may be of clinical importance because concentrations of 10 nmol/L are consistent with the one that results in proteasome inhibition at therapeutic doses of bortezomib (4).
We further studied bortezomib effects on SK-BR-3 and BT-474 cells because they share expression of EGFR and overexpression of HER-2, whereas their sensitivity to bortezomib was markedly different (Fig. 1A). In cells treated with 10 nmol/L bortezomib, effects on cell viability were markedly more pronounced in SK-BR-3 cells compared with BT-474 cells (Fig. 2A). The differences on cell viability among the two cell lines were statistically significant ($P < 0.05$, two sided) at all the time points tested. We also assessed by fluorescence-activated cell sorting the effects of bortezomib on cell cycle and on apoptosis. Apoptosis was clearly induced in SK-BR-3 cells at 10 nmol/L of bortezomib, whereas effects on BT-474 cells were very slight (Table 1). High concentration and exposure to bortezomib for at least 24 hours were required to observe apoptosis in BT-474 cells. On the other hand, bortezomib effects on the cell cycle were more pronounced in BT-474 cells. At a 10 nmol/L concentration, bortezomib induced a moderate cell cycle arrest and cell accumulation in G2-M phase in BT-474 cells (Table 2). The difference in the assays used (MTT and fluorescence-activated cell sorting) and the important role of duration and dose on the effects of bortezomib on the resulting effect on cell proliferation and apoptosis contributed to the fact that the magnitude of the effects observed in MTT assays (a composite effect on cell activation, proliferation, and death) were more pronounced than the proapoptotic effects observed by fluorescence-activated cell sorting analysis.

**Effects of Bortezomib on Proteasome Activity and on Proteasome Substrates in Sensitive and Resistant Cell Lines**

We next compared the effects of bortezomib on proteasome activity in SK-BR-3 versus BT-474 cell lines. Cells were exposed to various concentrations of bortezomib for 2 hours, a time point used to measure bortezomib-induced proteasome inhibition in clinical studies (9). In both cell lines, relative proteasome activity inhibition was similar (Fig. 2B). At 10 nmol/L, relative proteasome activity inhibition was consistent with the level of inhibition achieved in patients. To study the durability of proteasome activity in both cell lines, cells were exposed to bortezomib 10 nmol/L for 72 hours. Relative proteasome activity inhibition at this time point was 85.1 ± 8.7% in SK-BR-3 cells and 59.5 ± 28% in BT-474 cells.

To test whether the proteasome inhibition induced by bortezomib resulted in accumulation of well-known proteasome substrates, we examined the effects on p21 and p27 and, indirectly, on the transcription factor NF-κB. Assays for p27 and p21 protein levels were done at 24 hours of bortezomib exposure. Increased levels of p27 protein were detected in both SK-BR-3 and BT-474 cells treated at concentrations of ≥10 nmol/L (Fig. 2C). Like p27, p21 levels were also substantially increased after 24 hours of treatment in BT-474 cells (Fig. 2C). We were not able to detect basal p21 in SK-BR-3 cells, and no p21 accumulation was detected following bortezomib exposure. Levels of p21 (in BT-474) and p27 (in both cell lines) proteins accumulated as early as 2 hours (data not shown) and remained elevated up to 24 hours of bortezomib exposure.

NF-κB/Rel transcription factors is another system regulated by the proteasome because proteasome inhibition prevents IκB degradation and therefore abrogates NF-κB/p65 nuclear translocation. To test whether bortezomib exhibited a similar effect on NF-κB in both SK-BR-3 and

