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Proteasome Inhibition Activates Epidermal Growth Factor Receptor (EGFR) and EGFR-Independent Mitogenic Kinase Signaling Pathways in Pancreatic Cancer Cells

Callum M. Sloss,¹ Fang Wang,¹ Rong Liu,¹ Lijun Xia,¹ Michael Houston,¹ David Ljungman,¹ Michael A. Palladino,² and James C. Cusack, Jr.¹

Abstract **Purpose:** In the current study, we investigate the activation of antiapoptotic signaling pathways in response to proteasome inhibitor treatment in pancreatic cancer and evaluate the use of concomitant inhibition of these pathways to augment proteasome inhibitor treatment responses. **Experimental Design:** Pancreatic cancer cell lines and mouse flank xenografts were treated with proteasome inhibitor alone or in combination with chemotherapeutic compounds (gemcitabine, erlotinib, and bevacizumab), induction of apoptosis and effects on tumor growth were assessed. The effect of bortezomib (a first-generation proteasome inhibitor) and NPI-0052 (a second-generation proteasome inhibitor) treatment on key pancreatic mitogenic and antiapoptotic pathways [epidermal growth factor receptor, extracellular signal-regulated kinase, and phosphoinositide-3-kinase (PI3K)/AKT] was determined and the ability of inhibitors of these pathways to enhance the effects of proteasome inhibition was assessed *in vitro* and *in vivo*. **Results:** Our data showed that proteasome inhibitor treatment activates antiapoptotic and mitogenic signaling pathways (epidermal growth factor receptor, extracellular signal-regulated kinase, *c-Jun*-NH₂-kinase, and PI3K/AKT) in pancreatic cancer. Additionally, we found that activation of these pathways impairs tumor response to proteasome inhibitor treatment and inhibition of the *c-Jun*-NH₂-kinase and PI3K/AKT pathways increases the antitumor effects of proteasome inhibitor treatment. **Conclusion:** These preclinical studies suggest that targeting proteasome inhibitor – induced antiapoptotic signaling pathways in combination with proteasome inhibition may augment treatment response in highly resistant solid organ malignancies. Further evaluation of these novel treatment combinations in clinical trials is warranted.

Pancreatic tumors remain one of the most lethal forms of cancer with a 5-year survival rate of <5%. Late diagnosis and early development of metastasis to the regional lymph nodes and liver limit curative resection to <10% of patients. To date, chemotherapy and radiation treatments have failed to significantly improve long-term survival; with 37,000 new diagnoses and 33,000 deaths estimated in the United States for 2007, it is imperative to find more effective therapies for pancreatic carcinomas (1).

Monotherapy with single chemotherapeutic agents and targeted compounds is rarely able to surmount the divergent multipathway survival and growth signaling pathways that are

critical to the survival of cancer cells. As a result, researchers and clinicians have resorted to investigating the potential of combination therapies (2). Kim and Kaelin have suggested that the optimal combination of molecular anticancer therapies may be achieved by either, "horizontal combinations" in which numerous differing signaling pathways downstream of a known oncogenic molecule or pathway are inhibited, or "vertical combinations" in which only one key oncogenic pathway is targeted but at multiple levels to obtain a greater level of inhibition (3). To accurately predict the best potential combinations, it is therefore necessary to identify the specific aberrant signaling pathways which are responsible for a cancer phenotype.

Aberrant expression and activity of a number of molecules have been shown to be involved in cellular transformation and tumor development of pancreatic neoplasms including the oncogenic K-ras mutation (in >95% of analyzed tumors), the loss of the tumor suppressors p53 (~75%) and p16^{Ink4A} (>90%), and overexpression of growth factor receptors such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor, and platelet-derived growth factor receptor along with many of their ligands (4). Several intracellular signaling pathways downstream of K-ras and the growth factor receptors have been associated with antiapoptotic responses and chemoresistance in pancreatic

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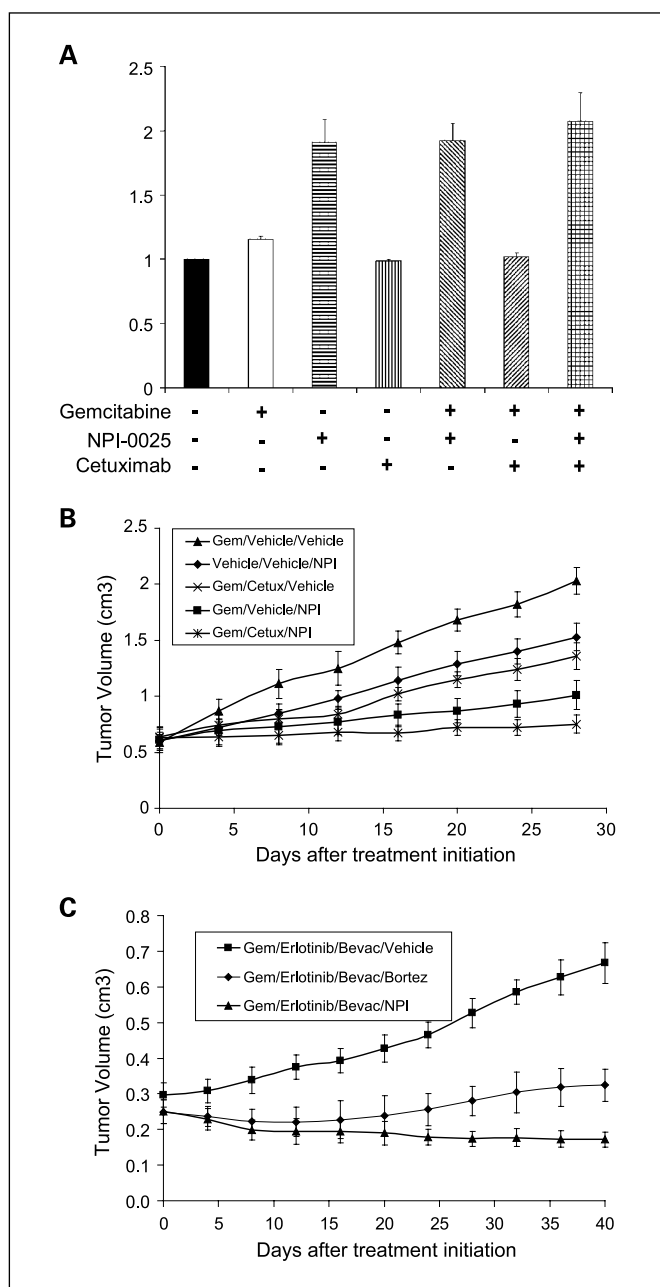


