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Bortezomib Targets Sp Transcription Factors in Cancer Cells

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Running Title: Bortezomib targets Sp transcription factors in cancer cells

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Abbreviations: EGFR, epidermal growth factor receptor; FADD, Fas-associated death domain; FBS, fetal bovine serum; GSH, glutathione; MM, multiple myeoloma; NAC, *N*-acetylcysteine; OKA, okadaic acid; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; Sp1, specificity protein 1

ABSTRACT

Bortezomib alone and in combination with other anticancer agents are extensively used for chemotherapeutic treatment of multiple myeloma (MM) patients and are being developed for treating other cancers. Bortezomib acts through multiple pathways and in this study with ANBL-6 and RPMI 8226 MM cells, we show that bortezomib inhibited growth and induced apoptosis and this was accompanied by downregulation of Sp1, Sp3 and Sp4 transcription factors that are overexpressed in these cells. Similar results were observed in pancreatic and colon cancer cells. The functional importance of this pathway was confirmed by showing that individual knockdown of Sp1, Sp3 and Sp4 in MM cells inhibited cell growth and induced apoptosis and this correlates with results of previous studies in pancreatic colon and other cancer cell lines. The mechanism of bortezomib-mediated downregulation of Sp transcription factors in MM was due to induction of caspase-8 and upstream factors including Fas-associated death domain (FADD). These results demonstrate that an important underlying mechanism of action of bortezomib was due to activation of caspase-8-dependent downregulation of Sp1, Sp3, Sp4 and pro-oncogenic Sp-regulated genes.

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INTRODUCTION

Multiple myeloma (MM) is a B-cell malignancy associated with terminally differentiated plasma cells which proliferate in the bone marrow and complications from MM include bone marrow failure, renal disease and osteolytic bone disease (Anderson, 2016; Cook, 2008; Dimopoulos et al., 2015; Paiva et al., 2015). It is estimated that 30,770 new cases of myeloma will be diagnosed in 2018 and 12,770 patients will die of this disease in the United States (Siegel et al., 2018). Among all cancers, FDA approvals for new therapies are among the highest for MM (Anderson, 2016; Bates, 2016; Neri et al., 2016; Orlowski and Lonial, 2016), and this has contributed to the improvements in overall survival from this disease from 3 years in the 1960's to 8-10 years (Anderson, 2016). Among the most recent and prominent therapies for treating MM patients include the class of proteasome inhibitors such as bortezomib (Velcade®) and immunotherapies which are being developed for many cancers (Anderson, 2016; Bates, 2016; Neri et al., 2016; Orlowski and Lonial, 2016).

One of the major advances in treatment of multiple myeloma was the development and clinical applications of bortezomib a proteasome inhibitor which is used extensively in MM chemotherapy (Hideshima et al., 2003; Hideshima et al., 2001; LeBlanc et al., 2002; Mitsiades et al., 2002; Richardson et al., 2005; San Miguel et al., 2008) and in drug combination therapies. Bortezomib and related analogs target the ubiquitin proteasome pathway by interacting with N-terminal threonine residues in the active site of the proteasome catalytic region, thereby blocking the function of the 26S proteasome (Pandit and Gartel, 2011). Although bortezomib was initially characterized as a proteasome inhibitor, there is evidence that this agent induces anticancer activities

in MM and other cancer cell lines through many other pathways including induction of reactive oxygen species (ROS), intracellular stress and apoptosis (Laussmann et al., 2011; Lipchick et al., 2016; Xian et al., 2017). Treatment of MM cells and other cancer cell lines with bortezomib has also been linked to downregulation of specificity protein 1 (Sp1) transcription factor. For example, bortezomib induces microRNA-29b in MM cells and acute myeloid leukemia cells and this is associated with downregulation of Sp1 and Sp1-regulated genes/responses (Amodio et al., 2012; Blum et al., 2012; Liu et al., 2008), and in MM cells, miR-29b and Sp1 are part of feedback loop where decreased expression of Sp1 induces miR-29b (Amodio et al., 2012). Another study showed that bortezomib decreased expression of Sp1 in MM cells and this was accompanied by decreased expression of Sp1-regulated survival factors IRF4 and cMyc (Bat-Erdene et al., 2016).

Research in our laboratory has focused on Sp transcription factors as drug targets and it has been demonstrated that Sp1, Sp3 and Sp4 are overexpressed in multiple cancer cell lines (Chadalapaka et al., 2012; Hedrick et al., 2015; Hedrick et al., 2017; Jutooru et al., 2014; Karki et al., 2017; Kasiappan et al., 2016; Mertens-Talcott et al., 2007; Nair et al., 2013; Safe et al., 2018), whereas levels of Sp2 and Sp5 were either low or variable and were not further investigated. RNA interference studies show that Sp1, Sp3 and Sp4 individually regulate cell proliferation, survival and migration of cancer cells (Hedrick et al., 2016). Bortezomib is a widely used anticancer agent and it is essential to understand its mechanism of action, particularly in MM, since insights on mechanisms are important for designing drug combination therapies. Results of this study demonstrate that not only Sp1 but also Sp3 and Sp4 are highly expressed and

exhibit pro-oncogenic activities in MM cells as previously observed in other cancer cell lines. Moreover, bortezomib induces caspase-8-dependent downregulation of Sp1, Sp3 and Sp4 in MM cells, suggesting that an important mechanism of action of this drug is due to targeting downregulation of Sp1, Sp3 and Sp4.

MATERIALS AND METHODS

Cell lines, antibodies, and reagents

The multiple myeloma cell lines (ANBL-6 and RPMI 8226) were generous gifts from Robert Z. Orlowski (Department of Lymphoma and Myeloma, The University of Texas MD Anderson Cancer Center, Houston, TX, USA). Panc1, L3.6pL, and SW480 cells were purchased from American Type Culture Collection (Manassas, VA). ANBL-6 and RPMI 8226 were maintained at 37°C in the presence of 5% CO_2 in RPMI 1640 medium with 10% fetal bovine serum (FBS) and for ANBL-6, IL6 (1 ng/ml) was added. Similarly, Panc1, L3.6pL, MiaPaCa2 and SW480 cells were grown and maintained at 37°C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% FBS. DMEM, RPMI 1640 medium, fetal bovine serum (FBS), formaldehyde, sodium orthovanadate, and trypsin were purchased from Sigma-Aldrich (St. Louis, MO). cMyc (cat# 9402s), survivin (cat# 2808s), cleaved poly (ADP-ribose) polymerase (cPARP, cat# 9541T), cyclin D1 (cat# 2978s), phospo-FADD (cat# 2781s), caspase-3 (cat# 9662s), cleaved caspase-3 (cat# 9661L), caspase-8 (cat# 9746T), FADD (cat# 2782T) and cleaved caspase-8 (cat# 9496T) antibodies were obtained from Cell Signaling (Boston, MA). Sp1 (cat#ab13370) antibody was purchased from Abcam (Cambridge, MA); Sp3 (cat# sc-644), Sp4 (cat# sc-645),

epidermal growth factor receptor (EGFR) (cat# sc-373746), and bcl2(cat# sc-7382) antibodies were obtained from Santa Cruz (Santa Cruz, CA), and β-actin (cat# A5316) antibody from Sigma-Aldrich (St. Louis, MO). Chemiluminescence reagents (Immobilon Western) for western blot imaging were purchased from Millipore (Billerica, MA), and bortezomib was purchased from LC Laboratories (Woburn, MA). Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from Biotium (Hayward, CA); ROS Determination Kit was purchased from Invitrogen (Grand Island, NY); and XTT cell viability kit was obtained from Cell Signaling (Boston, MA). Caspase-8 inhibitor (Z-IETD-FMK, cat# 51-69401U) and Interleukin-6 (IL6, cat# 10395-HNAE-25) recombinant human protein mixture were purchased from BD Bioscience (San Jose, CA) and Invitrogen (Carlsbad, CA), respectively. The caspase-3 inhibition was obtained from R & D Systems (Minneapolis, MN).

Cell viability assay

Cells were plated in 96-well plates at a density of 10,000 per well with RPMI and DMEM containing 2.5% charcoal-stripped FBS. Cells were treated with DMSO (solvent control) and different concentrations of bortezomib with DMEM containing 2.5% charcoal-stripped FBS for 0 to 48 hr. After treatment, 25 μL (XTT with 1% of electron coupling solution) was added to each well and incubated for 4 hr as outlined in the manufacturer's instructions (Cell Signaling, Boston, MA). Absorbance was measured at a wavelength of 450 nm in a 96-well plate reader after incubation for 4 hr in 5% CO_2 at 37°C.