![Figure 1](mct053-0025f1.jpg) **Figure 1.** A, sensitivities of breast cancer cells to bortezomib. Cells were seeded into 96-well tissue culture plates (1 × 10^4 per well) and allowed to attach overnight. Bortezomib (0.01 – 10^4 nmol/L) was then added, and cells were allowed to grow for an additional 72 h. Cell viability was assayed using CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay kit and expressed as relative to untreated controls cells. Viability for BT-474 (○), SK-BR-3 (△), MDA-MB-453 (▲), MDA-MB-231(■), MCF-7(□), and MDA-MB-468 (▲). Representative of three experiments. Points, means of at least four replicate wells; bars, SD. The final concentration of DMSO never exceeded 0.1%, and this concentration showed no effect on the proliferation of all tested cell lines (data not shown). B, proteasome basal-specific activity in breast cancer cells. Cell extracts (50 μg) were incubated for 1 h at 37°C with 20 μmol/L fluorogenic peptide substrate in the absence of bortezomib for the proteasomal chymotrypsin-like activity followed by measurement of free AMC groups. Columns, mean (n = 3); bars, SD. C, expression levels of signal transduction molecules in breast cancer cells. Cells lysates were generated, and the levels of indicated proteins were detected by Western blotting. Simultaneous immunoblotting of tubulin was used as an internal control for equivalent protein loading. Assays were done twice. Representative results.
and BT-474 cells, we examined by immunofluorescence the cellular localization of the NF-κB/p65 subunit in cells stimulated with TNF-α (a classic activator of NF-κB) with or without bortezomib pretreatment (Fig. 2D). Cells were exposed for 24 h to increase concentrations of bortezomib (1, 10, or 100 nmol/L). Lysates were prepared and assayed for p21 and p27 protein levels by Western blot; one representative experiment of three. D, nuclear translocation of NF-κB/p65 in response to treatment with TNF-α is prevented by bortezomib. Cells were left untreated or incubated with bortezomib (1, 10, or 100 nmol/L) for 90 min and then stimulated with 10 ng/mL TNF-α. Four hours after stimulation with TNF-α, cells were fixed, stained with anti-p65 antibody, and analyzed using confocal microscopy. a, control cells; b, 10 nmol/L bortezomib; c, TNF-α; d, TNF-α + 1 nmol/L bortezomib; e, TNF-α + 10 nmol/L bortezomib; f, TNF-α + 100 nmol/L Bortezomib. Two independent experiments with the same results were done.

Table 1. Percentage of apoptosis induced by bortezomib in SK-BR-3 and BT-474 cells

<table>
<thead>
<tr>
<th>Bortezomib (nmol/L)</th>
<th>SK-BR-3</th>
<th></th>
<th>BT-474</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 h</td>
<td>24 h</td>
<td>48 h</td>
<td>15 h</td>
</tr>
<tr>
<td>0</td>
<td>4.59 ± 0.28</td>
<td>6.33 ± 0.15</td>
<td>6.09 ± 0.11</td>
<td>0.99 ± 0.21</td>
</tr>
<tr>
<td>1</td>
<td>5.29 ± 1.16</td>
<td>5.26 ± 0.21</td>
<td>6.12 ± 1.50</td>
<td>0.81 ± 0.50</td>
</tr>
<tr>
<td>10</td>
<td>9.16 ± 0.14</td>
<td>17.3 ± 0.88</td>
<td>25.1 ± 0.96</td>
<td>1.01 ± 0.29</td>
</tr>
<tr>
<td>100</td>
<td>9.27 ± 1.14</td>
<td>23.5 ± 1.96</td>
<td>25.9 ± 0.89</td>
<td>1.72 ± 0.80</td>
</tr>
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NOTE: Results represent means of three experiments ± SD.
PI3K and the levels of PTEN were unaffected by HER-2 and EGFR. Total levels of the p85 subunit of survival PI3K/AKT pathway, which is regulated by HER-2, protein receptor modestly decreased in SK-BR-3 cells. In BT-474 cells, bortezomib reduced slightly HER-2 phosphorylation, and effects on total HER-2 receptor were minor (Fig. 3A). Total levels of EGFR remained essentially unchanged in both cell lines during bortezomib exposure. However, in SK-BR-3 cells a marked inhibition of EGFR phosphorylation occurred at bortezomib concentrations of ≥10 nmol/L. These effects were less evident in BT-474 cells.

To assay whether receptor protein changes observed in cells treated with bortezomib for 24 hours were related to a shift in their subcellular compartments, which may result in their presence in detergent-insoluble fractions, we studied receptor protein levels in detergent-soluble and insoluble fractions, following a methodology previously reported by others (22). Bortezomib minimally attenuated EGFR levels, and accumulation in the detergent-insoluble fraction was undetected. In contrast, treatment with bortezomib resulted in an evident decline of HER-2 protein in the detergent (NP40)–soluble fraction, with a concomitant increase in the detergent-insoluble fraction (Fig. 3C).