Fig. 1. EGFR inhibition enhances proteasome inhibitor – induced apoptosis *in vivo* but not *in vitro*. **A**, Panc-1 cells were treated with either gemcitabine (1 μ mol/L), NPI-0052 (200 nmol/L), or cetuximab (5 μ g/mL) alone or in combination. When combination drug treatments were used, the drug treatments were administered concurrently. Levels of apoptosis were measured using the cell death ELISA assay. Results are expressed as fold of basal (columns, mean of three individual experiments; bars, SE). **B**, mice bearing 8 to 10 mm Panc-1 xenograft tumors were randomized and treated with 80 mg/kg of gemcitabine, 33 mg/kg of cetuximab, and 0.25 mg/kg of NPI-0052 (orally) alone or in combination twice weekly. **C**, mice were treated with 80 mg/kg of gemcitabine, 2.5 mg/kg of bevacizumab, 50 mg/kg of erlotinib, and either 1 mg/kg of bortezomib or 0.125 mg/kg of NPI-0052 (i.v.) twice weekly except for erlotinib which was administered orally five times weekly. Tumor growth was assessed by measuring tumor volume every 4 d ($n \geq 7$; bars, SE).

cancer including the phosphoinositide-3-kinase (PI3K)/AKT pathway (5), the Ras/Raf/extracellular signal-regulated kinase (ERK) pathway (6), and the transcription factor nuclear factor κ B (NF- κ B), which has been found to be constitutively

active in both pancreatic cancer cell lines and tumor tissue (7).

It has been established that the proteasome inhibitor bortezomib is effective as monotherapy treatment of hematologic malignancies such as multiple myeloma (8). In addition, we and others have shown in preclinical studies of pancreatic and colon cancer that bortezomib (9–14), and more recently, the second-generation proteasome inhibitor NPI-0052 (15) are effective additions to multidrug treatment. Disappointingly, these results from promising preclinical studies have not translated into significant responses in the clinic when proteasome inhibitors were combined with conventional anticancer therapies for the treatment of solid organ malignancies. Recent reports examining cell lines refractory to proteasome inhibitors attributed resistance in hematologic and renal malignancies to high levels of heat shock protein-27 (Hsp27; ref. 16) or Hsp70 (17, 18). In breast cancer cells, proteasome inhibition was found to induce the expression of mitogen-activated protein kinase phosphatase-1, which led to the inhibition of *c-Jun*-NH₂-kinase (JNK) activity and the suppression of apoptosis (19).

In the current study, we hypothesize that proteasome inhibition in pancreatic cancer may induce survival signaling pathways that ultimately protect against the apoptotic effects of proteasome inhibition. To evaluate this hypothesis, we examined the activation of several key mitogenic and anti-apoptotic signaling pathways including EGFR, ERK, JNK, and PI3K/AKT in response to proteasome inhibition. We found that proteasome inhibitors activate these pathways, and furthermore, that inhibition of JNK and PI3K/AKT pathways augments the apoptotic response to proteasome inhibitor treatment both *in vitro* and *in vivo*. These findings suggest that proteasome inhibition should be coupled with inhibitors of these stress response pathways in order to optimize the therapeutic response to proteasome inhibition. This finding will have major effects on the design of clinical trials that target highly resistant solid organ malignancies using proteasome inhibition.

Materials and Methods

Reagents. All reagents were of the highest commercial grade possible and were purchased from Sigma-Aldrich unless otherwise stated. Bortezomib (Millennium Pharmaceuticals), cetuximab (Bristol-Myers Squibb), gemcitabine (Eli Lilly), bevacizumab (Genentech), and erlotinib (Genentech) were purchased from the Massachusetts General Hospital Pharmacy Department (Boston, MA). NPI-0052 was generously provided by Nereus Pharmaceuticals (San Diego, CA). PD98059 and LY290042 were purchased from EMD Biosciences and SP600125 was purchased from AG Scientific.

Cell culture. The human pancreatic adenocarcinoma cell lines Panc-1, BxPC3, and Capan2 were obtained from the American Type Culture Collection. Panc-1 and Capan2 cells were grown in DMEM and BxPC3 in RPMI-1640, with 10% fetal bovine serum, 100 μ g/mL of streptomycin, and 100 μ g/mL of penicillin at 37°C with 5% CO₂.