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Measurement of ROS

ROS levels were measured using the cell permeable probe CM-H2DCFDA [5-(and 6)-chloromethyl-2-,7- dichlorodihydrofluorescein diacetate acetyl ester] as outlined in the manufacturer's instructions (Life Technologies, Carlsbad, CA). Cells were seeded at density of $1.5x10^5$ per ml in 6-well plates and pretreated with glutathione (GSH) for 30 min and then treated with vehicle (DMSO), bortezomib alone or in combination with GSH, N-acetyl cysteine and catalase for 30 min and incubated for up to 9 hr. ROS levels were measured by flow cytometry as previously described (Karki et al., 2017).

Measurement of apoptosis (Annexin V staining)

Cancer cells were seeded at density of $1.5x10^5$ per ml in 6-well plates and pretreated with the caspase-8 inhibitor (Z-EITD-FMK) for 30 min and then treated with either vehicle or bortezomib alone or combination with 15 μM of the caspase-8 inhibitor. Cells were stained and analyzed by flow cytometry using the Dead Cells Apoptosis Kit and Alexa Fluor 488 assay kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Western blot analysis

ANBL-6 and RPMI 8226, Panc1, L3.6pL and SW480 cells were seeded at density of $1.5x10^5$ per ml in 6-well plates and treated with various concentrations of bortezomib alone or in combination with caspase-8 inhibitors, and whole cell proteins were extracted using RIPA lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM

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NaCl, 1 mM EDTA, 1% Triton X-100 (w/v), 0.5% sodium deoxycholate and 0.1% SDS with protease and phosphatase inhibitor cocktail. Protein concentrations were measured using the Lowry's method and equal amounts of protein were separated in 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. PVDF membranes were incubated overnight at 4°C with primary antibodies in 5% skimmed milk and incubated for 2-3 hr with secondary antibodies conjugated with HRP. Membranes were then exposed to HRP-substrate and immune-reacted proteins were detected with chemiluminescence reagent. The same Sp3 antibody detects both the high and low molecular weight forms of Sp3 as previously reported (Karki et al., 2017; Kasiappan et al., 2016)

Small interfering RNA interference assay

siRNA experiments were conducted using siRNA by electroporation (ECM-830, Harvard Apparatus). ANBL-6 cells ($2x10^6$) in 400 μl serum free media in a 4 mm gap cuvette were electroporated using the following conditions: 100V and 3 pulses for 30 ms. RPMI 8226 cells ($2x10^6$) in 400 μl serum free media in a 4 mm gap cuvette were also electroporated at voltage of 250V, one pulse with a pulse length of 10 ms. One μl (100 μM) of the siRNAs were used during electroporation and the complexes used in the study are as follows:

iNS-5′: CGU ACG CGG AAU ACU UCG A (non-specific)

siSp1: SASI_Hs02_00333289 [1] SASI_Hs01_ 00140198 [2] SASI_Hs01_00070994 [3]

siSp3: SASI_Hs01_00211941 [1] SASI_Hs01_ 00211942 [2] SASI_Hs01_00211943 [3]

siSp4: SASI_Hs01_00114420 [1] SASI_Hs01_ 00114421 [2] SASI_Hs01_00114420 [3]

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siCaspase-8: SASI_Hs02_0031422 [1] SASI_Hs02_ 00314221_AS

Effects of knockdown were determined 72 hr after initial transfection.

Statistical analysis

One way ANOVA and Dunnett's test were used to determine the statistical significance between two groups. In order to confirm the reproducibility of the data, the experiments were performed at least three independent times, and results are expressed as means ± SD. P-values < 0.05 were considered to be statistically significant.

RESULTS

1. Bortezomib decreases expression of Sp transcription factors in MM cells and other cancer cell lines

In this study, we used human ANBL-6 and RPMI 8226 multiple myeloma cell lines, and treatment with 1-20 nM bortezomib decreased growth of both cell lines with significant growth inhibition observed at 1 nM bortezomib after treatment for 24 and 48 hr (Figs. 1A and 1B). Bortezomib also induced Annexin V staining (apoptosis marker) in both cell lines (Fig. 1C and Supplemental Fig. 1A) and statistically significant induction was observed after treatment with 5-10 nM bortezomib. This concentration range also induced caspase-3 cleavage in ANBL-6 and RPMI 8226 cells (Fig. 1D). Previous studies show that Sp1 is expressed in MM cells, and bortezomib decreased Sp1 levels and results in Figure 1E confirms that bortezomib downregulated Sp1 in ANBL-6 and RMPI 8226 cells. However, it is also evident that Sp3 and Sp4 are highly expressed in

both cell lines, and bortezomib decreased levels of Sp3 and Sp4 proteins and the effects were observed in cells after treatment with 1-5 nM bortezomib. *Cyclin D1*, *survivin* and *bcl-2* are typical Sp-regulated genes (Safe et al., 2018) and bortezomib decreased levels of these proteins in MM cells.

Since bortezomib is being developed to treat multiple cancers, we next investigated the effects of this compound in Panc1 and L3.6pL pancreatic and SW480 colon cancer cell lines. Figures 2A-2C demonstrate that 5-100 nM bortezomib treatment for 24-48 hr decreased growth of all 3 cell lines. Western blot analysis also showed that bortezomib induced caspase-3 cleavage in these cell lines (Fig. 2D). In addition, bortezomib also decreased expression of Sp1, Sp3 and Sp4 in Panc1, L3.6pL and SW480 cells and levels of Sp-regulated c-Myc and EGFR were also decreased (Fig. 2E). Previous studies have reported several other anticancer agents induce similar responses in these cell lines (Jutooru et al., 2014; Kasiappan et al., 2016; Nair et al., 2013; Safe et al., 2018).

RNA interference studies in Panc1, L3.6pL and SW480 cells and other cancer cell lines show that knockdown of Sp1, Sp3 and Sp4 individually and in combination resulted in growth inhibition and induction of apoptosis (Hedrick et al., 2016). Therefore ANBL-6 and RPMI 8226 cells were transfected with oligonucleotides that target Sp1 (iSp1), Sp3 (iSp3), Sp4 (iSp4) and their combination (iSp1/3/4) and effects on Sp knockdown were determined by western blots of whole cell lysates (Fig. 3A). Knockdown of Sp1 decreased expression of Sp1 and also Sp4 in both cell lines; Sp3 knockdown decreased Sp3 and also Sp1 (ANBL-6 and RPMI 8226) and Sp4 (ANBL-6), and knockdown of Sp4 primarily decreased only the target protein. The specificity of Sp

knockdown is cell context-dependent (Hedrick et al., 2016) and their self-regulation is due, in part, to common GC-rich *cis*-elements in the 5'-promoter regions of Sp1, Sp3 and Sp4 genes (Lou et al., 2005; Nicolas et al., 2001; Song et al., 2001). Transfection of these oligonucleotides individually also decreased proliferation (Fig. 3B) and induced Annex V staining (Fig. 3C) in ANBL-6 and RPMI 8226 cells. Thus, Sp1, Sp3 and Sp4 individually regulate MM cell growth and survival, and knockdown of one Sp transcription factor is not compensated by the other two and this has previously been observed in other cancer cell lines including Panc1, L3.6pL and SW480 cells (Hedrick et al., 2016). Thus, bortezomib-mediated downregulation not only of Sp1 but also Sp3 and Sp4 contributes to the effects of this compound on growth inhibition and induction of apoptosis.

