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14. ABSTRACT The purpose of this past year's work was to investigate the in vivo anti-cancer efficacy of combining proteasome inhibition with recombinant human TRAIL protein on prostate cancer. This combined treatment regimen was well tolerated and prevented the growth of prostate tumors in a LNCaP xenograft model. Each agent used singly demonstrated no effect on tumor volume. To elucidate the mechanism of the anti-tumor activity, angiogenesis, proliferation, and apoptosis was examined. No detectable effects on blood vessel density were observed. A wave of caspase-8 activity and tumor cell apoptosis was noted after the initial combined treatments. A decrease in proliferation was discovered only at the later time points. To determine if the activation of caspase-8 was responsible for the reported anti-cancer effect of proteasome inhibition as a single agent, PC3 cells were made deficient in death receptor signaling. The death receptor defective cells were resistant to the anti-cancer effects of Velcade. This study provides a proof-of-principle in animals that proteasome inhibition combined with a death receptor agonist is a viable treatment for human prostate cancer.					
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INTRODUCTION:

Apoptosis is initiated by two major pathways, the extrinsic (death receptor) pathway or the intrinsic (mitochondrial) pathway. The majority of chemotherapeutics used in the clinic today target the intrinsic pathway. This is primarily due to the fact that the majority of cancer cells are resistant to death receptor agonists. We reported (1) that the resistance to apoptosis initiated by the death receptor agonists TRAIL or Fas ligand can be removed with a low dose of proteasome inhibitor. Because a proteasome inhibitor (Velcade) is currently used in the clinic today for the treatment of myeloma, the combined strategy of Velcade with a death receptor agonist has promise. The scope of this past year's research was first to determine whether combining TRAIL with Velcade has efficacy in vivo and, if so, determine the mechanism by which anti-tumor activity occurs. Second, we wished to determine if the extrinsic pathway plays a major role in the anti-cancer efficacy observed in some tumors treated with Velcade as a single agent.

BODY:

As this work has resulted in a publication (2), this document will be routinely referenced. The manuscript has been included in the appendix. The original statement of work is listed in italics. Below each point, the progress has been described in detail.

Task 1. Test the extent to which proteasome inhibition can act as an anti-neoplastic agent in vivo by sensitizing cancer cells to cell death ligands in the tumor microenvironment. (Months 1-18).

a. Induce LNCaP xenografts in nude mice and follow tumor growth over time after treatment with saline or Velcade and GST or GST-TRAIL. (Months 1-6). 144 mice will be used for this study. LNCaP cells will be injected with Matrigel into the inguinal fat pad of nude mice. The study will consist of 4 treatment arms or groups. Once cells reach 100 mm³ (about 21 days) the mice will be injected with Velcade (1 mg/kg) or saline. The next day the mice will be treated with recombinant GST or GST-TRAIL (all of the recombinant protein needed for this study is already prepared and stored at -80°C). This injection cycle will be repeated every 4 days for 4 total cycles. Tumor volume will be measured with a caliper 3 times per week. At the end of this period, we will have documented the tumor growth rate in the 4 treatment arms that will allow for statistical comparisons between the groups. From this, we will be able to conclude if Velcade can render tumors susceptible to TRAIL.

1a. This experiment was largely conducted as described. Approximately 90% of the mice injected with the LNCaP/Matrigel mixture formed tumors in nude mice. The tumors reached 100 mm³ after 30-40 days. Upon reaching the set size they were randomized into four groups and two groups treated intra-peritoneal with GST-TRAIL (4 mg/kg) or two groups treated with GST (4 mg/kg) as a control. The following day, one of the two GST-TRAIL cohorts were either treated intra-peritoneal with Saline or Velcade (1 mg/kg) and one of the two GST cohorts also treated with Saline or Velcade (described in Figure 1C). After four days this cycle was repeated for a total of five cycles. No decrease in body weight was noted over this period (Supplemental Figure

1A). Tumor volume was measured bi-weekly over the treatment period. As demonstrated in Figure 2A, the tumors did not grow in the group treated with both Velcade and GST-TRAIL, while the single agents had no anti-tumor effect. In addition, we assayed mouse blood after a single GST-TRAIL injection to determine the half-life of this agent. This study showed the GST-TRAIL half-life to be 7 hours (Figure 1B). This experiment demonstrates that combining a death receptor agonist with Velcade is a viable treatment option that could be pursued in human patients. This study is consistent with that of Shanker et al. who recently demonstrated that providing an antibody to induce death receptor activation with Velcade could block the growth of lung cancer (3).

