MAP17 (PDZKIP1) Expression Determines Sensitivity to the Proteasomal Inhibitor Bortezomib by Preventing Cytoprotective Autophagy and NFκB Activation in Breast Cancer

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Abstract

MAP17 is a small nonglycosylated membrane protein that is overexpressed in a high percentage of carcinomas. High levels of MAP17 enhance the tumorigenic properties of tumor cells by increasing oxidative stress, which is dependent on Na⁺-coupled cotransport. Here, we show that MAP17 is associated with proteins involved in protein degradation and that proteasome inhibition induces autophagy. To analyze whether MAP17 could also alter this process, we used the proteasome inhibitor bortezomib (Velcade, PS-341), which is approved for the treatment of multiple myeloma and mantle cell lymphoma, although it has a high rate of resistance emergence and poor efficacy in solid tumors. We provide evidence that bortezomib induces a cytoprotective effect by activating autophagy and NFκB nuclear translocation, responses that are repressed in the presence of high levels of MAP17 both in vitro and in vivo. Indeed, patients with multiple myeloma treated with bortezomib showed higher response rates and a longer time to progression associated with increased levels of MAP17 expression. The MAP17-induced sensitivity to bortezomib is dependent on the oxidative status of the cells and the activity of Na⁺-coupled transporters because treatment with antioxidants or the inhibitor furosemide restores the cytoprotective activity induced by bortezomib. Therefore, bortezomib induces a pro-survival response through cytoprotective autophagy and NFκB nuclear translocation, which is repressed by high levels of MAP17. We propose that the levels of MAP17 could be used as a prognostic marker to predict the response to bortezomib in hematologic malignancies and in other tissues that are not commonly responsive to the drug. Mol Cancer Ther; 14(6); 1–12. ©2015 AACR.

Introduction

Bortezomib (Velcade, PS-341) is a proteasome inhibitor that has been approved for the treatment of multiple myeloma and mantle cell lymphoma (MCL; refs. 1, 2). This drug acts on the 20S proteasome (3, 4) and possesses potent antitumor activity both in vitro and in xenograft models of a variety of tumors, including prostate, pancreatic, and squamous cell carcinomas (5–9). It has been proposed that proteasome inhibition by bortezomib induces endoplasmic reticulum (ER) stress, and sustained ER stress causes calcium release, leading to cytochrome C release from the mitochondria and subsequent apoptosis (10, 11). Accordingly, proteasome inhibition by bortezomib leads to the activation of proapoptotic genes and the repression of antiapoptotic genes (12). It has been reported that inhibition of the proteasome by bortezomib suppresses the NFκB pathway, which is an important survival mechanism in tumor cells (13, 14). NFκB is a heterodimeric transcription factor that remains inactive in the cytoplasm by binding to its inhibitory protein, IkB. Ubiquitination and degradation of IkB lead to the release of NFκB, which then translocates into the nucleus (15). It has been reported that the levels of NFκB activity are different in bortezomib-sensitive versus bortezomib-resistant cells (16). Furthermore, the relapse of multiple myeloma tumors observed in patients is correlated with higher levels of NFκB (17). It has also been shown that bortezomib induces IkBα degradation, which is removed by the autophagic process, and activates NFκB transcriptional activity (18). These and other results suggest that NFκB is a key target of bortezomib. However, other bortezomib targets have been proposed. Bortezomib induces a 20- to 60-fold increase in the level of the proapoptotic protein NOXA in cancer cells but not in normal cells (19). NOXA can also be induced by bortezomib in the absence of p53 activity (20). Moreover, in prostate cancer cell lines, bortezomib was reported to induce HIF1α protein synthesis through suppression of the PI3K and MAPK pathways (21).

Despite its efficacy, bortezomib treatment has toxic effects in patients, preventing the administration of more efficacious doses. Additionally, approximately 60% of patients eventually stop responding to the drug due to the emergence of resistance (2). Bortezomib has little efficacy in solid tumors, possibly due to stress granule formation involving the phosphorylation of elf2a (22). This situation has encouraged a search for selective markers to identify patients for whom bortezomib treatment might be more efficacious. It was found that cyclin D1 expression levels were correlated with the overall response to bortezomib treatment.
among patients with multiple myeloma (23, 24); thus, cyclin D1 has been proposed as a marker that can predict the response to treatment (25).