In vitro measurement of apoptosis. Apoptosis was measured using the Cell Death Detection Plus kit from Roche Applied Science (Indianapolis, IN) as described in the manufacturer's protocol and by detection of Annexin-V and 7-AAD binding (BD Pharmingen). Briefly, cells were seeded into six-well plates and allowed to adhere and grow overnight before treatment. After treatment, both floating and adherent cells were collected by trypsinization and washed twice in PBS. Cells (5×10^5) were resuspended in 500 μ L of Annexin-V binding buffer

(BD PharMingen) containing 5 μ L of Cy5-Annexin-V. Cells were incubated for 5 min at room temperature in the dark before the addition of 5 μ L of 7-AAD and analysis in a BD FACSCalibur flow cytometer. The apoptotic fraction was defined as cells with low 7-AAD and high Annexin-V fluorescence.

Western blotting. Equal amounts of proteins were resolved on denaturing polyacrylamide gels, before being transferred to polyvinylidene difluoride membrane (Bio-Rad) using the Bio-Rad Mini Trans-Blot cell system. Membranes were blocked in 5% nonfat milk dissolved in NaTT buffer [50 mmol/L Tris/HCl (pH 7.4), 150 mmol/L NaCl, 0.02% (v/v) Tween 20] for 2 h. Membranes were incubated with primary antibodies for 16 h at room temperature in NaTT containing 0.5% nonfat milk. Membranes were washed in NaTT before the addition of appropriate horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature in NaTT containing 0.5% nonfat milk. Membranes were again washed using NaTT before visualization using enhanced chemiluminescence (Pierce Biotechnology, Inc.).

Antibodies were acquired from Santa Cruz Biotechnology unless otherwise stated and used at the following dilutions: p-EGFR-Y1173 (1:2,000), EGFR (1:1,000; Cell Signaling Technologies), p-ERK (1:5,000), ERK (1:3,300), p-JNK (1:10,000; Biosource), JNK1-FL (1:5,000), p-AKT (1:3,300), glyceraldehyde-3-phosphate dehydrogenase (1:10,000), and rabbit and mouse horseradish peroxidase secondary antibody (1:3,300).

In vivo evaluation of tumor inhibition. Tumors were established by injecting 5×10^6 cells into the flank of 6-week-old female *nu/nu* mice. Treatment was initiated once the tumors reached a mean diameter of 8 to 10 mm. Mice were then randomized into treatment groups and treated with the various inhibitors either orally (NPI-0052 and erlotinib), via i.p. injection (bortezomib, bevacizumab, cetuximab, PD98059, SP600125, and LY294002), or tail vein injection (NPI-0052 and gemcitabine) twice weekly on days 1 and 4 unless otherwise stated. Tumor size was measured every 4 days and calculated using the formula $TV = 4/3\pi r^3$ (where r is half of the mean tumor diameter, measured in at least two directions). All experiments were done in full compliance with institutional guidelines and with the approval of the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Statistics. Statistical significance was analyzed by one-way ANOVA with a Dunnett's post-test. Statistics were done using the WINKS SDA software (Texasoft) and Microsoft Excel. P values of 0.05 were used unless otherwise stated.

Results

Tumoricidal response to proteasome inhibition is augmented by EGFR inhibition. Initially, we assessed the effectiveness of combining a proteasome inhibitor and an EGFR inhibitor to the clinical pancreatic cancer treatment gemcitabine. To achieve this *in vitro*, we used Panc-1 cells, a poorly differentiated pancreatic adenocarcinoma cell line, and measured the induction of apoptosis using an ELISA for DNA fragmentation, an early indicator of apoptosis. Treatment with NPI-0052 induced an increase in apoptosis. Neither gemcitabine treatment nor inhibition of EGFR with the monoclonal antibody cetuximab had any major effect on apoptosis either alone or when combined with NPI-0052. The addition of all three compounds together also had little additive effect on the apoptosis observed with NPI-0052 treatment alone (Fig. 1A).

To assess these treatment combinations *in vivo*, a Panc-1 mouse xenograft model was used (Fig. 1B). We have previously determined the dosing and schedule of proteasome inhibitor treatment in mice to achieve effective proteasome inhibition (9, 15). Similar to our findings *in vitro*, NPI-0052 was significantly more effective as a monotherapy compared with

gemcitabine, with the average tumor size being 25% smaller in mice treated with NPI-0052 compared with gemcitabine ($P < 0.05$, ANOVA). In combination with gemcitabine, NPI-0052 was able to reduce tumor size by 50% more than gemcitabine treatment alone. Interestingly, as part of a combination therapy, EGFR inhibition had little effect *in vitro* (Fig. 1A), but the addition of cetuximab to gemcitabine *in vivo* resulted in a significant reduction in tumor volume (33%) when compared with gemcitabine treatment alone ($P < 0.05$, ANOVA). Staining of tumor sections from mice treated with combination erlotinib and NPI-0052 showed no microvasculature or stromal-specific apoptosis that could explain this discrepancy (data not shown). The addition of NPI-0052 significantly increased the antitumor effect of combined gemcitabine and cetuximab ($P < 0.05$, ANOVA), and although the addition of cetuximab to combined gemcitabine and NPI-0052 improved the tumoricidal response, this improvement did not reach statistical significance (Fig. 1B).