Previous studies show that drug-induced downregulation of Sp transcription factors is due to induction of proteolytic enzymes or phosphatases, or through induction of ROS (Safe et al., 2018) Results illustrated in Figure 4A demonstrate that the phosphatase inhibitor okadaic acid (OKA) did not affect bortezomib-induced Sp downregulation, whereas the caspase-8 inhibitor FMK-ZEITD but not Z-FA-FMK (non-specific) inhibited degradation of Sp1, Sp3 and Sp4 in ANBL-6 and RPMI 8226 cells. Similar results were observed in L3.6pL and SW480 cells (Fig. 4B) and we did not observe any inhibitory effects by the caspase-3 inhibitor DEVD (Figs. 4C and 4D). Bortezomib-induced downregulation of Sp1, Sp3 and Sp4 was not inhibited by antioxidants glutathione (GSH) or N-acetylcysteine (NAC) in ANBL-6 and RPMI 8226 (Fig. 4E) and L3.6pL and SW480 (Fig. 4F) cells and therefore not ROS-dependent, and this was consistent with the failure of bortezomib to induce ROS in ANBL-6 and RPMI

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8226 cells (Supplemental Fig. 2). Interestingly, previous studies have reported that bortezomib induces activation of caspase-8 (Amodio et al., 2012; Bat-Erdene et al., 2016; Bullenkamp et al., 2014; Laussmann et al., 2011; Liu et al., 2007) and bortezomib-mediated Sp1 degradation is blocked by FMK-ZEITD (Bat-Erdene et al., 2016). A role for caspase-3-dependent cleavage of Sp1 has also been reported (Torabi et al., 2018). The inhibition of bortezmib-induced effects by FMK-ZEITD is consistent with the observed induction of caspase-8 in MM cells (ANBL-6 and RPMI 8226) (Fig. 5A) and the L3.6pL pancreatic and SW480 colon cancer cells (Fig. 5B). We also investigated possible upstream activators of caspase-8 and the extrinsic apoptosis pathway and observed that Fas-associated death domain (FADD) was induced by bortezomib in MM cells (Fig. 5C) as previously observed in Hela cervical cancer cells (Laussmann et al., 2011). FADD was also induced in pancreatic and colon cancer cells (Fig. 5D). The role of caspase 8 in mediating bortezomib-induced Sp downregulation was confirmed in MM, SW480 and L3.6pL cells since treatment of the cell lines with bortezomib plus an oligonucleotide targeting caspase-8 blocked downregulation of Sp1, Sp3 and Sp4 (Fig. 5E).

The role of bortezomib-induced caspase-8 on the effects of this compound on MM cell proliferation was determined in ANBL-6 and RPMI 8826 cells treated with FMK-ZEITD, Z-FA-FMK and bortezomib alone and in combination. Bortezomib-mediated inhibition of MM cell proliferation was inhibited by FMK-ZEITD but not Z-FA-FMK (Fig. 6A) and similar results were observed for Annexin V staining (Fig. 6B and Supplemental Fig. 1B) and not surprisingly, the effects of FMK-ZEITD were more pronounced for the Annexin V assay. We also observed similar effects in L3.6pL and SW480 cells (Figs.

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6C and 6D), demonstrating that bortezomib-induced activation of caspase-8 plays an important role in mediating the growth inhibitory and pro-apoptotic effects of this compound in MM and other cancer cell lines.

We also examined the time course-dependent effects of bortezomib on FADD, caspase-8, Sp1, Sp3 and Sp4 expression in the MM cell lines. In ANBL-6 and RPMI 8226 cells, loss of Sp proteins was observed after 2 or 4 hr; cleaved caspase-8 was induced within 1-2 hr and pFADD was enhanced after 2 hr, suggesting that in addition to FADD, other factors upstream from caspase-8 may also be activated by bortezomib (Figs. 7A and 7B). Similar results were observed for L3.6pL and SW480 cells (Figs. 7C and 7D). Figure 7E summarizes a possible mechanism of bortezomib-induced downregulation of Sp1, Sp3 and Sp4 which involves caspase-8-dependent proteolysis. Western blots at early time points show some cleavage products (data not shown), and the cleavage sites and rates of Sp1, Sp3 and Sp4 degradation will be investigated in future studies. Current studies are investigating the mechanisms associated with bortezomib-dependent induction of FADD and other upstream factors that activate caspase-8.

DISCUSSION

Bortezomib and related proteasome inhibitors are used in drug combination therapies for treatment of MM and are being evaluated for treatment of other tumors (Anderson, 2016; Bates, 2016; Neri et al., 2016; Orlowski and Lonial, 2016). Therefore, it is essential that the mechanism of action of this compound be understood so that combination therapies can take advantage of mechanism-based drug-drug interactions.

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Bortezomib works through multiple pathways in MM and other cancer cells, and our initial hypothesis was that bortezomib induced ROS which results in an ROS-dependent pathway resulting in repression of Sp1, Sp3 and Sp4 mRNAs/proteins and pro-oncogenic Sp-regulated genes. This pathway has been worked out for several ROS-inducing anticancer agents including benzyl- and phenethylisothiocyanates, histone deacetylase inhibitors, piperlongumine and penfluridol (Hedrick et al., 2015; Hedrick et al., 2017; Jutooru et al., 2014; Karki et al., 2017; Kasiappan et al., 2016). Treatment of MM cells with bortezomib decreased Sp1 protein expression as previously reported; however, this response was not affected by cotreatment with bortezomib plus GSH or NAC (Fig. 4E) and the effects of bortezomib appeared to be ROS-independent (Supplemental Fig. 2).

Previous studies have identified a miR-29b-Sp1 loop where miR-29b targets Sp1 (3'-UTR) to decrease gene expression; however, our results show that bortezomib not only decreased levels of Sp1 but also Sp3 and Sp4 in MM cells and other cancer lines, suggesting that bortezomib may induce degradation pathways that simultaneously targets all three Sp transcription factors. Individual knockdown of Sp1, Sp3 and Sp4 in lung, kidney, breast, pancreatic, colon and rhabdomyosarcoma cancer cell lines results in decreased cell proliferation, survival and migration/invasion and decreased expression of pro-oncogenic Sp-regulated genes, suggesting that Sp transcription factors are non-oncogene addiction genes (Hedrick et al., 2016). Similar results were observed in MM cells (Fig. 3) and this indicates that not only Sp1 but also Sp3 and Sp4 play an important role in the growth and survival of MM cells. Moreover, among these three Sp transcription factors which target GC-rich *cis*-elements, the loss of one factor is

not compensated by the other two in MM cells and this is consistent with their differential regulation of gene expression previously demonstrated in pancreatic, colon and other cancer cells (Hedrick et al., 2016).

Among those agents that inhibit drug-induced Sp downregulation including antioxidants, proteasome inhibitors, phosphatase inhibitors (okadaic acid), zinc ions and caspase inhibitors (rev. in Safe et al., 2018), only the caspase-8 inhibitor FMK-ZEITD inhibited bortezomib-induced downregulation of Sp1, Sp3 and Sp4 (Fig. 4). This observation is consistent with previous reports showing that bortezomib activates caspase-8 and the extrinsic pathway of apoptosis (Bat-Erdene et al., 2016; Bullenkamp et al., 2014; Laussmann et al., 2011; Liu et al., 2007) and one of those reports also show that inhibition of caspase-8 blocked bortezomib-induced cell death (Bullenkamp et al., 2014) as observed in this study (Fig. 5).

Previous studies showed that the NSAID tolfenamic acid also induced degradation of Sp1, Sp3 and Sp4 in SW480 colon cancer cells (Pathi et al., 2014) and this response was blocked in cells cotreated with tolfenamic acid plus the caspase-8 inhibitor FMK-ZEITD. Thus, at least in SW480 cells, both bortezomib (Figs. 2 and 5) and tolfenamic acid exhibited a common mechanism of action involving caspase-8 (Fig. 7E) and this was also observed for bortezomib in pancreatic cancer and MM cell lines. Moreover, in these same cell lines, bortezomib induced FADD which is upstream from caspase-8, and this was previously reported in Hela cells where bortezomib also induced FADD protein expression (Laussmann et al., 2011). Current studies in this laboratory are investigating the mechanisms associated with bortezomib-mediated

induction of FADD and other genes upstream from caspase-8 and the mechanisms of caspase-8-dependent degradation of Sp1, Sp3 and Sp4.

In summary, this study shows for the first time the bortezomib-dependent activation of caspase-8 results in degradation not only of Sp1 but also Sp3 and Sp4 which are important non-oncogene addiction genes (Hedrick et al., 2016) that are highly expressed in MM cells. Moreover, individual knockdown of Sp1, Sp3 and Sp4 induced inhibition of MM cell growth and survival (Fig. 3), suggesting that Sp transcription factors are also pro-oncogenic in MM cells as previously observed in colon, pancreatic, breast, lung and kidney cancer cells (Hedrick et al., 2016). Thus, the contributions of bortezomib-induced downregulation of Sp1, Sp3 and Sp4 to the overall efficacy of this drug should be considered in the development of clinical applications of bortezomib since several Sp-regulated genes are associated with drug and radiation resistance (Safe et al., 2018).

Author Contributions:

Participated in research design: Karki and Safe

Conducted experiments: Karki and Harishchandra

Contributed new reagents or analytic tools: Safe

Performed data analysis: Karki and Harishchandra

Wrote or contributed to the writing of the manuscript: Karki and Safe

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CONFLICT OF INTEREST: The authors declare that there are no competing financial interests.