MAP17 (DD96, PDZKIP1) is a small, nonglycosylated membrane-associated protein of 17 kDa that is located on the plasma membrane and in the Golgi apparatus (26–28). MAP17 contains a hydrophobic N-terminus composed of 13 amino acids that encode a PDZ-binding domain and two transmembrane regions (29, 30). MAP17 binds several PDZ domain–containing proteins, including PDZK1 (NHRF3) and other NHRF proteins, such as NaPilla and NHe3. Together with NHRF3 and NHRF4, overexpression of MAP17 in opossum kidney cells leads to internalization of NaPilla in the trans-Golgi network (30–32). The physiologic role of MAP17 in proximal tubules is not known; however, MAP17 stimulates specific Na-dependent transport of mannose and glucose in Xenopus oocytes (33) and some human tumor cells (28, 34).

MAP17 enhances the tumorigenic properties of tumor cells by increasing the levels of reactive oxygen species (ROS; refs. 35, 36). At the molecular level, MAP17 protects Rat1a fibroblasts from Myc-induced apoptosis through ROS-mediated activation of the PI3K/AKT signaling pathway (28). Notably, modulation of ROS by antioxidant treatment prevented the activation of AKT and restored the level of apoptosis in serum-starved Rat1a/c-Myc fibroblasts (28). MAP17 is overexpressed in a wide variety of human carcinomas (37). In cervical, breast, prostate, and ovarian carcinomas, MAP17 overexpression is strongly correlated with tumor progression (34, 37). Furthermore, high levels of MAP17 predict a good response and survival among patients with cervical carcinoma treated with cisplatin and radiotherapy (34).

Because high MAP17 levels seem to correlate with a higher grade and poorer differentiation of tumors, the search for therapies that can counteract MAP17 expression may lead to beneficial application of known treatments to tumors with a poor prognosis. To explore the role of MAP17 as a predictive biomarker of the response to antitumor treatments, we performed a search for MAP17 partners to identify a functional relationship between MAP17 and a cellular process suitable for targeting. In this search, we identified 184 proteins, most of which belong to the proteasomal degradation pathway. Because the inhibition of proteasomal function has been described as a suitable antitumor strategy for some cancers, we tested whether interfering with proteasomal function might constitute a valuable therapeutic strategy in MAP17-expressing cells. We found that these cells are more sensitive to bortezomib, and patients with higher MAP17 mRNA levels respond better to this therapy and exhibit prolonged survival. We also showed that MAP17 determines the bortezomib sensitivity by inhibiting the cytoprotective effects related to bortezomib-induced NFκB nuclear translocation and autophagy. Furthermore, inhibition of oxidative stress abolishes the sensitivity to bortezomib induced by MAP17. The MAP17-induced increase in ROS is functionally related to Na+–coupled membrane transporters (NHeRF). Inhibition of these transporters also impairs the response to bortezomib by increasing the resistance to this drug in MAP17-overexpressing cells. Therefore, high levels of MAP17 could be used as a prognostic marker to predict the response of patients with diseases that can be clinically treated with bortezomib. Additionally, high MAP17 levels might be used to select some patients with other tumors for which bortezomib is not currently indicated, such as breast cancer.

Materials and Methods

Cell lines

The T47D and MDA-MB-231 cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC) commercial repository at the beginning of the study (2012). No further authentication was performed by the authors. Cytotoxicity studies were performed as previously described (38).

In vivo xenograft studies

Tumorigenicity was assayed by the s.c. injection of 4 × 10⁶ MDA-MB-231 or T47D cells expressing either the empty vector or MAP17 into the back legs of 4-week-old female athymic nude mice. Cohorts of 5 mice each were either treated with bortezomib (1.0 mg/kg body weight, in 0.9% NaCl) or saline serum (0.9% NaCl) when the tumor reached a diameter of 0.5 cm. A total of 5 doses per week were given for 4 weeks. The animals were examined weekly and incubated. The survival rates were analyzed. All animal experiments were performed according to the experimental protocol approved by HUVR Ethics Animals (04/12-2012P/115).

Immunodetection of MAP17

Cells were seeded onto glass coverslips, fixed with 4% paraformaldehyde for 20 minutes, and permeabilized with 0.5% Triton X-100 for 5 minutes. The coverslips were incubated with a MAP17 antibody for 1 hour. Detection was performed with an Alexa Fluor 594 antibody (red; Life Technologies), the nuclei were stained with DAPI, and the coverslips were mounted with Prolong Gold Antifade (Life Technologies). A confocal ultraspectral microscope (Leica TCS-SP2-AOBS-UV) with sequential scanning of the emission channels was used for image detection. The monoclonal MAP17 antibody was generated from a bacterially expressed and purified GST-MAP17 protein (37).