NPI-0052 is more effective than bortezomib as part of a multidrug regimen in vivo. Recent evidence from our lab and others has suggested both mechanistic and compound stability differences between the two proteasome inhibitors NPI-0052 and bortezomib (15, 20–22). Having established

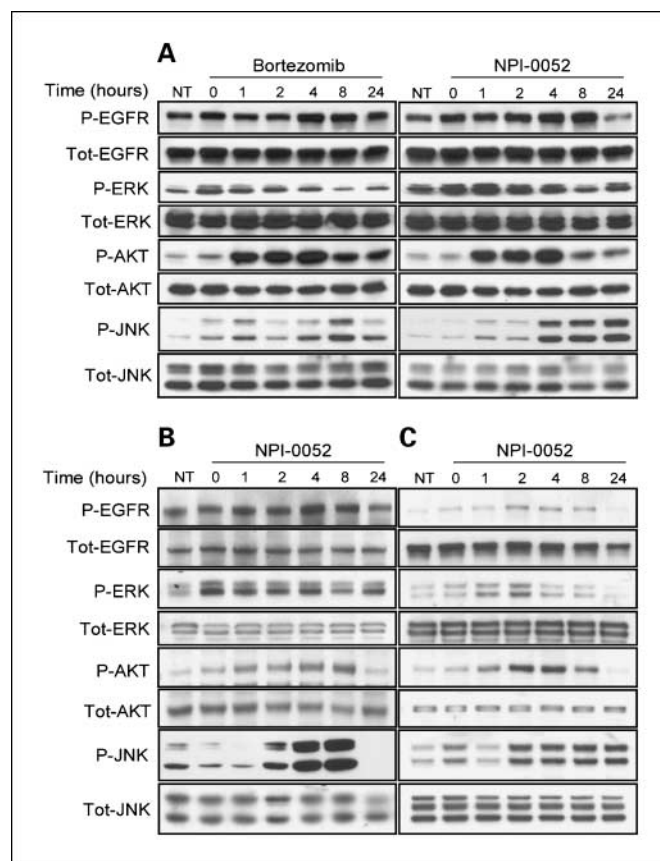


Fig. 2. Proteasome inhibition activates several mitogenic signaling pathways. **A.** Panc-1 cells were treated with bortezomib (1 μ mol/L) or NPI-0052 (200 nmol/L) for 1 h, cells were incubated for the indicated times in serum-free medium. No treatment (NT) indicates that cells were treated with vehicle for 1 h and sampled after 4 h. BxPC3 (**B**) and Capan2 (**C**) cells were treated with NPI-0052 (100 nmol/L) as above. Samples were prepared and subjected to Western blotting with antibodies for both the active, phosphorylated protein and the total level of protein. Blots are representative of at least three individual experiments.

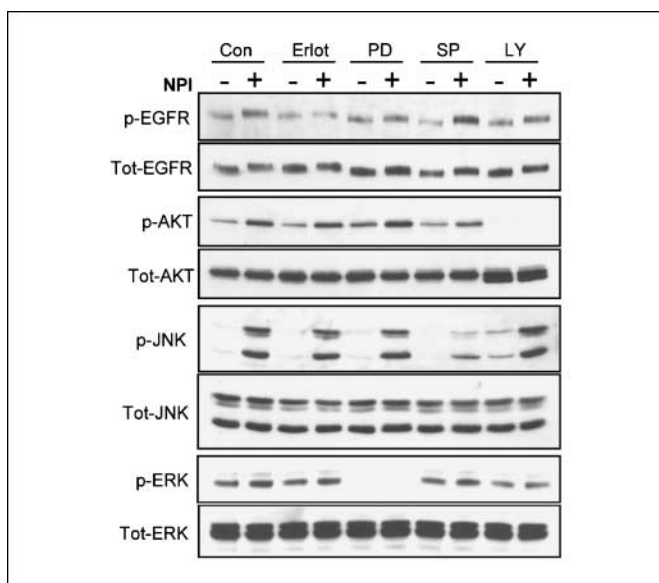


Fig. 3. Inhibition of EGFR signaling has no effect on NPI-0052-induced activation of ERK, AKT, or JNK. Panc-1 cells were treated with either vehicle (Con) or 200 nmol/L of NPI-0052 (NPI) for 1 h, and either sampled immediately (ERK) or incubated in serum-free medium for 4 h (EGFR, AKT, and JNK). Where indicated, mitogenic inhibitors were added for the entire course of the experiment: EGFR inhibitor, erlotinib (1 μmol/L); ERK pathway inhibitor, PD98059 (20 μmol/L); JNK inhibitor, SP600125 (20 μmol/L); and the PI3K/AKT inhibitor, LY294002 (20 μmol/L). Samples were prepared and subjected to Western blotting with antibodies to detect both the active, phosphorylated protein and the total protein levels. Blots are representative of at least two separate experiments.

that inhibition of EGFR and the proteasome makes an effective combination *in vivo*, we sought to identify which proteasome inhibitor would give the best response in our Panc-1 xenograft model. The differing proteasome inhibitors were used as part of a multidrug therapy including the EGFR inhibitor erlotinib and the vascular endothelial growth factor pathway inhibitor bevacizumab. In these combinations, NPI-0052 treatment resulted in a 73% decrease in tumor volume relative to control, compared with only a 49% decrease in the bortezomib-treated group (Fig. 1C; *P* < 0.05, ANOVA).

Proteasome inhibition leads to activation of NF-κB-independent antiapoptotic pathways. Our lab and others have shown that genotoxic drugs induce antiapoptotic survival signals that are mediated by the activation of the transcription factor NF-κB. Proteasome inhibition is one method by which this antiapoptotic response may be abrogated to promote chemosensitivity. Interestingly, little is known about the potential survival signals that are induced by inhibitors of the proteasome. Because we found EGFR inhibition to be potentially synergistic with proteasome inhibition, we sought to determine if proteasome inhibitor treatment was affecting EGFR activity and downstream mitogenic signaling. As proteasome inhibitors are rapidly cleared from the plasma *in vivo* (23), we used a 1-hour transient exposure of cells for all subsequent experiments. Panc-1 cells were treated with either bortezomib or NPI-0052 for 1 hour and the activation state of EGFR and several of the downstream signaling pathways (ERK, AKT, and JNK) was measured over 24 hours using antibodies against the phosphorylated active forms of the proteins and Western blot analysis (Fig. 2A). Exposure to either proteasome inhibitor induced an increase in phospho-EGFR levels that peaked at 4 to 8 hours. ERK activity

increased rapidly to a maximal level within 1 hour of treatment removal and AKT activation was observed to peak at 2 to 4 hours. Phospho-JNK levels increased in a time-dependant manner maximal at 8 to 24 hours post-drug treatment but showed slightly differing responses to the two proteasome inhibitors,