We also studied whether bortezomib was able to prevent ligand-induced EGFR phosphorylation. To this end, we exposed SK-BR-3 and BT-474 cells to EGF in the presence or absence of bortezomib (Fig. 3D). EGF addition, as expected, induced a marked increase in the phosphorylation of the EGFR and decreased the levels of total EGFR. The decrease in the levels of EGFR is in agreement with the ability of EGF to efficiently induce the degradation of the receptor (23). Pretreatment of cells with bortezomib did not prevent EGF-induced receptor phosphorylation and only slightly prevented total EGFR down-modulation. Under these experimental conditions, we also analyzed the NP40-insoluble fractions. EGF addition resulted in down-modulation of total EGFR levels without any detected increase in the NP40-insoluble fraction. However, bortezomib pretreatment (2 hours) prevented partially EGF-induced down-modulation of total EGFR (Fig. 3D), and EGFR accumulated in the NP40-insoluble fraction (data not shown).

We also examined the effects of bortezomib on the cell survival PI3K/AKT pathway, which is regulated by HER-2 and EGFR. Total levels of the p85 subunit of PI3K and the levels of PTEN were unaffected by bortezomib. A marked suppression of p-AKT was noted at 24 hours in SK-BR-3 cells at bortezomib concentrations of ≥10 nmol/L. Inhibition of AKT phosphorylation was less pronounced in BT-474 cells. Total level of AKT protein slightly decreased at a concentration of bortezomib of 100 nmol/L (Fig. 3A).

Time course experiments were then done (Fig. 3B). HER-2 phosphorylation decreased only at 24 hours of treatment. Inhibition of EGFR phosphorylation was observed at 4 hours (no effects were seen at earlier time points; data not shown) and remained inhibited for up to 24 hours (latest time point analyzed) in SK-BR-3 cells. AKT phosphorylation was transiently increased in SK-BR-3 cells, but a marked decrease in AKT phosphorylation was noted at 24 hours. Effects in BT-474 cells were less pronounced (Fig. 3B).

To study whether proteasome inhibitors other than bortezomib were more toxic to SK-BR-3 cells than BT-474 cells, we assayed cell viability by MTT and the effects on HER-2, EGFR, and AKT in cells treated with the proteasome inhibitor MG-132 (24). In these experiments, SK-BR-3 cell viability (IC<sub>50</sub> = 0.28 ± 0.05 μmol/L) was reduced to a greater extent than BT-474 cell viability (IC<sub>50</sub> = 1.5 ± 0.08 μmol/L; P < 0.05; data not shown). Similarly, the reduction in phosphorylation of HER-2, EGFR, and AKT induced by MG-132 was more pronounced in SK-BR-3 cells compared with BT-474 cells (Fig. 3E).

Bortezomib Activated the Raf/MEK/ERK1/2 Pathway and Affected MKP1/JNK

To study the effects of bortezomib on the Raf/MEK/ERK1/2 pathway, which is also regulated by HER-2 and EGFR, we did a series of Western blotting assays under the same culture conditions as indicated above. At 24 hours of exposure to bortezomib (≥10 nmol/L), there was an increased phosphorylation of Raf, MEK1/2, and ERK1/2 (Fig. 4A), which were more pronounced in SK-BR-3 cells than in BT-474 cells. Time course experiments, bortezomib-induced phosphorylation of ERK1/2 started 4 hours after exposure (Fig. 4B). We also analyzed bortezomib effects on JNK and MKP-1. The specific protein phosphatases MKP-1 (or CL100), a proteasome substrate, accumulated in both cell lines at concentrations of ≥10 nmol/L (Fig. 4A), and this was seen at 4 hours of exposure (Fig. 4B). Similar concentration-dependent and time-dependent effects were seen on p-JNK, without changes in total JNK protein levels (Fig. 4B).
Effects on Cell Viability of Combined Exposure of Bortezomib and Pharmacologic Inhibitors of HER Signaling Pathways