Western blotting was performed as previously described. The following primary antibodies were used: anti–NFκB-pp65 (1:2000; Abcam #ab16502), anti–NFκB-pp65 (phospho-Ser536) [93H11] (1:1000; Cell Signaling Technology #3033), anti-IKKα [Y463] (1:5000; Abcam #ab32041), anti-IKKβ (phospho-Ser32/36) [5A5] (1:1000; Cell Signaling Technology #9246), anti-p44/42 MAPK [137F5] (1:2000; Cell Signaling Technology #4695), anti-p44/42 MAPK (phospho-Thr202/Tyr204) [E10] (1:2000; Cell Signaling Technology #9106), anti-CyclinD1 [SP4] (1:200; Abcam #ab16663), anti-p21 [C-19] (1:200; Santa Cruz Biotechnology #sc-397), anti-p53 [FL-393] (1:200; Santa Cruz Biotechnology #sc-6243), anti-Stat3 [124H6] (1:1000; Cell Signaling Technology #9139), anti-Stat3 (phospho-Tyr705) [D3A7] (1:2000; Cell Signaling Technology #9145), anti-Hsp27 [G31] (1:1000; Cell Signaling Technology #2402), anti-Hsp70 (1:1000; Epitomics #1776-1), anti-Hsp90α [2G5.G3] (1:2000; Abcam #ab79849), anti-LC3B (1 µg/ml; Abcam #ab48394), and anti–α-tubulin (1:5000; Sigma 9026). Secondary antibodies used were horseradish peroxidase–labeled rabbit anti-mouse (1:3000; Amersham) and goat anti-rabbit (1:3000; Abcam #6721). The proteins were visualized using the ECL detection system (Amersham Biosciences).

Fluorescent ROS detection

Cells were grown in 96-well optical bottom black plates (Thermo Scientific Nunc). The CellROX green reagent (Life
Tissue microarray immunohistochemistry was performed as previously described (34, 35).

**Autophagy analyses**

Cells were seeded onto glass coverslips. After 24 hours, the cells were incubated with the corresponding drugs for 16 hours. Subsequently, the cells were fixed and permeabilized as described above. Then, the coverslips were coincubated with anti-LC3B (1 μg/mL; Abcam #ab48394) and anti-LC3A (1 μg/mL; Abcam #ab25631). Anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 633 were used as secondary antibodies. The nuclei were counterstained with DAPI, and the slides were mounted with Prolong Gold Antifade (Life Technologies). The samples were visualized with a confocal ultraspectral microscope (Leica TCS-SP2-AOBS-UV) by sequential scanning of the emission channels. Autophagy was defined as colocalization of LAMP2 and LC3B. A minimum of 200 cells per experiment were analyzed. The average of three independent experiments was plotted as a percentage. Statistical significance was calculated using the Student t test.

**NFκB nuclear translocation**

Cells were seeded onto glass coverslips, fixed, and permeabilized as described above. Then, the coverslips were incubated with anti–NFκB p65 (1:100; Abcam #ab16502) or anti–NFkB-p65 (phospho-Ser536) [93H1] (1:100; Cell Signaling Technology #3033). Alexa Fluor 488–conjugated secondary antibodies (green; Life Technologies) were used in both cases. The nuclei were counterstained with DAPI, and the slides were mounted with Prolong Gold Antifade (Life Technologies). The samples were visualized with a confocal ultramicroscope (Leica TCS-SP2-AOBS-UV) by sequential scanning of the emission channels. The mean intensity of the fluorescence was measured for a minimum of 400 cells per condition using Leica Confocal Software. The plotted values represent the mean (±SD) of each condition. Statistical significance was calculated using the Student t test.

**Biocomputational analysis**

The protein-to-protein interaction network was examined with the ToppGenet tool (http://toppgene.cchmc.org/) of the ToppGene suite (39) using databases that were updated at the end of 2012. MAP17 was used as a seed, and two steps were used as the distance to the seeds. The K-step Markov prioritization method was used as described above. Then, the coverslips were coincubated with anti–NHE3 (SLC9A3) and other well-described interactors, including SLC22A5, SLC15A2, SLC22A4, SLC1A2, ABC2C, and CFP (32). Furthermore, we found that a majority of the genes were involved in posttranslational modifications related to protein degradation. We performed GO analysis to determine the most represented cellular components among these genes, and we found that cullin-RING proteins, ubiquitin ligase complexes, and membrane transporters were enriched (Supplementary Fig. S1B). Consistently, analysis of the most highly represented molecular functions revealed an enrichment of ubiquitin–protein ligase activities among the MAP17-interacting genes (Supplementary Fig. S1C), and analysis of the biologic processes showed a similar biologic distribution within the ubiquitin/proteosomal pathway (Supplementary Fig. S2). Consistent with MAP17 function in the proximal tubules of the apical brush border of the kidney, we observed that MAP17 also appears to be functionally related to transmembrane transport of anions.