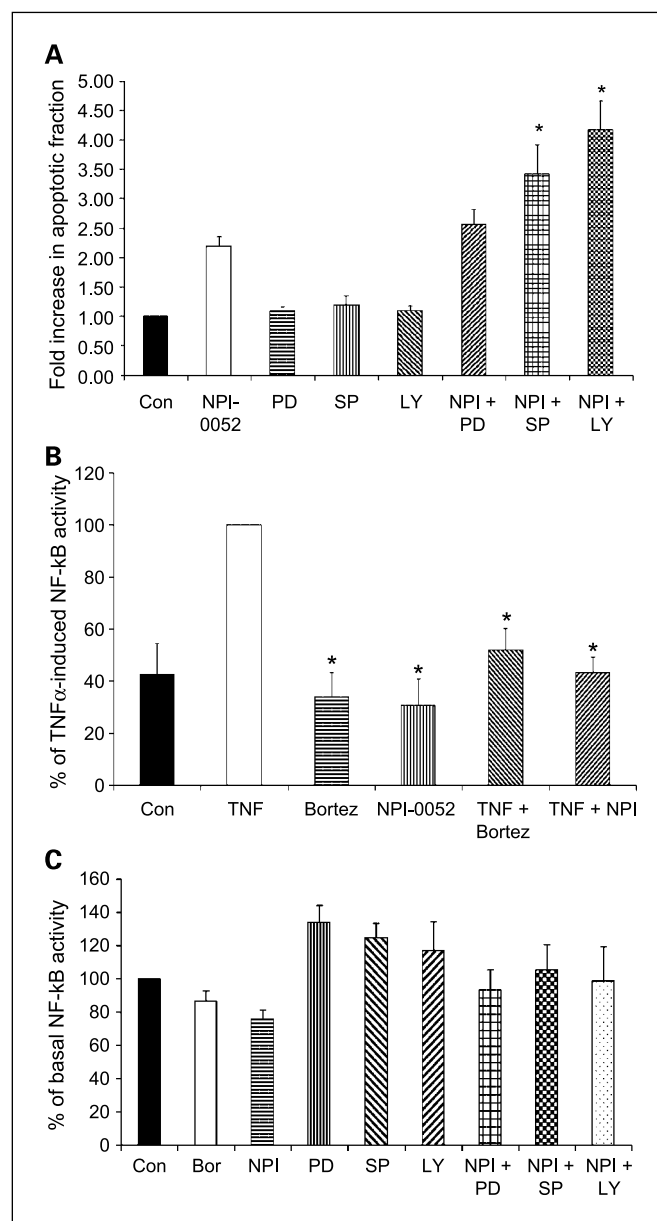


Fig. 4. Inhibition of JNK and AKT but not ERK signaling augments the apoptotic effects of NPI-0052 *in vitro*. **A**, Panc-1 cells were treated with NPI-0052 (200 nmol/L) for 1 h and then incubated for 24 h in serum-free medium. Where indicated, mitogenic inhibitors were added for the entire course of the experiment: PD98059 (20 μmol/L), LY294002 (20 μmol/L), and SP600125 (20 μmol/L). Levels of apoptosis were detected by flow cytometry with Annexin-V and 7-AAD staining. Results are expressed as fold increase in the apoptotic fraction (columns, mean of three separate experiments; bars, SE). **B**, Panc-1 cells were pretreated with bortezomib (1 μmol/L) or NPI-0052 (200 nmol/L) for 1 h before treatment with vehicle or hr-tumor necrosis factor-α (15 ng/mL) for 15 min. Nuclear extracts were collected and the activity of p65 NF-κB measured by ELISA. **C**, Panc-1 cells were treated with bortezomib (1 μmol/L) or NPI-0052 (200 nmol/L) for 1 h alone or in combination with PD98059 (20 μmol/L), LY294002 (20 μmol/L), or SP600125 (20 μmol/L). Nuclear extracts were collected and the activity of p65 NF-κB measured by ELISA (columns, mean of three separate experiments; bars, SE; *, *P* < 0.05, ANOVA).