FIGURE CAPTIONS

Figure 1. Bortezomib inhibits MM cell growth and survival and downregulates Sp1, Sp3 and Sp4. ANBL-6 (A), RPMI 8226 (B) and both cell lines (C) were treated with 1-20 nM bortezomib for 24, and effects on cell growth and Annexin V staining were determined as outlined in the Materials and Methods. ANBL-6 and RPMI 8226 cells were treated with 1-20 nM bortezomib for 24 hr, and whole cell lysates were analyzed for markers of apoptosis (D) and Sp transcription factors and Sp-regulated genes (E) by western blots. Results (A-C) are means ± SD for at least 3 replicated experiments and significant ($p<0.05$) effects of treatment compared to solvent-treated control are indicated (*). Both high (upper) and low (lower) molecular weight forms of Sp3 are shown in all western blots.

Figure 2. Bortezomib inhibits pancreatic and colon cancer cell growth, induces apoptosis and downregulates Sp1, Sp3 and Sp4. Panc1 (A), L3.6pL (B) and SW480 (C) cancer cell lines were treated with 5-100 nM bortezomib, and effects on cell proliferation and Annexin V staining were determined. The cells were treated with 5-100 nM bortezomib for 24 hr, and whole cell lysates were analyzed for markers of apoptosis (D) and Sp downregulation (E) by western blots. Results (A-C) are means ± SD for at least 3 replicate experiments and significant ($p<0.05$) effects of treatment (compared to solvent-treated controls) are indicated (*).

Figure 3. Functional effects of Sp knockdown in MM cells. (A) ANBL-6 and RPMI 8226 cells were transfected with oligonucleotides targeting Sp1 (iSp1), Sp3 (iSp3), Sp4 (iSp4)

or their combination (iSp1/3/4) or a non-specific control (iCtl), and whole cell lysates were analyzed by western blots. Cells were transfected with the same set of oligonucleotides and after 72 hr, effects of Sp knockdown on cell proliferation (B) or Annexin V staining (C) were determined as outlined in the Methods. Results (B and C) are expressed as means ± SD for at least 3 replicate experiments and significant ($p<0.05$) changes compared to iCtl (control) are indicated (*).

Figure 4. Potential Inhibitors of bortezomib-induced Sp downregulations in MM, colon and pancreatic cancer cells. ANBL-6 and RPMI 8226 (A) and L3.6pL and SW480 (B) cells were treated with bortezomib alone or in combination with Z-FA-FMK, FMK-ZEITD or OKA for 24 hr, and whole cell lysates were analyzed by western blots. Treatment with the inhibitors alone (Z-FA-FMK, FMK-ZEITD or OKA) did not affect Sp expression (data not shown). ANBL-6 and RPMI 8226 (C) and L3.6pL and SW480 (D) cells were also treated with bortezomib alone or in combination with DVED, and whole cell lysates were analyzed by western blots. ANBL-6 and RPMI 8226 (E) and L3.6pL and SW480 (F) cells were treated with bortezomib alone or in combination with antioxidants, and whole cell lysates were analyzed by western blots.

Figure 5. Bortezomib induces FADD and caspase-8 in cancer cells and caspase-8 is required for Sp degradation. ANBL-6 and RPMI 8226 (A) and L3.6pL and SW480 (B) cells were treated with different concentrations of bortezomib for 24 hr, and whole cell lysates were analyzed by western blots for caspase-8 activation (cleavage). ANBL-6 and RPMI 8226 (C) and Panc1, L3.6pL and SW480 (D) cells were treated as described

in (A) and (B), and whole cell lysates were analyzed by western blots for induction of FADD. (E) ANBL-6, RPMI 8226, L3.6pL and SW480 cells were treated with bortezomib alone and in combination with an oligonucleotide targeted to caspase-8, and whole cell lysates were isolated and analyzed by western blots.

Figure 6. Effects of caspase inhibitors on bortezomib-mediated inhibition of growth and induction of apoptosis. (A) ANBL-6 and RPMI 8226 cells were treated bortezomib, Z-FA-FMK and FMK-ZEITD alone or in combination for 24 hr, and effects on cell proliferation were determined as outlined in the Materials and Methods. (B) ANBL-6 and RPMI 8226 cells were treated as described in (A) and effects on induction of Annexin V staining were determined. L3.6pL and SW480 cells were treated as outlined in (A) and effects on cell proliferation (C) and Annexin V staining (D) were determined as outlined in the Materials and Methods. Results (A-C) are means $\pm$ SD for at least 3 replicate determinations, and significant ($p<0.05$) changes compared to control are indicated (*) and significant ($p<0.05$) reversal of the effects are also indicated (**).

Figure 7. Time-dependent effects of bortezomib. ANBL-6 (A), RPMI 8226 (B), L3.6pL (C) and SW480 (D) cells were treated with bortezomib and effects on expression of various proteins at different treatment times were determined by western blot analysis. (E) Proposed model for bortezomib-induced downregulation of Sp1, Sp3, Sp4 and Sp-regulated genes that play a role in cell proliferation and survival.