We next did a series of MTT assays to study potential interactions on cell viability between bortezomib and pharmacologic inhibitors of selected signaling molecules that were altered during bortezomib treatment. These experiments were done in SK-BR-3 cells due to their sensitivity to clinically relevant concentrations of bortezomib. The EGFR tyrosine kinase inhibitor AG 1478 reduced SK-BR-3 viability by \( \frac{1}{2} \) at 10 \( \mu \)mol/L following 72 hours of exposure. A combined treatment with AG1478 and bortezomib (both agents used at their estimated IC50s) resulted in an additional 8 ± 5% decrease in cell viability compared with each agent used alone. Similar results were observed with the PI3K inhibitor LY294002 (used at 40 \( \mu \)mol/L, a concentration that resulted in inhibition of AKT phosphorylation; data not shown). Combined treatment

Figure 3. A, Bortezomib dose-dependent effects in EGFR/HER-2 receptors and PI3K/AKT pathway in SK-BR-3 and BT-474 cells. The indicated breast cancer cell lines were exposed for 24 h to increased concentrations of bortezomib (1, 10, or 100 nmol/L). Equal amounts of protein from the cell lysates were resolved by SDS-PAGE and immunoblotted with a series of antibodies (p-HER-2, total HER-2, p-EGFR, total EGFR, PI3K (p85), PTEN, p-AKT (Ser473), total AKT, tubulin; from top to bottom). To detect p-EGFR, long exposures of the film were required. Bortezomib concentration values (above each bottom lane). Representative of three separate experiments. B, bortezomib time-dependent effects in EGFR/HER-2 receptors and PI3K/AKT pathway in SK-BR-3 and BT-474 cells. Exponentially growing SK-BR-3 and BT-474 cells (0 h) were treated with 10 nmol/L of bortezomib for the indicated hours, and then representative molecules were assayed by Western blot. C, bortezomib effects on EGFR and HER-2 in detergent NP40-insoluble fraction. Following treatment of SK-BR-3 at the indicated concentration of bortezomib for 24 h, cells were lysed in 1% NP40 buffer. Insoluble precipitates were resolubilized with SDS buffer (2% SDS). Equal amounts of protein were analyzed by Western blotting with anti-EGFR (A) or anti-HER-2 antibodies (B). Tubulin levels served as the control for equal protein loading. NP40 soluble (S) and insoluble (I) fractions. Representative of two independent experiments. D, bortezomib effects on EGF-induced receptor levels and phosphorylation. Cells were serum starved for 24 h and pretreated with the indicated concentrations of bortezomib or vehicle for 2 h. Cells were challenged for 15 min with EGF (100 ng/mL) or left untreated and lysed. Phosphorylated and total EGFR were assayed by Western blot. Tubulin is shown as loading control. One of two independent experiments with similar results. E, concentration-dependent effects of MG-132 in EGFR/HER-2 receptors and AKT in SK-BR-3 and BT-474 cells. Cells were cultured in the absence or presence of varying concentrations of MG-132 for 24 h, and protein extracts of cells were analyzed by Western blotting using antibodies as indicated. Tubulin was used as a loading control. Representative of two independent experiments.
with both agents further reduced by 10 ± 4% cell viability when compared with LY294002 alone. Finally, exposure of SK-BR-3 cells to a specific inhibitor of MEK, PD98059 (used at 40 μmol/L), a concentration that resulted in inhibition of ERK1/2 phosphorylation; data not shown), resulted in increased cell viability compared with untreated cells. This observation suggested that the hyperactivation state of Raf/MEK/ERK pathway in bortezomib-treated cells may contribute to growth inhibition and apoptosis induced by bortezomib in SK-BR-3 cells. Combined treatment with PD98059 did not appreciably increase the effects of bortezomib on cell viability (data not shown).