b. Test PC3 cells resistant to cell-extrinsic death. (Months 1-3). PC3 cells will be utilized that overexpress a dominant negative FADD protein or vector only as a control. These cells have been generated by transfecting cells with DNA constructs and selecting for those cells harboring the construct with hygromycin. These stable cell clones will be analyzed for truncated FADD expression and resistance to TRAIL-mediated cell death. This experiment will provide us with a reagent to carry out the remainder of Task 1.

1b. The object of this experiment was to use molecular reagents to generate cells that are resistant to extrinsic cell death (PC3 cells). These cells will be used as a reagent to complete 1c listed below. FADD is a critical adapter molecule in the death receptor pathway. By overexpressing a truncated form of the protein in cells one can make them more resistant to cell death ligands. Using retroviral techniques a dominant negative FADD (dn-FADD) was stably introduced into PC3 cells and a polyclonal population selected. These cells were found to robustly overexpress dn-FADD and were resistant to TRAIL (Supplemental Figure 1B). In addition, as a second genetic means to block death receptor signaling, c-FLIP (a death signaling inhibitory protein) was also introduced by the same means. This second set of death receptor resistant cells readily overexpressed c-FLIP and were even more resistant to death receptor signaling (Figure 4A,B). This experiment was successfully completed and provided us with two death receptor resistant models in which to carry out the subsequent in vivo experiments.

c. Generate PC3 xenografts in nude mice and follow tumor growth over time after treatment with saline or Velcade. (Months 7-12). 144 athymic nude mice will be purchased for this study. PC3 cells overexpressing dominant negative FADD or a vector control will be injected into the inguinal fat pad of nude mice. Once cells reach 100 mm³ (about 21 days) the mice will be injected with Velcade (1 mg/ml) or saline. This injection will be repeated every 4 days for 4 total cycles. Tumor volume will be measured with a caliper 3 times per week. At the end of this period, we will have documented the tumor growth rate in the 4 groups that will allow for statistical comparisons. From this, we will be able to conclude what percentage of the observed growth inhibition that Velcade causes to PC3 cells can be attributed to activation of death receptor pathways.

1c. The primary mechanism regarding how proteasome inhibition leads to anti-tumor activity in vivo is unknown. The data we generated in 1A suggested that much of this activity occurs through death receptor signaling initiated from ligands in the tumor microenvironment. PC3

prostate cancer cells are partially sensitive to TRAIL and respond to Velcade in vivo (1). The purpose of this experiment was to test in a cell line that is sensitive to Velcade alone in vivo, whether the anti-tumor activity was initiated through the extrinsic pathway. To test this hypothesis the engineered PC3 xenografts were propagated in nude mice. The groups consisted of PC3 Puro (control), PC3-dn-FADD, and PC3-cFLIP. Once tumors reached 100 mm³ PC3 xenografts were treated with and without Velcade (1 mg/kg). The animals were treated for five total cycles of Velcade and tumor volume was measured bi-weekly. As demonstrated in Figure 4D and supplementary Figure 1C, tumors overexpressing either c-FLIP or dn-FADD, respectively, did not respond to the Velcade treatment, while the Puro control cells were responsive. We would like to note that the only caveat is that the death receptor resistant cells grew at a slower rate in vivo when compared to the Puro control cells. This change in cell growth was not noted in vitro. We assume that this is due to a requirement of signaling through this pathway by multiple TNF family members that is due to ligands in the tumor microenvironment that are absent in vitro. The main caveat, therefore, is that the death receptor resistant tumors may have been less responsive to Velcade because they grew slower. In summary, this experiment was critical in showing for the first time that proteasome inhibition leads to an anti-tumor response by death receptor signaling. In the future, an alternative means to prove the role of death receptor signaling would be to also inject blocking/neutralizing antibodies for Fas ligand, TNF- α , and TRAIL and determine the sensitivity to Velcade with these regimens.