Figure 1

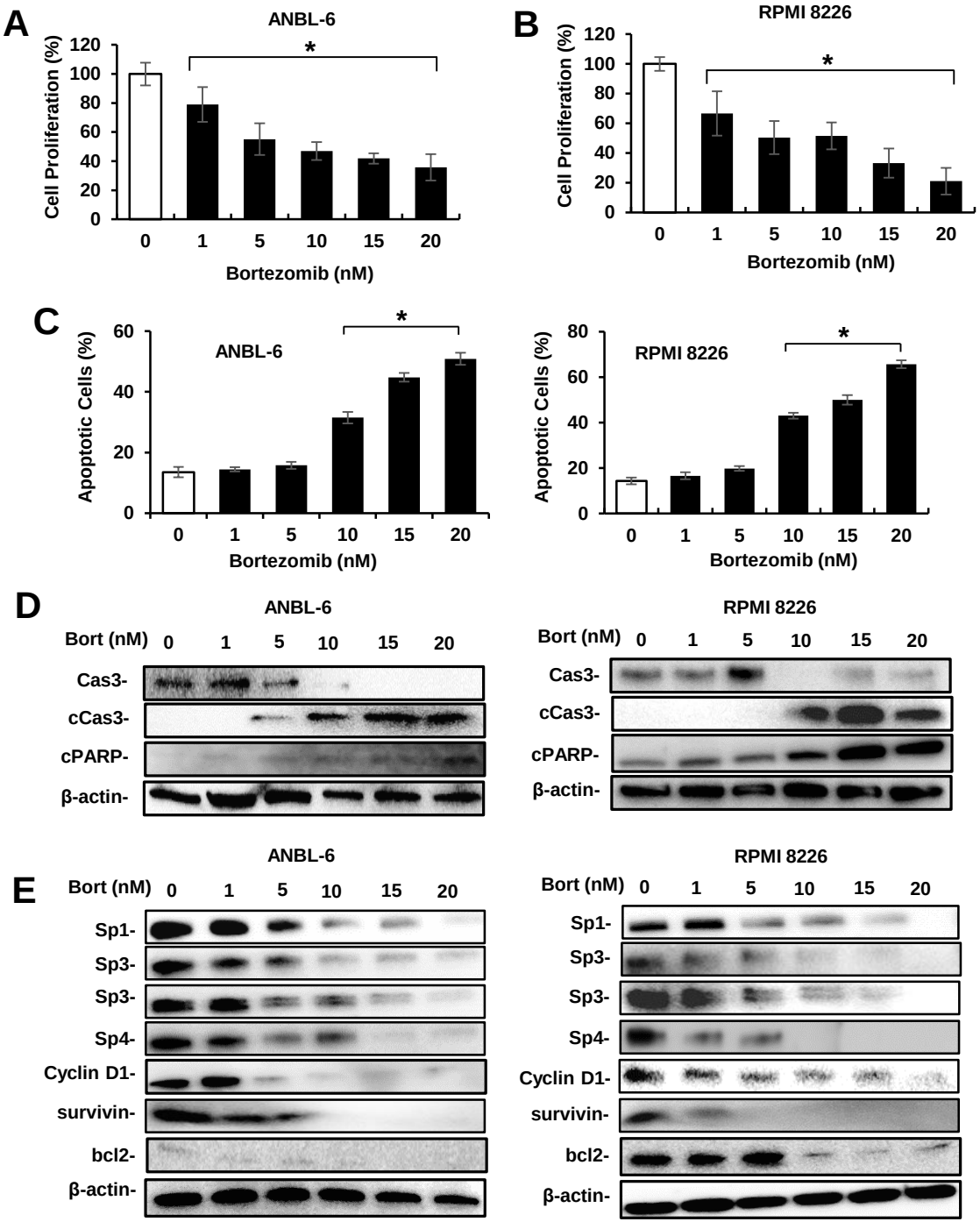
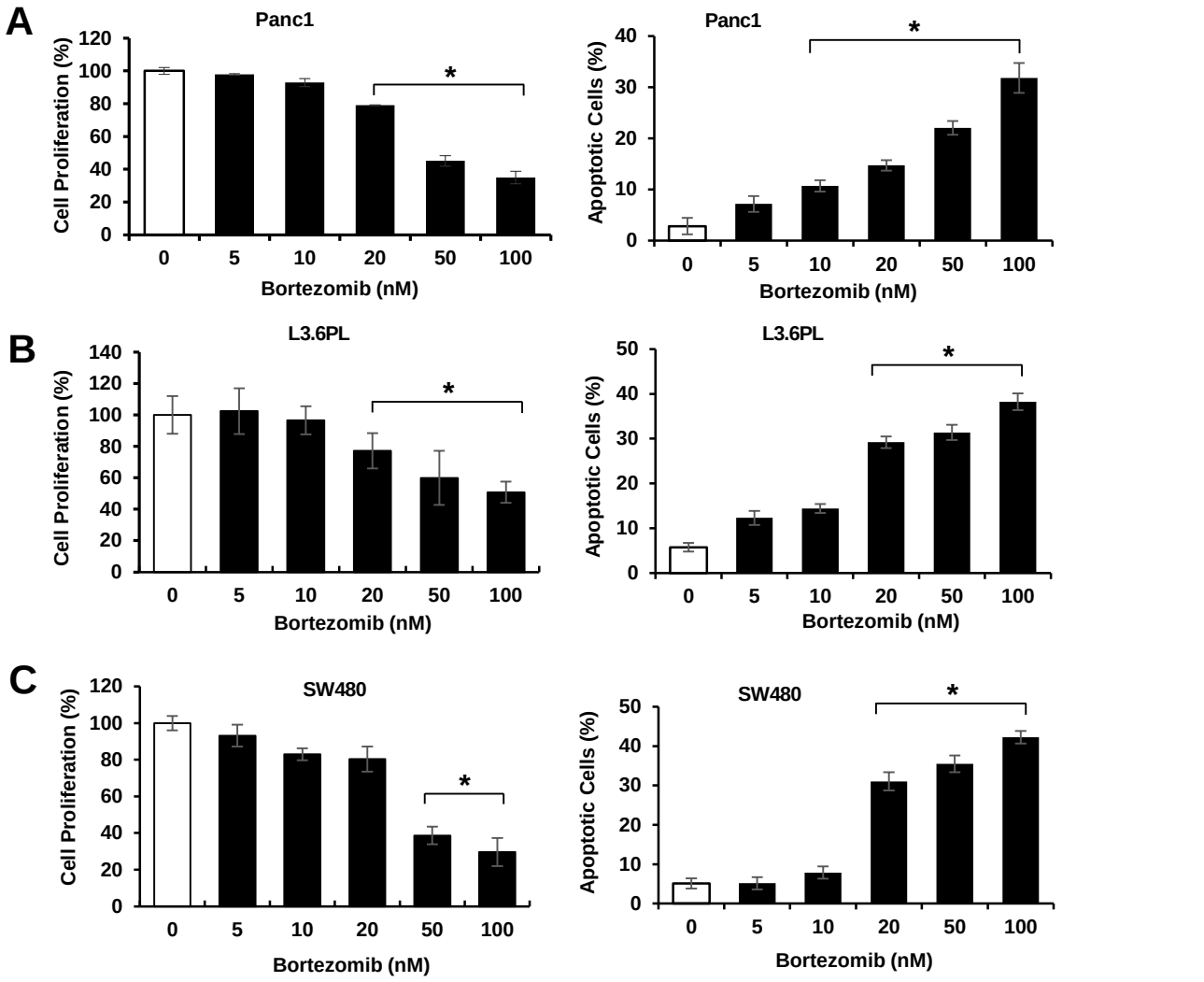
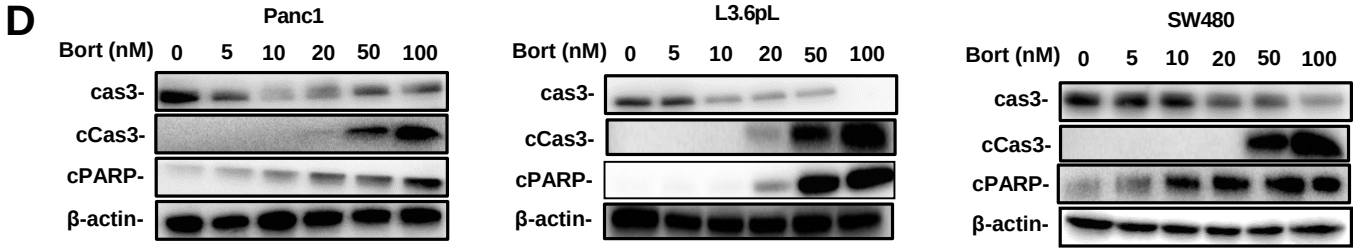

A
ANBL-6
Cell Proliferation (%)
120
100
80
60
40
20
0
*
0 1 5 10 15 20
Bortezomib (nM)
B
RPMI 8226
Cell Proliferation (%)
120
100
80
60
40
20
0
*
0 1 5 10 15 20
Bortezomib (nM)
C
ANBL-6
Apoptotic Cells (%)
60
40
20
0
*
0 1 5 10 15 20
Bortezomib (nM)
RPMI 8226
Apoptotic Cells (%)
80
60
40
20
0
*
0 1 5 10 15 20
Bortezomib (nM)
D
ANBL-6
Bort (nM) 0 1 5 10 15 20
Cas3-
cCas3-
cPARP-
β-actin-
RPMI 8226
Bort (nM) 0 1 5 10 15 20
Cas3-
cCas3-
cPARP-
β-actin-
E
ANBL-6
Bort (nM) 0 1 5 10 15 20
Sp1-
Sp3-
Sp3-
Sp4-
Cyclin D1-
survivin-
bcl2-
β-actin-
RPMI 8226
Bort (nM) 0 1 5 10 15 20
Sp1-
Sp3-
Sp3-
Sp4-
Cyclin D1-
survivin-
bcl2-
β-actin-

Figure 2


A
Panc1
Cell Proliferation (%)
120
100
80
60
40
20
0
0 5 10 20 50 100
Bortezomib (nM)
*
Panc1
Apoptotic Cells (%)
40
30
20
10
0
0 5 10 20 50 100
Bortezomib (nM)
*
B
L3.6PL
Cell Proliferation (%)
140
120
100
80
60
40
20
0
0 5 10 20 50 100
Bortezomib (nM)
*
L3.6PL
Apoptotic Cells (%)
50
40
30
20
10
0
0 5 10 20 50 100
Bortezomib (nM)
*
C
SW480
Cell Proliferation (%)
120
100
80
60
40
20
0
0 5 10 20 50 100
Bortezomib (nM)
*
SW480
Apoptotic Cells (%)
50
40
30
20
10
0
0 5 10 20 50 100
Bortezomib (nM)
*


D
Panc1
Bort (nM) 0 5 10 20 50 100
cas3-
cCas3-
cPARP-
β-actin-
L3.6pL
Bort (nM) 0 5 10 20 50 100
cas3-
cCas3-
cPARP-
β-actin-
SW480
Bort (nM) 0 5 10 20 50 100
cas3-
cCas3-
cPARP-
β-actin-