Discussion

The work presented here provides novel information on cellular and molecular effects of bortezomib in breast cancer cells. Bortezomib reduced cell viability in a concentration-dependent and time-dependent manner in breast cancer cell lines. Bortezomib’s effects showed marked heterogeneity in drug responsiveness, ranging from highly resistant (IC_{50} ≥ 100 nmol/L: MCF-7, MDA-MB-453, and BT-474) to highly sensitive (IC_{50} < 10 nmol/L: MDA-MB-468, MDA-MB-231, and SK-BR-3). Heterogeneous responses to bortezomib have been also recently reported in other tumor types such as prostate cancer (25).

A series of studies indicate that human tumors commonly have high proteasome expression levels (26). More recently, an analysis of transcriptional profiles from ~200 solid tumors indicated that mRNAs encoding proteasome subunits were highly coregulated in these cancers (27). These in vivo studies are also supported by findings in tumor cell lines. For instance, MCF-7 human breast cancer cells express elevated levels of one of the highly conserved proteasome subunits (28). In the present study, basal proteasome activity was 1.5- to 2-fold higher in the bortezomib-resistant MCF-7 and BT-474 cells compared with the other cell lines assayed. Although we cannot rule out that these differences may play a role in the differential sensitivity to bortezomib, a number of observations suggest that additional factors play a role in bortezomib effects in breast cancer cells. For instance, MDA-MB-453 cells had a basal proteasome activity similar to that of sensitive cells, whereas their IC_{50} was similar to the resistant MCF-7 cells. In addition, the degree of proteasome inhibition achieved during bortezomib exposure was similar in the tested cell lines, as reported in other models (18). Although relative proteasome inhibition by bortezomib measured at 2 hours was identical in SK-BR-3 (sensitive) and BT-474 (resistant) cells, the finding of a higher degree of proteasome inhibition at 72 hours of bortezomib exposure in SK-BR-3 cells raises the possibility that a quicker recovery from proteasome inhibition might contribute to the resistance of the BT-474 cells to bortezomib compared with SK-BR-3 cells.

Known proteasome substrates accumulated at a concentration of ≥10 nmol/L in cells with either relatively high or low basal proteasome activity. Bortezomib exposure in SK-BR-3 and BT-474 cells was associated with p27 accumulation and, in BT-474 cells, with p21 accumulation accompanied by induction of cell cycle arrest. p27 is a multifunctional protein, which, in addition to its cell cycle regulatory role, is a putative tumor suppressor (a proapoptotic protein; refs. 29, 30). In certain cancer cell lines treated with bortezomib, stabilization of p27 was observed in the induction of apoptosis (31, 32). In addition, by pretreating cells with bortezomib, the TNF-α-induced NF-κB/p65 translocation to the nucleus was attenuated to a similar extent in both cell lines. The inhibition of NF-κB by bortezomib is an effect well reported by others (33).

One of the best-characterized and clinically relevant signaling transduction systems in human breast cancer is the HER tyrosine kinase receptor system (34, 35). The HER receptor family is composed of four members [HER1 (also known as EGFR), HER-2, HER3, and HER4]. Signaling by the HER receptor tyrosine kinase family occurs through several downstream pathways, including the Raf/MEK/ERK1/2 and the PI3K/AKT pathways to promote cell proliferation and to inhibit apoptosis (36, 37). However, we did not observe any relationship between bortezomib effects on viability and expression/phosphorylation of HER-2, EGFR, AKT, or ERK1/2. We focused further studies in SK-BR-3 and BT-474 cell lines because both express similar levels of EGFR and HER-2 and respond similarly to...
anti-HER-2 (i.e., trastuzumab) or anti-EGFR agents (38, 39), whereas they exhibited markedly different responses to bortezomib. Apoptosis was quickly and strongly induced in SK-BR-3 cells when exposed to a clinically relevant concentration of bortezomib, whereas higher concentrations and a prolonged exposure to bortezomib were required to induce apoptosis in BT-474 cells. The relative resistance of BT-474 cells to bortezomib is in agreement with previous reports (40). The finding of similar results with another commonly used proteasome inhibitor (MG-132) supports the notion that the observed differential sensitivity of these two cell lines was not unique to bortezomib.