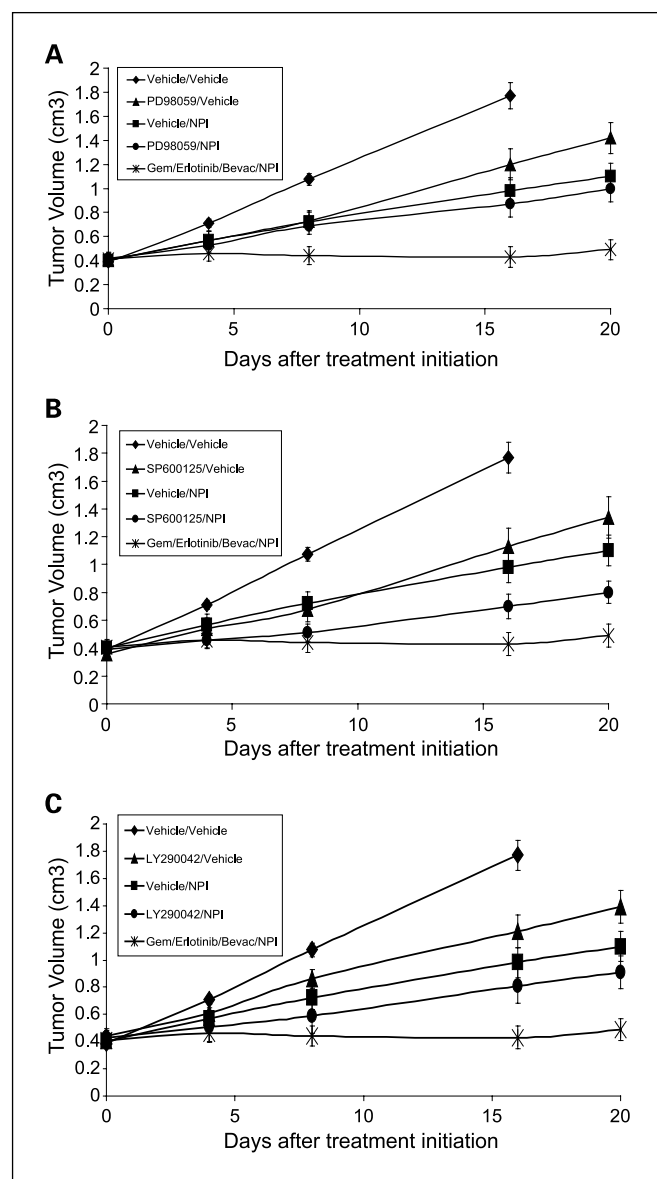


Fig. 5. Inhibition of the ERK, JNK, and AKT pathways enhances the antitumor effect of NPI-0052 treatment *in vivo*. Mice bearing 8 to 10 mm Panc-1 xenograft tumors were randomized and treated twice weekly either singly or with combinations of NPI-0052 (0.125 mg/kg) and PD98059 (4 mg/kg; A), SP600125 (60 mg/kg; B) or LY294002 (100 mg/kg; C) twice weekly on days 1 and 4. To allow a comparison between experiments, a group of mice were treated with gemcitabine/bevacizumab/erlotinib/NPI-0052 as in Fig. 3. Tumor growth was assessed by measuring tumor volume every 4 d ($n = 7$; bars, SE).

with a more robust response to NPI-0052 than bortezomib (Fig. 2A). To assess if the activation of these pathways was a cell line-specific response, these experiments were repeated using two other pancreatic cancer cell lines. BxPC3 (Fig. 2B) and Capan2 (Fig. 2C) were chosen, as unlike Panc-1 cells, these cell lines are wild-type for K-ras and p53, respectively. Both cell lines displayed activation of EGFR, ERK, AKT, and JNK in response to proteasome inhibitor treatment that was broadly similar to the responses observed in Panc-1 cells.

EGFR signaling is not responsible for activation of the ERK, AKT, and JNK pathways following proteasome inhibition. EGFR

activation has been shown to activate a number of cellular signaling pathways in both normal tissue and cancers, including those that we observed in Panc-1 cells treated with proteasome inhibitors (24). To assess if the proteasome inhibitor-induced activation of EGFR is responsible for the increased activity of the JNK, ERK, and AKT pathways, we used the EGFR inhibitor erlotinib to block EGFR signaling. As expected, treatment of Panc-1 cells with 1 $\mu\text{mol/L}$ of erlotinib reduced both the constitutive EGFR activity as well as the activation of EGFR induced by NPI-0052 treatment (Fig. 3). Interestingly, erlotinib did not significantly reduce the NPI-0052-induced activation of AKT, ERK, or JNK, suggesting that these pathways are activated through an EGFR independent mechanism (Fig. 3).

Inhibition of JNK and AKT augments the apoptotic effects of NPI-0052 *in vitro*. Our preliminary studies indicate that proteasome inhibition activates EGFR signaling and that EGFR inhibition is effective at augmenting proteasome inhibitor-induced apoptosis *in vivo*. However, we also found that activation of ERK, AKT, and JNK are largely independent of EGFR signaling. We next sought to determine if the activation of these pathways by proteasome inhibitor treatment influences the apoptotic response of these cells to proteasome inhibition. To do this, we used small molecule inhibitors of each signaling pathway: EGFR (erlotinib), ERK (PD98059), AKT (LY294002), and JNK (SP600125). To first confirm that the inhibitors were functional in our model, cells were treated with vehicle or NPI-0052 with or without the specific mitogenic inhibitors. The activation state of the pathways was then measured by Western blot analysis. All three inhibitors specifically inhibited the activity of their respective target proteins and had no cross-reactivity with the other pathways examined (Fig. 3). Induction of apoptosis 24 hours posttreatment was then assessed using Annexin-V and 7-AAD staining. Proteasome inhibitor treatment alone significantly increased the fraction of apoptotic cells whereas treatment with ERK, AKT, or JNK inhibitors alone had little effect. When used in combination with NPI-0052, inhibitors of AKT and JNK significantly increased levels of apoptosis (Fig. 4A), suggesting that they have proteasome inhibitor-induced antiapoptotic roles in pancreatic cancer cells ($P < 0.05$, ANOVA). Interestingly, ERK inhibition had no significant effect on induction of apoptosis by NPI-0052.