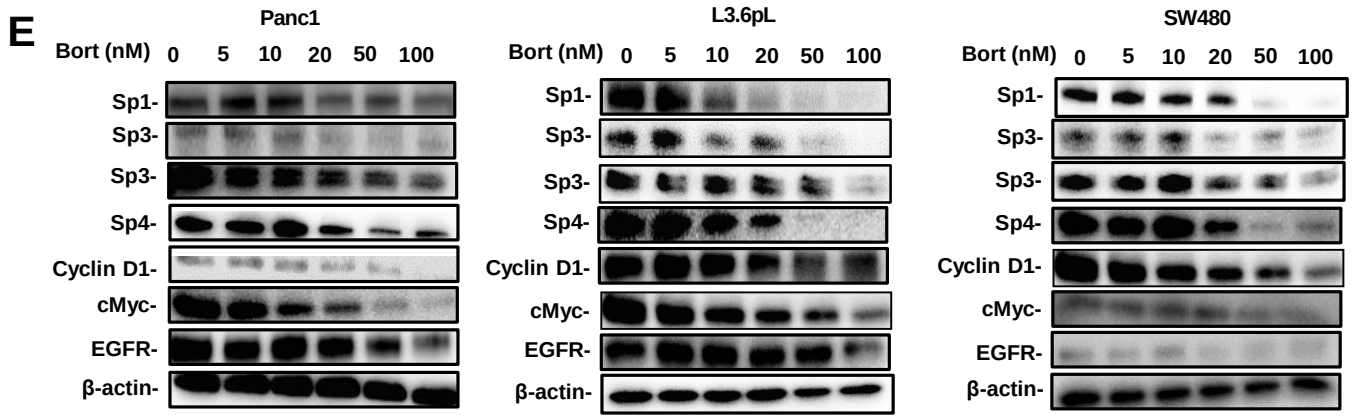
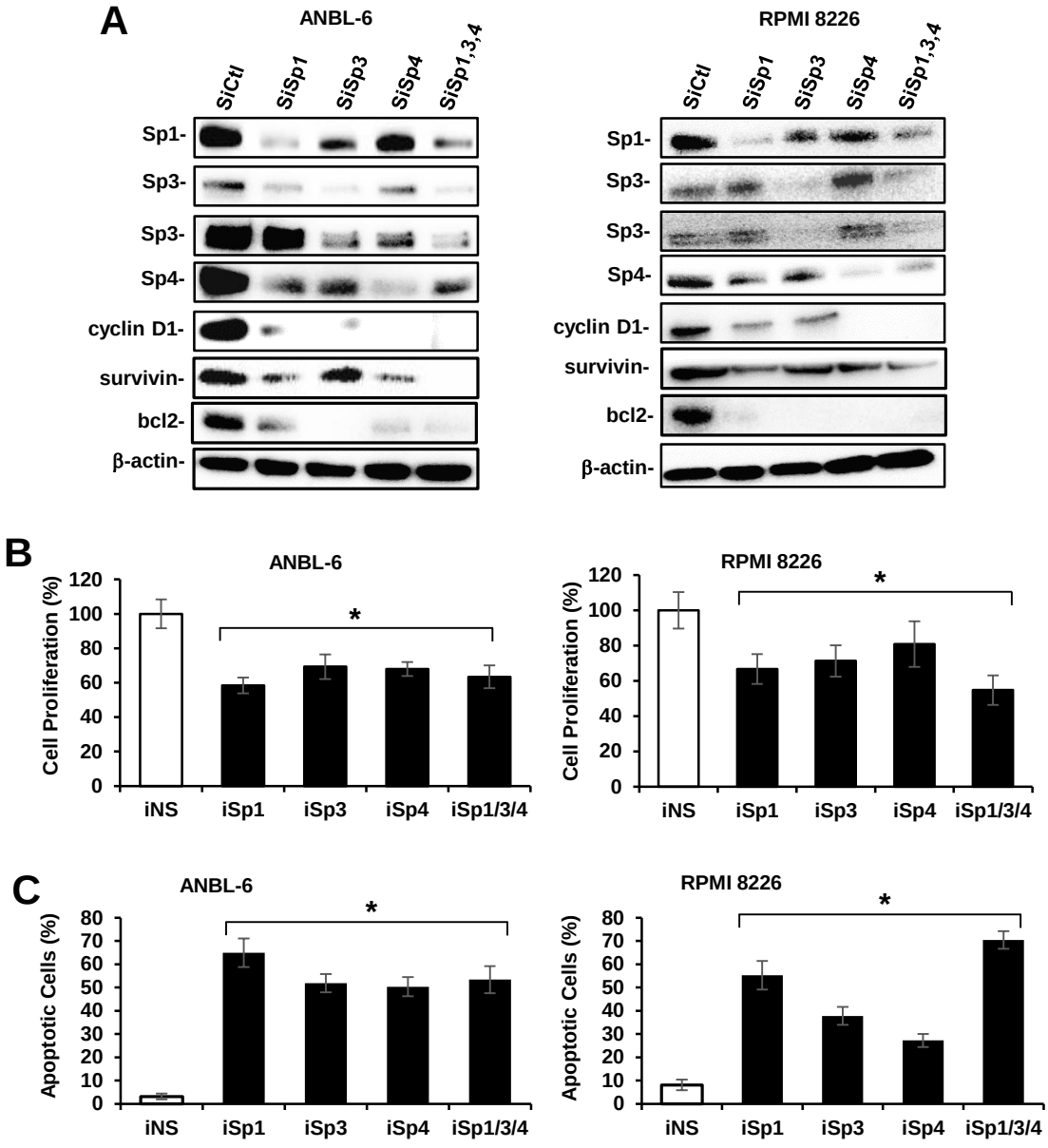

E
Panc1
Bort (nM) 0 5 10 20 50 100
Sp1-
Sp3-
Sp3-
Sp4-
Cyclin D1-
cMyc-
EGFR-
β-actin-
L3.6pL
Bort (nM) 0 5 10 20 50 100
Sp1-
Sp3-
Sp3-
Sp4-
Cyclin D1-
cMyc-
EGFR-
β-actin-
SW480
Bort (nM) 0 5 10 20 50 100
Sp1-
Sp3-
Sp3-
Sp4-
Cyclin D1-
cMyc-
EGFR-
β-actin-

Figure 3


A
ANBL-6
RPMI 8226
SiCtl
SiSp1
SiSp3
SiSp4
SiSp1,3,4
Sp1-
Sp3-
Sp3-
Sp4-
cyclin D1-
survivin-
bcl2-
β-actin-
B
ANBL-6
RPMI 8226
Cell Proliferation (%)
iNS
iSp1
iSp3
iSp4
iSp1/3/4
C
ANBL-6
RPMI 8226
Apoptotic Cells (%)