Bortezomib resulted in a marked inhibition of EGFR phosphorylation at 4 hours and remained inhibited for up to 24 hours (latest time point analyzed) in SK-BR-3 cells. In additional experiments, bortezomib did not affect EGFR-induced receptor phosphorylation. Notably, EGFR addition induced EGFR degradation, and this was modestly prevented by pretreatment with bortezomib (with a concomitant increase of receptor levels in NP40-insoluble fraction). This finding suggests that an intact proteasome function is required for the pathway of EGF-induced degradation of the receptor. Further experiments are needed to delineate the possibility that EGFR was degraded by lysosomal proteases in addition to the ubiquitin-independent proteasomal pathway.

HER-2 phosphorylation decreased only at 24 hours of treatment. The levels of total HER-2 also decreased during bortezomib exposure. The observation of down-modulation of HER-2 protein in HER-2-overexpressing cells exposed to bortezomib was intriguing, in light of their regulation by the proteasome. However, it should be noted that it is still unclear to what extent proteasomal processing is involved in down-regulation of receptors. With regard to HER-2, the down-modulation of protein levels observed in treated cells in our experiments is in keeping with findings by others that showed that proteasome inhibitors (MG-132 and bortezomib) repressed HER-2 promoter activity, destabilized mature cytoplasmic HER-2 transcripts, and induced a decline in HER-2 mRNA and protein levels in HER-2-overexpressing breast cancer cells (MDA-MB-453, SK-BR-3, and BT-474; ref. 40). Our results also showed that bortezomib treatment reduced the levels of HER-2 in the detergent (NP40)—soluble fraction. Moreover, we showed that this was associated to HER-2 recruitment in NP40-insoluble fraction in response to bortezomib. Studies of HER-2 ubiquitination and intracellular trafficking would be required to further characterize this finding (23). It is tempting to speculate that bortezomib caused HER-2 to accumulate as ubiquitinated proteins in aggresomes, which might then be found in the detergent-insoluble pellet fraction of cell lysates because bortezomib has been recently reported to affect ubiquitination (41). However, the down-modulation of HER-2 observed in the soluble fraction of cell extracts may explain, at least in part, the cooperative antitumor effects reported by others between bortezomib and the anti-HER-2 antibody trastuzumab (that down-modulates HER-2 levels; refs. 42, 43) or bortezomib- and hsp90-targeting agents (44) or HDAC inhibitors (40). With regard to the EGFR, at 24 hours of exposure, we observed a slight decrease in EGFR levels and no accumulation of receptor in the detergent insoluble fraction. This finding may be related to a limited role of the proteasome in EGFR degradation in the absence of ligand activation. Supporting this view, in cells exposed to cycloheximide, to facilitate studies of EGFR degradation by inhibiting protein synthesis, there was no evidence of constitutive decrease of total EGFR levels for the time points studied (up to 7 hours; ref. 23). Additional experiments pointed that the EGFR as such was not a direct proteasomal target in the absence of ligand-induced activation. These data provide possible explanations to our results of lack of EGFR accumulation during bortezomib exposure under our experimental conditions. Bortezomib treatment resulted in an early increase of AKT phosphorylation followed by a strong blockade of AKT phosphorylation. This was not associated to parallel changes in the total AKT or pI3K/p85 or PTEN protein levels. Taken together, it is tempting to speculate that the inhibition of AKT phosphorylation is associated to the upstream inhibition of EGFR and HER-2. The degree of the effects observed on phosphorylation of AKT in SK-BR-3 cells was much less evident in BT-474 cells.