Increased cell death was not due to the effects of NF- κ B signaling. One of the principal rationales for using proteasome inhibitors as therapeutics is their ability to inhibit NF- κ B signaling. It has been previously shown that many pancreatic tumors and cell lines including Panc-1 have a high level of constitutive NF- κ B activity (25). We assessed the ability of both bortezomib and NPI-0052 to inhibit both the constitutive and tumor necrosis factor- α -induced activity of the NF- κ B pathway in Panc-1 cells. Interestingly, despite both compounds' ability to significantly reduce tumor necrosis factor- α -induced NF- κ B activity ($P < 0.05$, ANOVA), neither was able to reduce the basal activity of this pathway (Fig. 4B). Additionally, we assessed whether effects on the NF- κ B pathway were responsible for the enhanced apoptosis observed with the combination of mitogenic and proteasome inhibition. The addition of PD98059, SP600125, or LY294002 to NPI-0052 had no significant effect on basal NF- κ B activity, suggesting that the proapoptotic effect of these combinations is not dependent on NF- κ B signaling (Fig. 4C).

Inhibition of ERK, JNK, or AKT increases the antitumor effects of NPI-0052 in vivo. We have shown that the cellular response to proteasome inhibition treatment involves the activation of ERK, JNK, and AKT signaling. We also showed that inhibition of the AKT and JNK pathways *in vitro* enhances the apoptotic response to proteasome inhibition. To assess if these combinations were effective *in vivo*, a Panc-1 mouse xenograft model was again used. Mice were treated with PD98059, SP600125, or LY294002 alone or in combination with NPI-0052 (Fig. 5). A gemcitabine/erlotinib/bevacizumab/NPI-0052 group was included to allow comparison between this and the previous experiments. NPI-0052, PD98059, SP600125, or LY294002 treatment alone all significantly reduced tumor growth relative to control mice ($P < 0.05$, ANOVA). NPI-0052 treatment alone reduced tumor size by 45%. ERK inhibition proved to be the least effective with PD98059 alone reducing tumor size by 32%. When used in combination with NPI-0052 the response was 50% (Fig. 5A). The JNK inhibitor SP600125 was able to decrease tumor size by only 36% when used alone, but when used in combination with NPI-0052, the tumor reduction was >60% (Fig. 5B). Similarly, PI3K/AKT inhibition with LY294002 alone was able to reduce tumor size by 32%, but when combined with NPI-0052, the mean tumor volume was 54% smaller than in control animals (Fig. 5C). These data indicate that all three signaling pathways studied are effective targets for monomolecular therapy. Furthermore, combining these targeted therapies with proteasome inhibition is additive in increasing the tumoricidal response to treatment. It is noteworthy that the gemcitabine/erlotinib/bevacizumab/NPI-0052 combination group was by far most effective in these studies validating the expanded horizontal blockade hypothesis.

Discussion

Proteasome inhibitors represent a class of drugs that have anticancer activity through a variety of cellular mechanisms, including induction of apoptosis, interference with cell cycle progression, inhibition of angiogenesis, and the suppression of NF- κ B (26). As single-agent therapies, proteasome inhibitors such as bortezomib, a reversible inhibitor of the chymotrypsin-like and caspase-like activity of the proteasome, have shown clinical response rates as high as 38% in the management of relapsed/refractory multiple myeloma. When combined with dexamethasone for the treatment of multiple myeloma, response rates have been reported to be as high as 88% (27). In contrast, treatment of solid organ malignancies such as breast or lung cancers with either single-agent bortezomib or bortezomib in combination with conventional chemotherapy agents has yet to yield significant improvements in treatment response (28–30). We therefore hypothesized that a more extensive investigation of the survival signals induced by proteasome inhibition may offer further insight into the poor responses of solid malignancies. It is these survival signals that are the focus of the current report.

In our preliminary studies, we sought to determine if inhibition of both EGFR signaling and proteasome function in combination would increase the apoptotic response of pancreatic cancer cells to gemcitabine treatment. Our results show that proteasome inhibition as a single agent was highly effective at inducing apoptosis *in vitro* and markedly reduced tumor growth in mice when used in combination with gemcitabine and/or

EGFR inhibition. Intriguingly, the addition of EGFR inhibition only enhanced proteasome inhibitor effects *in vivo* but not *in vitro*, suggesting that some other cell type and/or paracrine response may be involved in the effects of EGFR inhibition *in vivo*. An and Rettig have described the importance of the sequence of drug administration when bortezomib is used in combination with an EGFR tyrosine kinase inhibitor in renal cell carcinoma cells (31). They establish that pretreatment of renal cell carcinoma cells with the EGFR tyrosine kinase inhibitor prior to bortezomib was cytotoxic, whereas an antagonistic interaction resulted from bortezomib pretreatment. They concluded that NF- κ B activation in renal cell carcinoma was EGFR-mediated and likely occurs through the PI3K/AKT pathway (31). In the data shown in this report, proteasome and EGFR inhibitor treatments were administered concurrently but additional *in vitro* studies using a 2 hour pretreatment and posttreatment with erlotinib prior to NPI-0052 treatment failed to show any change in the level of apoptosis compared with proteasome inhibitor alone (data not shown).

As EGFR inhibition enhanced the response to proteasome inhibitor treatment *in vivo*, we hypothesized that proteasome inhibitor treatment may be activating antiapoptotic signaling pathways that require EGFR signaling as described by An and Rettig (31). We therefore sought to determine whether the stress response to proteasome inhibition could include the activation of EGFR and the antiapoptotic and mitogenic signaling pathways downstream of EGFR including NF- κ B, AKT, ERK, and JNK (all shown to be important in either the tumorigenesis or chemoresistance of pancreatic cancer; ref. 24). As one of the principal rationales for proteasome inhibitor use in cancer therapy is the ability to inhibit NF- κ B activation, NF- κ B activation is unlikely to play a significant role in the antiapoptotic response to proteasome inhibitor. As expected, we found that both bortezomib and NPI-0052 did not lead to the activation of NF- κ B in pancreatic cells, and both bortezomib and NPI-0052 were able to inhibit tumor necrosis factor- α -induced activation of NF- κ B. More importantly, we found that various pancreatic cancer cell types with a range of oncogenic genotypes all displayed similar mitogenic signaling responses when treated with proteasome inhibition, resulting in the phosphorylation of EGFR, ERK, AKT, and JNK. Interestingly, when we tested whether activation of these pathways was EGFR-dependant, we found that erlotinib treatment had little effect on downstream signaling, suggesting that unlike the renal cell carcinoma cell response (31), the proteasome inhibitor-induced activation of these pathways is EGFR-independent.