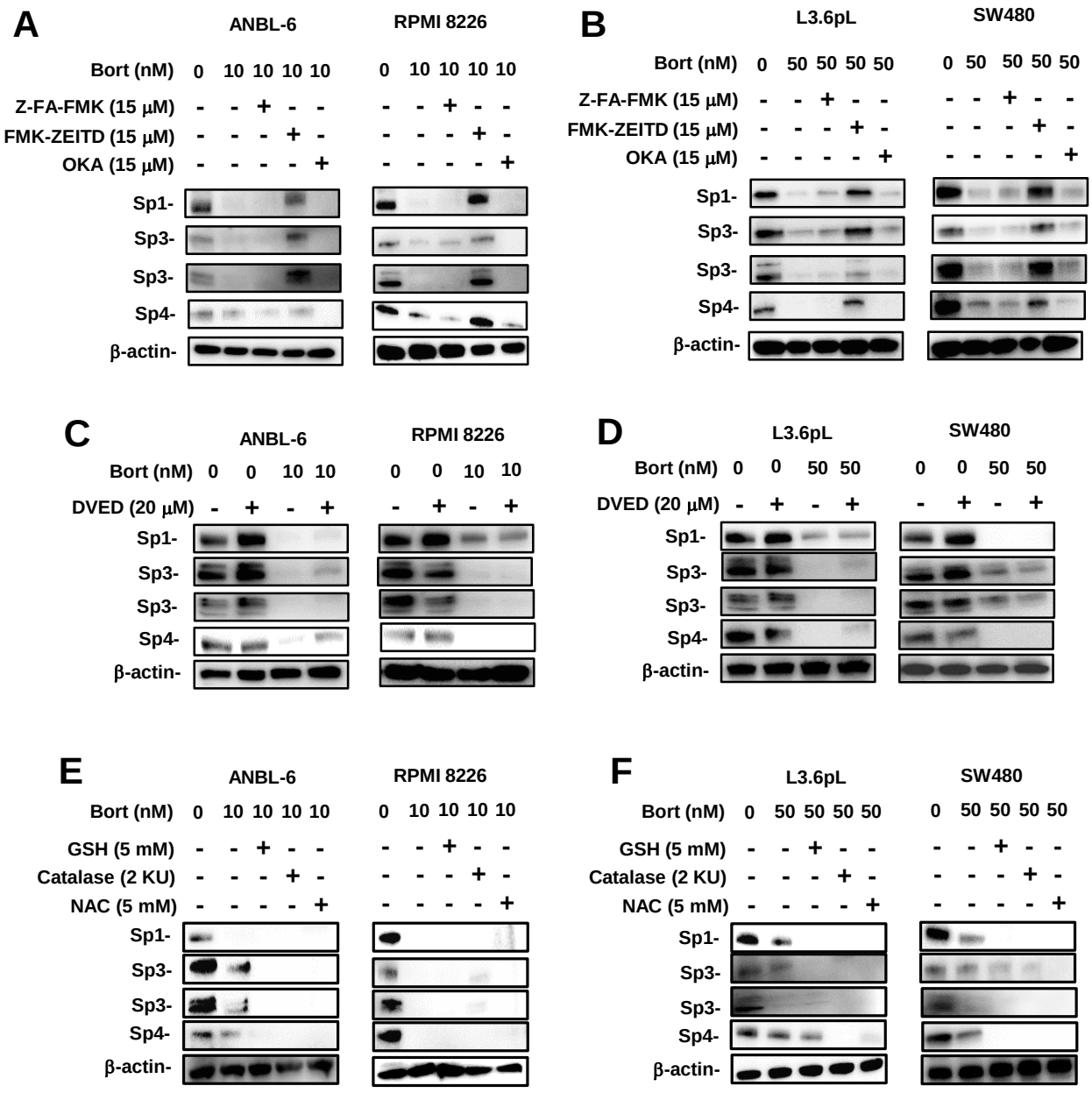

A
ANBL-6
RPMI 8226
Bort (nM) 0 10 10 10 10 0 10 10 10 10
Z-FA-FMK (15 μM) - - + - - - - + - -
FMK-ZEITD (15 μM) - - - + - - - - + -
OKA (15 μM) - - - - + - - - - +
Sp1-
Sp3-
Sp3-
Sp4-
β-actin-
B
L3.6pL
SW480
Bort (nM) 0 50 50 50 50 0 50 50 50 50
Z-FA-FMK (15 μM) - - + - - - - + - -
FMK-ZEITD (15 μM) - - - + - - - - + -
OKA (15 μM) - - - - + - - - - +
Sp1-
Sp3-
Sp3-
Sp4-
β-actin-
C
ANBL-6
RPMI 8226
Bort (nM) 0 0 10 10 0 0 10 10
DVED (20 μM) - + - + - + - +
Sp1-
Sp3-
Sp3-
Sp4-
β-actin-
D
L3.6pL
SW480
Bort (nM) 0 0 50 50 0 0 50 50
DVED (20 μM) - + - + - + - +
Sp1-
Sp3-
Sp3-
Sp4-
β-actin-
E
ANBL-6
RPMI 8226
Bort (nM) 0 10 10 10 10 0 10 10 10 10
GSH (5 mM) - - + - - - - + - -
Catalase (2 KU) - - - + - - - - + -
NAC (5 mM) - - - - + - - - - +
Sp1-
Sp3-
Sp3-
Sp4-
β-actin-
F
L3.6pL
SW480
Bort (nM) 0 50 50 50 50 0 50 50 50 50
GSH (5 mM) - - + - - - - + - -
Catalase (2 KU) - - - + - - - - + -
NAC (5 mM) - - - - + - - - - +
Sp1-
Sp3-
Sp3-
Sp4-
β-actin-

Figure 5

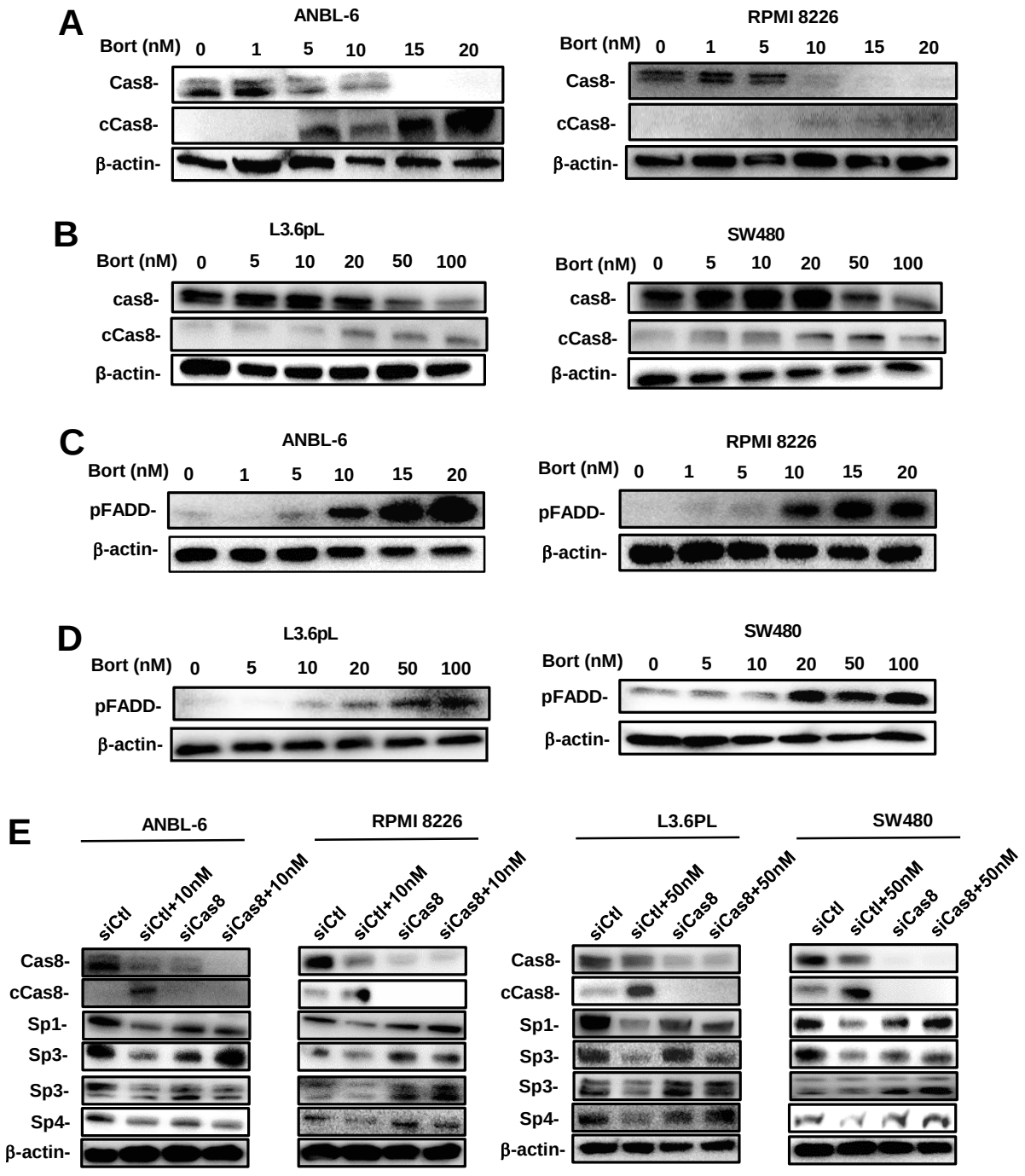

A
ANBL-6
Bort (nM) 0 1 5 10 15 20
Cas8-
cCas8-
β-actin-
RPMI 8226
Bort (nM) 0 1 5 10 15 20
Cas8-
cCas8-
β-actin-
B
L3.6pL
Bort (nM) 0 5 10 20 50 100
cas8-
cCas8-
β-actin-
SW480
Bort (nM) 0 5 10 20 50 100
cas8-
cCas8-
β-actin-
C
ANBL-6
Bort (nM) 0 1 5 10 15 20
pFADD-
β-actin-
RPMI 8226
Bort (nM) 0 1 5 10 15 20
pFADD-
β-actin-
D
L3.6pL
Bort (nM) 0 5 10 20 50 100
pFADD-
β-actin-
SW480
Bort (nM) 0 5 10 20 50 100
pFADD-
β-actin-
E
ANBL-6
siCtl
siCtl+10nM
siCas8
siCas8+10nM
RPMI 8226
siCtl
siCtl+10nM
siCas8
siCas8+10nM
L3.6PL
siCtl
siCtl+50nM
siCas8
siCas8+50nM
SW480
siCtl
siCtl+50nM
siCas8
siCas8+50nM
Cas8-
cCas8-
Sp1-
Sp3-
Sp3-
Sp4-
β-actin-