In contrast to the inhibition of AKT phosphorylation, bortezomib treatment resulted in an enhanced phosphorylation of the Raf/MEK/ERK1/2 pathway. ERK1/2 is activated by phosphorylation on Tyr(185) and Thr(183) by a dual specificity kinase MEK, which can be activated by Raf-1. Raf-1 activation can be achieved by the GTP-bound activated form of Ras. An increased phosphorylation of ERK1/2 occurred at 4 hours of bortezomib exposure and remained sustained throughout the experiment. Moreover, these effects were associated with stimulation of MEK1/2 and Raf phosphorylation, thus showing that upstream components of the ERK signaling cascade participate in this process. As observed for EGFR, HER-2, and AKT, the magnitude of the effects on Raf/MEK/ERK1/2 were greater in SK-BR-3 cells than in BT-474 cells. Phosphorylation of JNK, a common mediator of bortezomib cell kill, was also observed (45). MKP-1 levels were increased following bortezomib treatment, in agreement with results recently reported by Small et al. (45). MKP-1 belongs to a family of inducible nuclear dual-specificity phosphatases exerting catalytic activity to phosphotyrosine-containing and phosphothreonine-containing proteins. However, it seems not to be a direct link between the increase in MKP-1 and the decline in p-HER-2, p-EGFR, and p-AKT after bortezomib treatment, because in our time-response studies, MKP-1 increase preceded the decline in the phosphorylation of HER-2, EGFR, and AKT. This view is further supported by the fact that these molecules are not known substrates for MKP-1. MKP-1 is known to inactivate ERK1/2 (among other ERKs); however, in our assays, we could not find an association between MKP-1 accumulation and ERK1/2 phosphorylation. Proteasome inhibitor-mediated induction of MKP-1 has been reported to have
an antiapoptotic role. We observed that MKP-1 was para-
doxically induced more strongly by bortezomib in SK-BR-3
bortezomib-sensitive cells than in the BT-474 bortezomib-
resistant cells. The potential role of MKP-1 accumulation
induced by bortezomib in the sensitivity of SK-BR-3 and
BT-474 cells to this agent remains to be characterized.

Notably, in other cell lines tested, such as MCF-7 and
MDA-MB-231, ERK1/2 phosphorylation decreased during
bortezomib exposure (data not shown). This later observa-
tion is in agreement with the findings observed with other
nonclinical proteasome inhibitors in MDA-MB231 cells (46)
or in other tumor systems (47). The reasons behind the
opposite effects on ERK1/2 between the HER-2 over-
expressing cells (i.e., ERK1/2 activation in SK-BR-3 and BT-
474) and the non–HER-2-overexpressing cells (i.e., ERK1/2
inhibition in MDA-MB-231 and MCF-7) are yet uncharac-
terized. In general, Raf/MEK/ERK1/2 pathway activation
is coupled with cell proliferation and survival (48),
although a prolonged activation may exert a proapoptotic
influence depending upon the cellular context (36, 49).
Here, exposure of SK-BR-3 cells to PD98059, a specific
inhibitor of MEK, resulted in increased cell viability. Thus,
pharmacologic blockade of MEK and the resulting inhi-
bition of ERK1/2 phosphorylation were possibly associ-
ated to the release of proapoptotic stimuli to the cells. This
observation suggests, indirectly, that the hyperactivation
state of the Raf/MEK/ERK1/2 pathway observed in
bortezomib-treated SK-BR-3 cells may contribute to growth
inhibition and apoptosis in SK-BR-3 cells. Interestingly,
combined treatments with bortezomib and PD98059 in
these cells resulted in an antitumor effect similar to that
achieved with bortezomib alone. Collectively, these results
show that bortezomib has distinct and opposite effects on
the phosphorylation of EGFR, HER-2, and AKT (suppres-
sion) and of the Raf/MEK/ERK1/2 pathway (activation) in
HER-2-overexpressing breast cancer cells. Such effects were
more pronounced in SK-BR-3 than in BT-474 cells. Whether
such molecular effects play a role in the response to
bortezomib in breast cancer cells or reflects differing
degrees of residual proteasome activity is yet unknown.

In conclusion, the data presented here provide novel
evidence for differential cellular and molecular effects of
bortezomib in a panel of human breast cancer cells. The
observed molecular effects of bortezomib on important
signaling transduction molecules regulated by the HER
receptor system may be relevant to help to design mechanistic-based combination treatments against breast
cancer. Future studies on other proteasome inhibitors that
act on similar and different parts of the proteasome
pathway might be important for delineating the role of
this pathway in mediating anticarcinogenic responses. The
results presented here show differential responses that are
dependent on cell context, and their interaction with other
agents were also variable. These results illustrate the
complex mechanism of bortezomib action. Mechanistic
studies will be needed to assess the role of the proteins
affected by bortezomib reported here in the antitumor
effects of this agent.

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