This is the first report on the effects of NPI-0052 and bortezomib on mitogenic signaling pathways in pancreatic cancer, and only recently have the potential therapeutic effects of the second-generation proteasome inhibitor NPI-0052 been described (15, 20). However, several prior studies have looked at the effects of bortezomib or MG-132 treatment on molecular signaling in differing cancer types. In squamous cell carcinoma, bortezomib treatment increased EGFR activity similar to our observations in pancreatic cells, whereas in breast cancer cells, EGFR activity was decreased (32, 33). Likewise, AKT activity was decreased in SK-BR3 breast cancer cells, but increased in both A1N4-myc mammary epithelial cells and our pancreatic cells following bortezomib treatment (29, 33). In liver and breast cancer cells treated with bortezomib, JNK and ERK showed very

similar patterns of activation to those described here in pancreatic cancer cells (33–35). Although the reported results to date may seem contradictory, they may more likely represent tissue and cell type–specific differences in the response to proteasome inhibition.

Similar to the *in vitro* response to single-agent EGFR inhibition that we observed, single-agent inhibition of the individual ERK, JNK, and AKT pathways had little cytotoxic effect *in vitro*, whereas *in vivo*, the single-agent targeting of the mitogenic signaling pathways resulted in decreased tumor growth, although it should be noted that none were as effective as NPI-0052 treatment alone. When combined with NPI-0052 treatment, inhibition of JNK and AKT increased the level of apoptosis *in vitro* and reduced tumor size *in vivo* relative to proteasome inhibition alone. ERK inhibition had no significant effect on proteasome inhibitor–induced apoptosis *in vitro* and although there was a small but significant effect *in vivo*, ERK inhibition was the least effective of the pathways tested. This suggests that in our pancreatic cancer models, JNK and AKT have an antiapoptotic role in response to proteasome inhibitor treatment, whereas the *in vivo* response to ERK inhibition is through a mechanism unrelated to the proteasome inhibitor–induced induction of ERK phosphorylation. These findings are supported by similar experiments that describe the antiapoptotic role of PI3K/AKT (36) and ERK (37) in pancreatic cancer cells. We had presumed, however, that the proteasome inhibitor–induced JNK activation would be proapoptotic as described by Meriin et al. in lymphoid and kidney tumors (17). However, the finding that JNK inhibition increases proteasome inhibitor–induced apoptosis *in vitro* and enhances the tumoricidal response *in vivo* suggests that, under these conditions, JNK functions in an antiapoptotic role in this cell type. Although predominantly proapoptotic in many tumor models, JNK has been shown to be activated and/or overexpressed in an antiapoptotic manner in various cancers and cell types, and in Panc-1 cells, JNK inhibition has been shown to stop cellular proliferation, suggesting a mitogenic role for JNK in this cell type (38, 39).

Similar to our findings, several other distinct antiapoptotic responses to proteasome inhibitor treatments have been described in the literature. The Orłowski group found that in breast cancer cells, proteasome inhibitor–induced apoptosis was dependent on the activation of the JNK pathway and that proteasome inhibition increased cellular levels of mitogen-

activated protein kinase phosphatase-1, a specific deactivator of proapoptotic JNK activity (29, 40). Others have found that proteasome inhibitor–induced antiapoptotic signaling involves the induction of heat shock proteins Hsp27, Hsp70 (Hsp72), and Hsp90 (16–18, 41, 42). Similar to our results, the suppression of these various responses by small molecule inhibitors or small interfering RNA significantly increased the apoptotic response of various cell types to proteasome inhibition (16–18, 41, 42).

The specific mechanism linking proteasome inhibition to the activation of mitogenic signaling pathways is unknown. In contrast to findings in other tumor models, our results suggest that proteasome inhibitor–induced EGFR activation is not responsible for the activation of the other mitogenic signaling pathways studied. Due to the broad spectrum of cellular effects caused by proteasome inhibition, there are many possible mechanisms that could lead to the activation of the mitogenic kinase cascades. Obvious examples would include dysregulation of the phosphatases responsible for maintaining these kinase cascade pathways in a basal state, or possibly, the accumulation of upstream activator complexes. The mechanism of proteasome inhibitor–induced mitogenic signaling is currently under investigation in our laboratory.

In summary, our data indicate that in highly treatment-resistant pancreatic adenocarcinoma, proteasome inhibition in combination with EGFR inhibition is an effective addition to gemcitabine-based regimens. Furthermore, we have shown that proteasome inhibitor treatment activates several mitogenic signaling pathways that blunt the full potential of the apoptotic response to proteasome inhibitor treatment. As an alternative or addition to EGFR inhibition, selective inhibition of these downstream mitogenic signaling pathways may increase the apoptotic response to proteasome inhibition and further overcome the drug resistance mechanisms in pancreatic adenocarcinoma. These studies identify new drug combinations that require further study and show potential for use in new clinical trials.

Disclosure of Potential Conflicts of Interest

J.C. Cusack, Jr., has an unrestricted grant and is a paid consultant to Nereus Pharmaceuticals. M.A. Palladino has an ownership interest in Nereus Pharmaceuticals.

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