Figure 6

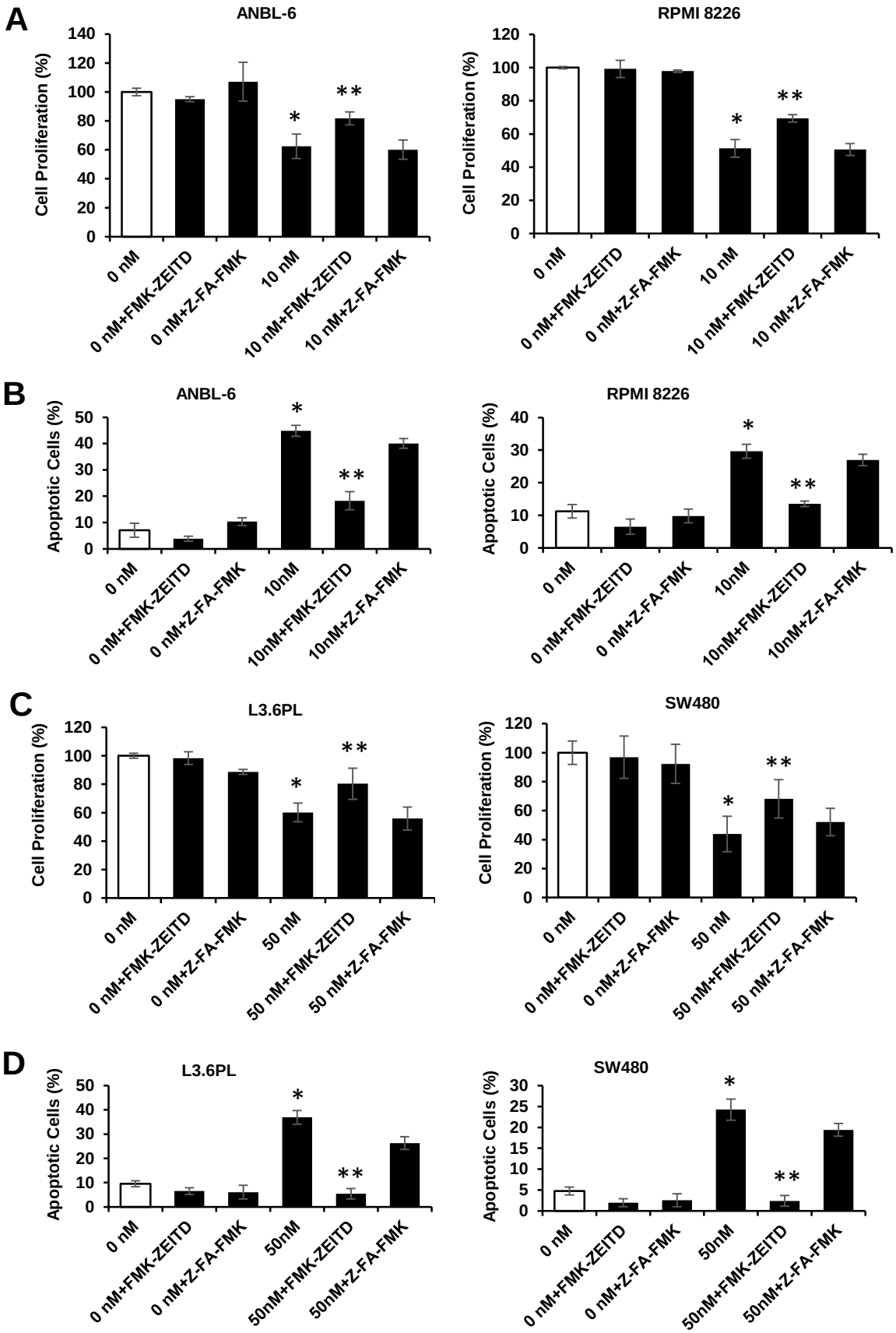
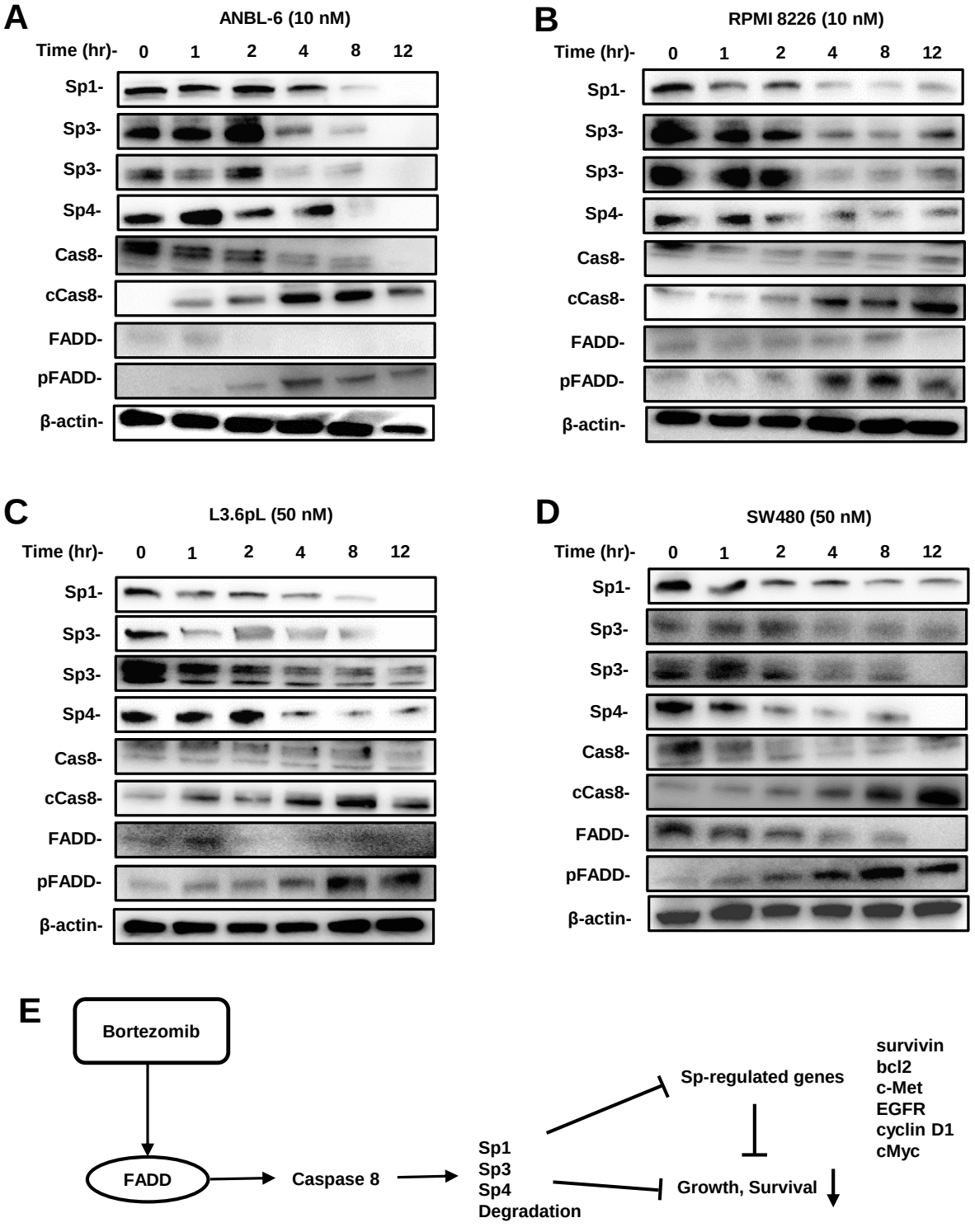

A
ANBL-6
Cell Proliferation (%)
0 nM
0 nM+FMK-ZEITD
0 nM+Z-FA-FMK
10 nM
10 nM+FMK-ZEITD
10 nM+Z-FA-FMK
RPMI 8226
B
Apoptotic Cells (%)
10nM
10nM+FMK-ZEITD
10nM+Z-FA-FMK
C
L3.6PL
SW480
50 nM
50 nM+FMK-ZEITD
50 nM+Z-FA-FMK
D
50nM
50nM+FMK-ZEITD
50nM+Z-FA-FMK

Figure 7


A
ANBL-6 (10 nM)
Time (hr)- 0 1 2 4 8 12
Sp1-
Sp3-
Sp3-
Sp4-
Cas8-
cCas8-
FADD-
pFADD-
β-actin-
B
RPMI 8226 (10 nM)
Time (hr)- 0 1 2 4 8 12
Sp1-
Sp3-
Sp3-
Sp4-
Cas8-
cCas8-
FADD-
pFADD-
β-actin-
C
L3.6pL (50 nM)
Time (hr)- 0 1 2 4 8 12
Sp1-
Sp3-
Sp3-
Sp4-
Cas8-
cCas8-
FADD-
pFADD-
β-actin-
D
SW480 (50 nM)
Time (hr)- 0 1 2 4 8 12
Sp1-
Sp3-
Sp3-
Sp4-
Cas8-
cCas8-
FADD-
pFADD-
β-actin-
E
Bortezomib
FADD
Caspase 8
Sp1
Sp3
Sp4
Degradation
Sp-regulated genes
Growth, Survival
survivin
bcl2
c-Met
EGFR
cyclin D1
cMyc