These results prompted us to determine whether MAP17 levels had any effect on the cellular response to proteasome inhibition or to the response to the clinically used inhibitor bortezomib, which is currently indicated as a treatment for patients with multiple myeloma.

**MAP17 overexpression leads to high bortezomib sensitivity in breast tumor cells**

To explore the correlation between MAP17 levels and the response to bortezomib, we ectopically overexpressed MAP17 in cell lines from commonly unresponsive mammary tumors. In these tumors, MAP17 is overexpressed mainly in the advanced stages (36), and it is barely detectable or not expressed in non-tumoral tissues and intraductal neoplasias (MIN; Supplementary Fig. S3). Therefore, we generated MAP17-overexpressing T47D and MDA-MB-231 cells (Fig. 1A and D). Furthermore, we confirmed that was MAP17 overexpressed by immunofluorescence (Fig. 1B and E). Next, we subjected the cells to different doses of bortezomib to calculate the IC50 (38). Interestingly, we found that the tumor cells overexpressing MAP17 had a 10-fold lower IC50 than the control cells (Fig. 1C and F), indicating that overexpression of MAP17 increases the sensitivity of breast tumor cells to bortezomib. Similar sensitivity increases were observed, to varying extents, in cell lines of other tumor types, i.e., cervical cancer (HeLa) and colon cancer (HCT116) cells (Supplementary Table S2).

To confirm these data in vivo, we generated xenografts of T47D and MDA-MB-231 cells expressing the empty vector or MAP17. Cohorts of 5 mice each were treated either with bortezomib or with saline serum once the tumor reached a diameter of 0.5 cm. Bortezomib treatment caused the regression of 80% of the tumors in MAP17-expressing T47D cells, and the mice survived at least 6 months longer than mice that were xenografted with T47D cells
expressing the empty vector (Fig. 1G). Likewise, in MAP17-expressing MDA-MB-231 cells, bortezomib treatment caused the regression of 40% of the tumors, and the mice survived at least 6 months longer than the untreated mice (Fig. 1I). However, T47D and MDA-MB-231 cells expressing the empty vector alone caused death in mice at the same rate, independently of the treatment with bortezomib (Fig. 1H and J). These data confirm the ability of MAP17 to induce sensitivity to bortezomib in vitro and in vivo.

To further confirm whether MAP17 has any effect on the in vivo response to bortezomib, we sought to validate our findings in patients treated with bortezomib. This inhibitor has been mainly used to treat multiple myeloma; therefore, data regarding this correlation were available. To determine whether multiple myeloma patients expressing MAP17 responded better to bortezomib, we examined previously published clinical data analyzing the overall transcription in tumors from patients with multiple myeloma treated with bortezomib (40). We investigated whether the MAP17 mRNA levels were specifically correlated with the progression of the illness and the patient's response (as defined by the clinical parameters described in ref. 40). We found a positive and significant correlation between the MAP17 mRNA levels and the number of days until tumor progression ($P<0.05$; Supplementary Fig. S4A). In addition, we observed higher levels of MAP17 mRNA in patients responding to bortezomib treatment compared with
those that did not respond to the treatment ($P < 0.05$; Supplementary Fig. S4B). We concluded that patients with higher levels of MAP17 showed a longer time to progression and had a better response to bortezomib treatment.