d. Analyze treated PC3 xenografts for markers of cell division, cell death, angiogenesis and caspase-8 activation. (Months 10-18). Following the last injection cycle in (c), the tumors will be excised and prepared for histology and biochemical analysis. Five tumors in each group will be analyzed. Sections of the tumors will be made using histologic techniques. The proliferative capacity will be determined by Ki-67 staining, the apoptotic index quantified by in situ TUNEL staining, and angiogenesis measured by CD31 staining. Caspase-8 activation will be assayed by assaying the tumor homogenate for procaspase-8 cleavage by Western blot analysis. Following the completion of Task 1, we will know how Velcade alters angiogenesis, cell proliferation, apoptosis and the extent to which these processes are affected by cell-extrinsic death stimuli.

1d. Tumors obtained from the LNCaP Velcade plus TRAIL efficacy study (1a) were harvested from the mice in an effort to elucidate the mechanism of how Velcade plus TRAIL was leading to an anti-tumor outcome. First, it was obvious when the tumors were obtained from the mice only the doubly treated tumors lacked the characteristic hemorrhagic phenotype (Figure 2B). This phenotype was attributed to the loss of blood pools within the tumors (Figure 2B). Tumors were then analyzed to identify whether the anti-tumor effects were due to a change in angiogenesis, apoptosis, or proliferation. Blood vessel recruitment was examined by identifying CD31 positive cells using immunohistochemical techniques. This assay revealed CD31 positive endothelial cells, however the density did not differ among the groups (Figure 2C). Next, apoptosis was examined. In tumors treated with one round of chemotherapy a wave of caspase-8 activity (Figure 3D, denoted by HDAC7 cleavage) and apoptosis (Figure 3B, identified by TUNEL staining) was observed only in the doubly treated tumors. Importantly, this was the first time that HDAC7 was ever used to assess caspase-8 activity in vivo. Monitoring the HDAC7 processing (a caspase-8 specific target) was accomplished by Western blot analysis (4). Due to

the high background activity normal caspase-8 enzymatic assays do not yield specific activity. The HDAC7 assay described here is a breakthrough in monitoring the activity of the death receptor pathway in vivo. Importantly, at the end of the study no difference in apoptosis could be observed between the groups. Finally, proliferation was analyzed. Two hours prior to euthanasia the mice had been injected with BrdU intra-peritoneal (100 mg/kg) to label dividing cells. The BrdU incorporated into DNA was detected by immunohistochemistry. Tumors analyzed at the end of the study demonstrated much slower proliferation in the doubly treated groups than those singly treated or untreated (Figure 3C).

The mechanistic study described here has led us to a model for how the combined proteasome inhibition and TRAIL efficacy is achieved. First, the proteasome inhibition sensitizes the tumors to allow the subsequent administration of TRAIL to induce death receptor signaling (noted by caspase-8 activity) and apoptosis (TUNEL staining). These reagents are readily accessible due to the leaky vasculature. After a number of rounds of treatment, the tumor cells become more differentiated and lose the leaky vasculature. This prevents the accessibility of TRAIL and resulting effects on apoptosis. However, the Velcade can still reach the tumors and prevents cell growth. In the future to improve the efficacy of this therapy one would need to make the recombinant protein more accessible to the tumors to completely wipe out all cancer.

Task 2. Define the mechanism whereby proteasome inhibition enhances sensitivity to cell death ligand-induced apoptosis.

The remainder of Year 1 was spent working out the conditions and models necessary to complete the most difficult tasks (Task 2-c and d) in this proposal. This work will be highlighted below.

c. Identify the region of caspase-8 necessary and sufficient for degradation. Various caspase-8 constructs will be fused to the C-terminus of GFP. The DNA constructs will be transfected into LNCaP cells and the relative fluorescence quantified by flow cytometry. The goal is to identify the amino acids in caspase-8 necessary for degradation.