Levels of the MAP17 oncogene determine the sensitivity to bortezomib by regulating the nuclear translocation of cytoprotective NFkB

To explore the mechanism by which MAP17 increases the sensitivity to bortezomib, we first treated T47D and MDA-MB-231 cells expressing high levels or wild-type levels of MAP17 with different concentrations of the drug. In control cells, bortezomib induced dose-dependent phosphorylation of NFkB-p65 at Ser536, increased IkBalpha phosphorylation at Ser32/36, and decreased total IkBalpha (Fig. 2A and Supplementary Fig. S5), which is consistent with previous reports (41, 42). We also observed a dose-dependent inactivation of ERK1/2, measured as a decrease in the phosphorylation of p42/p44 at Thr202/Tyr204 residues. Additionally, we found a decrease in the activation of STAT3, as its phosphorylation at Tyr705 decreased. However, cells overexpressing MAP17 showed lower levels of IkBalpha and phospho-NFkB-p65 (Fig. 2A). These cells also showed decreased ERK1/2 phosphorylation levels at lower concentrations of bortezomib (Fig. 2A). In addition, the response of STAT3 was similar in control cells of both cell lines (Fig. 2A). A direct comparison of MAP17-overexpressing and control cells showed lower levels of basal phospho-NFkB-p65 and phospho-IkBalpha in MAP17-overexpressing cells, which may

Figure 2.

MAP17 levels determine the sensitivity to bortezomib by reducing the activation and regulating the nuclear translocation of cytoprotective NFkB. A, Western blots showing the levels of different proteins and modified proteins in T47D and MDA-MB-231 cells overexpressing MAP17 or empty vector only and treated with increasing concentrations of bortezomib. B, Western blots showing chaperone levels in T47D and MDA-MB-231 (D) cells overexpressing MAP17 and treated with increasing concentrations of bortezomib. NFkB-p65 phosphorylated at Ser536 is shown in green. DAPI nuclear staining is shown in blue. Cells were seeded onto glass coverslips, treated with bortezomib, fixed, and permeabilized. Immunofluorescence was performed following standard procedures (see Material and Methods). The mean fluorescence intensity was measured for at least 400 cells per condition using Leica Confocal Software. Quantification of nuclear phospho-NFkB-p65 from C or D, respectively, plotted as a percentage of signal in the nucleus versus total signal in the cell. The average and standard deviation of at least 400 cells in each condition are shown. Values, mean ($\pm$ SD). Statistical significance was calculated using the Student t test: **, $P < 0.01$; ***, $P < 0.001$. 

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Figure 3. MAP17 levels determine the sensitivity to bortezomib regulating autophagy. Cells were treated with the appropriate drugs and fixed for immunofluorescence as described (see Materials and Methods). Autophagy was defined as the percentage of cells showing colocalization of LAMP2 and LC3B. At least 200 cells per experiment were analyzed. Colocalization of the autophagosomal marker LC3B (green) and the lysosomal marker LAMP2 (red) were observed by immunofluorescence in T47D (A) and MDA-MB-231 (C) cells overexpressing MAP17 and treated with increasing concentrations of bortezomib. DAPI nuclear staining is shown in blue. B and D, quantification of autophagic cells from A and C, respectively, measured as a percentage of LC3B and LAMP2 colocalization. The average and standard deviation of at least 200 cells in each condition are shown. *, P < 0.05 (Student t test).
account for the differences observed. We also found that other proteins that might be involved in the degradation process, such as components of the HSP complex, showed no variations in the presence of MAP17 (Fig. 17). These results suggest that MAP17 overexpression leads to a decrease in the activation of the NFκB pathway in response to bortezomib.

For full activation, NFκB must translocate to the nucleus, where it can act as a transcription factor. This translocation occurs as a consequence of the phosphorylation of the p65 subunit. Therefore, we measured the nuclear translocation of the p65 subunit by immunofluorescence. We observed dose-dependent phospho-NFκB-p65 accumulation in the nucleus after bortezomib treatment (Fig. 2C and D). This accumulation doubled at 20 μmol/L bortezomib and increased 4-fold at 40 μmol/L bortezomib (Fig. 2C and D). However, the nuclear accumulation was significantly reduced in MAP17-expressing cells (Fig. 2C and D). Similar results were obtained when we measured the total NFκB-p65 nuclear accumulation (Supplementary Fig. S6). The increase in the nuclear accumulation induced by bortezomib suggests that NFκB-p65 has a cytoprotective effect, and its inhibition in MAP17-overexpressing cells may partially explain the increase in sensitivity.

Levels of the MAP17 oncogene determine the sensitivity to bortezomib and regulate cytoprotective autophagy

In addition to the NFκB pathway, previous studies have indicated that bortezomib also induces autophagy (43–45). Therefore, we analyzed whether MAP17 could also alter this process. Autophagy is characterized by intracellular formation of a double-membrane vesicle, the autophagosome. Effective autophagy requires fusion of the autophagosome with lysosomes, which then digest and recycle cellular components. Therefore, we analyzed autophagy using coimmuno-fluorescence staining of the autophagosomal marker LC3 and the lysosomal marker LAMP-2 (Fig. 3A and C). This indicates that MAP17-induced autophagy in a dose-dependent manner (Fig. 3A and C), and this response was reduced by 30% in MAP17-overexpressing cells (Fig. 3B and D). To confirm these data, we have analyzed total LC3B by Western blot after bortezomib treatment in parental (expressing only vector) and MAP17-ectopically expressing cells (Supplementary Fig. S7). We found that treatment with bortezomib clearly increases LC3B in parental cells, but not in MAP17-expressing cells (Supplementary Fig. S7). These data confirmed the role of MAP17 in preventing the autophagy activated by bortezomib. Because it has been reported that autophagy is often elicited as a cytoprotective measure against cellular stress, our data suggest that MAP17-induced inhibition of this process may also contribute to the sensitivity to bortezomib.

Increased sensitivity of MAP17-overexpressing cells to bortezomib depends on ROS generation

To further explore the mechanisms underlying the increased sensitivity of MAP17-overexpressing cells to bortezomib, we investigated the known molecular properties of MAP17. The increased tumorigenesis induced by MAP17 is associated with an increase in ROS (35). MAP17 greatly alters the mRNA levels of genes involved in oxidative stress and increases the endogenous ROS levels (Supplementary Fig. S8). Additionally, the treatment of MAP17-expressing cells with antioxidants leads to a reduction in their tumorigenic properties (35, 36). Furthermore, ROS have been found to be directly related to NFκB activation, and ROS production is deregulated in autophagy-deficient cells. Therefore, we questioned whether ROS were mediating the effects observed in MAP17-expressing cells.

To evaluate whether ROS play a role in the response to bortezomib at a physiologic level, we measured the IC50 for bortezomib in the presence of the ROS scavengers N-acetyl cysteine (NAC) and glutathione (GSH). We found that ROS scavenging caused the cells to become more resistant to bortezomib—particularly the MAP17-overexpressing cells—thus restoring the resistance to the drug (Table 1). To evaluate whether MAP17-induced ROS might contribute to the cytoprotective responses induced by bortezomib, we treated parental and MAP17-overexpressing cells with different concentrations of bortezomib and analyzed the behavior of the drug targets. We found that treatment with ROS scavengers abolished the phosphorylation of IkBα and the subsequent activation of NFκB (Fig. 4A–D). This result indicates that NFκB-induced cytoprotection is dependent on ROS activity. Thus, full activation of phospho-NFκB requires its translocation to the nucleus, where it can act as a transcription factor. Therefore, we analyzed the nuclear translocation of phospho-NFκB-p65 in the presence of ROS scavengers in cells either expressing or not expressing MAP17 and found that ROS scavengers recovered the levels of dose-dependent phospho-NFκB-p65 accumulation in the nucleus after bortezomib treatment in MAP17-expressing cells (Supplementary Fig. S9). Similar results were obtained by measuring total NFκB-p65 nuclear accumulation (Supplementary Fig. S10). These results indicate that the inhibition of NFκB-p65 nuclear translocation and the subsequent activation of NFκB in MAP17-expressing cells is mediated by ROS. On the other hand, the cytotoxicity data (Table 1) indicated that these cells were more resistant to bortezomib, suggesting that this treatment induced two independent effects: a cytoprotective effect involving NFκB and an effect on the apoptotic response that is independent of NFκB.

The basal levels of autophagy in parental and MAP17-overexpressing cells treated with ROS scavengers were similar (Fig. 4E and F). However, bortezomib treatment induced a clear increase

### Table 1. Determination of the IC50 for T47D and MDA-MB-231 cells overexpressing MAP17 as well as control cells that were treated (or not treated) with bortezomib and/or NAC, GSH, or furosemide.

<table>
<thead>
<tr>
<th>IC50 (nmol/L)</th>
<th>T47D</th>
<th>MAP17</th>
<th>MDA-MB-231</th>
<th>MAP17</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>21.8 ± 8</td>
<td>2.9 ± 2.25</td>
<td>8.1 ± 1.5</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td>NAC &gt;30,000</td>
<td>36.1 ± 8</td>
<td>18 ± 3.9</td>
<td>38 ± 6.9</td>
<td>28.2 ± 1</td>
</tr>
<tr>
<td>GSH &gt;30,000</td>
<td>45 ± 3</td>
<td>27.7 ± 17.7</td>
<td>21.5 ± 0.5</td>
<td>15.2 ± 9.2</td>
</tr>
<tr>
<td>Furosemide</td>
<td>1,078</td>
<td>1,154 ± 40</td>
<td>11 ± 2</td>
<td>1,154 ± 40</td>
</tr>
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in the rate of cytoprotective autophagy only in parental cells and not in cells overexpressing MAP17. Treatment with ROS scavengers restored the activation of cytoprotective autophagy in MAP17-overexpressing cells (Fig. 4E and F). Therefore, the ROS levels may influence autophagy by restoring its activation in MAP17-overexpressing cells or by releasing the inhibition induced by MAP17 expression.

The increased sensitivity to bortezomib observed in MAP17-overexpressing cells depends on the active transport machinery at the membrane

Through NHHeRF1/3 binding, MAP17 may alter the membrane localization of pumps and transporters, particularly Na⁺-coupled transporters (32). Thus, MAP17-overexpressing cells may contain different membrane-associated Na⁺-coupled transporters

Figure 4.
MAP17-induced sensitivity to bortezomib depends on ROS generation. Western blots showing the levels of different proteins and modifications in T47D (A) and MDA-MB-231 (B) cells overexpressing MAP17 and untransformed cells that were treated (or not) with increasing concentrations of bortezomib and 10 mmol/L N-acetylcysteine (NAC) or (C and D) glutathione (GSH). E and F, quantification of autophagic cells, measured as colocalization of LC3B and LAMP2, in T47D and MDA-MB-231 cells overexpressing MAP17 and untransformed cells that were treated (or not) with 20 nmol/L bortezomib and 10 mmol/L NAC or GSH. The average and standard deviation of at least 200 cells in each condition are shown. *, P < 0.05; **, P < 0.01 (Student t test).
Na⁺-K⁺-ATPase pumps return reabsorbed Na⁺ to the systemic circulation and maintain the cellular Na⁺ concentration at relatively low levels. The latter effect is particularly important because it allows filtered Na⁺ to enter the cells down a favorable concentration gradient via carrier-mediated transport. By inhibiting the transporter, furosemide reduces the reabsorption of NaCl and also diminishes the positive potential derived from K⁺ recycling. By reducing this potential, furosemide causes an increase in Mg²⁺ and Ca²⁺ excretion.

Among other nonspecific inhibitory effects, furosemide also inhibits PG dehydrogenase, an enzyme that degrades PGE2, carbonic anhydrase, and GABA-A receptors.

We treated the cells with furosemide in the presence of different concentrations of bortezomib and measured the molecular and physiologic responses to the treatments. We found that furosemide specifically inhibited the activation of cytoprotective NFκB only in MAP17-overexpressing cells (Fig. 5A and B). Furthermore, furosemide increased the basal levels of autophagy in MAP17-overexpressing cells and maintained this cytoprotective effect in the presence of bortezomib (Fig. 5C and D). Therefore, the inhibition of oxidative stress abolished the MAP17-induced sensitivity to bortezomib. The MAP17-induced increase in ROS is functionally related to NHEFRs. Inhibition of these transporters by furosemide impairs the response to bortezomib, thus leading to the recovery of bortezomib resistance in MAP17-overexpressing cells.

**Discussion**

Using bioinformatic analyses, we found that MAP17 interacts with 184 proteins, and most of them are related to the proteasomal degradation pathway. This finding led us to test whether MAP17-overexpressing cells displayed differential sensitivity to the proteasomal inhibitor bortezomib, an anticancer drug that is used clinically. To explore the response to MAP17 expression, we overexpressed this gene in bortezomib-resistant tissue. We chose breast tissue because MAP17 is expressed in 60% of human mammary tumors and is not expressed in normal breast tissue or benign neoplasias. MAP17 overexpression in mammary carcinoma cells induced a 10-fold increase in the sensitivity to bortezomib, both in vitro and in vivo. Therefore, we focused on the mechanism that causes the resistance to bortezomib. We found that the MAP17-dependent increase in sensitivity was correlated with inhibition of the cytoprotective effect induced by the nuclear translocation of phosphorylated NFκB and the autophagy induced by bortezomib. Furthermore, the inhibition of oxidative stress abolished the MAP17-induced sensitivity to bortezomib. The MAP17-induced increase in ROS is functionally related to NHEFRs. Inhibition of these transporters by furosemide impairs the response to bortezomib, thus leading to the recovery of bortezomib resistance in MAP17-overexpressing cells.
It is generally thought that autophagy has two opposing functions in tumor cells in response to chemotherapy-induced stress: a cytoprotective role and a cytotoxic role. Recent efforts have been dedicated to exploiting cytoprotective autophagy inhibition as a therapeutic strategy to sensitize cells to chemotherapy or radiation (46). However, these efforts are hampered by several factors, including the inability to predict the cytoprotective effect of each treatment, the efficacy of the treatment as opposed to the induction of cytotoxic autophagy, and the expected toxic effects of inhibiting autophagy in organisms. Indeed, many severe diseases, such as Parkinson’s disease, are associated with defective autophagy (47). Furthermore, there are doubts about the suitability of the tested autophagy inhibitors because it is not clear whether the contributions of each cytoprotective effect (NFκB phosphorylation) to bortezomib resistance is not clear. It is clear that the presence of MAP17 could functionally substitute for an autophagy inhibitor, there is no need to include an autophagy inhibitor in the treatment to decrease the expected toxic effect on the host. MAP17 is expressed only in normal kidney tissue and in tumors; thus, no other secondary effects are expected to arise due to bortezomib treatment.

As described earlier, bortezomib-induced NFκB phosphorylation promotes its nuclear translocation and thus its activity as transcription factor. NFκB is known to activate antiapoptotic genes and promote survival, and the NFκB pathway has been described as an important survival mechanism in tumor cells (13, 14, 18). High levels of MAP17 reduced NFκB translocation upon bortezomib treatment, reducing its cytoprotective role in tumors, and this reduction is dependent on ROS because treatment with ROS scavengers restores the resistance. However, the relative contribution of each cytoprotective effect (NFκB activation and autophagy) to bortezomib resistance is not clear. It is clear that the presence of MAP17 renders tumor cells unable to exploit these cytoprotective effects.

Therefore, high levels of MAP17 could be of prognostic use for predicting the treatment response in patients with diseases that clinically treated with bortezomib. Additionally, high MAP17 levels could be used to select some patients with other tumors for which bortezomib is not currently an indication.

MAP17 is overexpressed in malignant tumors (stages II and III) but not in normal, benign, or early-stage human breast tumors. Therefore, MAP17 can be considered an independent marker of malignancy in human breast tumors. We have previously shown that MAP17 overexpression is correlated with malignant stages of ovarian, cervical, and prostate tumors (37). Mammary carcinoma cells, but not nontumoral epithelial cells that stably express MAP17, show an enhanced tumor phenotype, which is characterized by enhanced proliferative capabilities. The increased tumorigenic properties induced by MAP17 are associated with high levels of ROS, and treatment of MAP17-overexpressing cells with antioxidants leads to a reduction in their tumorigenic properties. Inhibition of ROS with antioxidant treatments also inhibits the effect of MAP17 on NFκB activation and autophagy, restoring resistance to bortezomib and indicating that ROS are involved in this process.

It has been shown that bortezomib favors the unfolded protein response, which is activated in response to alterations in the ER physiologic environment (48–50). This ER stress stimulates ROS production, which alters the responses to bortezomib treatment in patients with MCL (50) and multiple myeloma (51).

Similarly, furosemide, a nonspecific inhibitor of membrane transport, specifically inhibits the activation of cytoprotective NFκB only in MAP17-overexpressing cells. Furthermore, furosemide increases the basal levels of autophagy in MAP17-overexpressing cells and maintains this cytoprotective effect in the presence of bortezomib. Therefore, the inhibition of membrane transport by furosemide restores the cytoprotective response induced by bortezomib. Therefore, we should take into consideration certain additional treatments, such as antioxidants or diuretics, which may influence the response to bortezomib treatment in patients.

In summary, MAP17 determines the sensitivity to bortezomib by inhibiting the cytoprotective effects resulting from bortezomib-induced NFκB nuclear translocation and autophagy. Furthermore, the inhibition of oxidative stress abolishes the MAP17-induced sensitivity to bortezomib. The MAP17-induced increase in ROS is functionally related to NHeRFs, and the inhibition of these transporters also impairs the bortezomib response by triggering bortezomib resistance recovery in MAP17-overexpressing cells. Therefore, high levels of MAP17 could be of prognostic use for predicting treatment responses in patients with diseases that clinically treated with bortezomib. Additionally, some patients with other tumors for which bortezomib is not currently an indication could be selected as potential candidates for bortezomib treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Writing, review, and/or revision of the manuscript: S. Muñoz-Galván, G. Gutierrez, M. Perez, A. Camero
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Gutierrez, M. Perez
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