This aim was pursued exactly as described. Multiple caspase-8 constructs were cloned downstream of GFP. However, in contrast to the p18 subunit alone, the GFP-caspase-8 constructs were not dependent on proteasome inhibition for expression. As GFP is a very stable protein, we concluded that it is likely stabilizing the p18 subunit. Therefore, we proceeded as described in the ‘alternatives’ section of the proposal and cloned multiple caspase-8 residues upstream of the hemagglutinin (HA) tag. We were excited to see that the caspase-8 pro domain and the unprocessed active subunits did not require proteasome inhibition for expression, but the p18 fragment did (identical to the endogenous protein) (See Figure 5 below). However, not only was proteasome inhibition required for the p18 fragment to be expressed, but also the p10 fragment and overlapping p18 fragments. It became clear that all of these constructs are recognized for degradation by the ubiquitin proteasome system. Normally in a cell, the p18 and p10 subunits exist as a heterotetramer. The p18 and p10 subunits are not found apart. Therefore although this system replicates the necessity of proteolytic processing for proteasomal targeting, it also introduces a construct that is unstable in a cell, not due to regulated degradation, but to non-specific degradation of an unstable protein. As a second alternative we will be

immunoprecipitating caspase-8 and examining it by mass spectrometry to identify the ubiquitination sites.

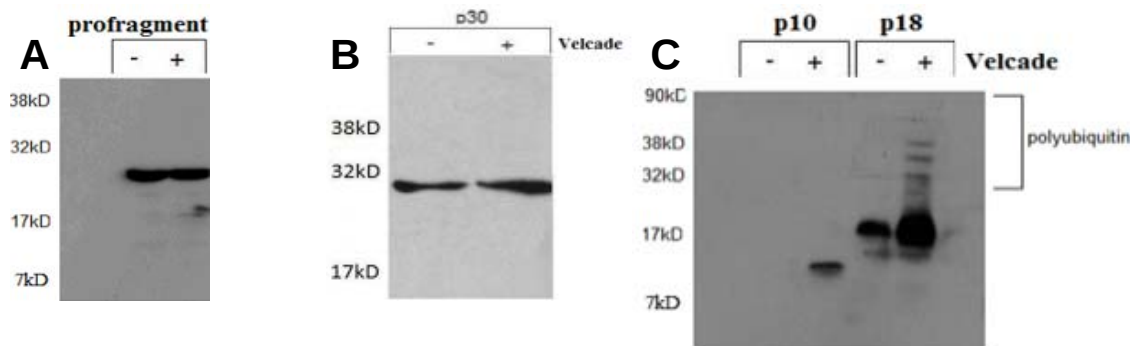


Figure 5. Caspase-8 HA approach to identify ubiquitinated residues. The caspase-8 profragment (A), p30 (contains unprocessed p18 and p10 subunits)(B), and processed p18 and p10 subunits (C) were expressed in PC3 cells and treated with or without Velcade for 24 hours. Cell extracts were analyzed for protein expression by HA immunoblotting. The pro and p30 construct did not require Velcade for expression in contrast to the processed p18 and p10 fragments. Note the high molecular weight smear indicative of ubiquitin incorporation only with the p18 fragment.

d. Examine the enzyme activity of degradation-resistant caspase-8 constructs. The mutations that yielded a stable caspase-8 in (c) will be introduced into the full-length caspase-8 cDNA. The mutant and wild-type cDNA constructs will be transfected into the caspase-8 negative SY5Y cells. Transfected SY5Y cells will be assayed for caspase-8 activity. This step will test if the degradation-resistant mutation has affected caspase-8 activity.

The critical preliminary studies necessary to complete Task 2 require a system to study the degradation resistant caspase-8 constructs. Our goal was to verify that the SY5Y cell system would work and, if not, identify a new system. First, wild-type caspase-8 was stably expressed in the SH-SY-5Y line. SH-SY-5Y caspase-8 deficient neuroblastoma cells were successfully transduced and expressed wild-type caspase-8. However, this could not be used as an expression system because despite caspase-8 expression, the SH-SY-5Y cells were incapable of undergoing TRAIL mediated apoptosis (data not shown). After having little success with the SH-SY-5Y cells we sought to use the I9.2 caspase-8 deficient Jurkat cell line.

Our initial data confirmed that the caspase-8 expressing wild-type Jurkat cell line behaved similarly to the LNCaP cell line in terms of caspase-8 cleavage, p18 stability, and the apoptotic response upon treatment with proteasome inhibitor and TRAIL (Figures 6A,B), however their ability to undergo TRAIL-mediated apoptosis upon restoring caspase-8 needed to be tested. The I9.2 cells were transduced either with an empty pBABE-puro expression vector (with puromycin selection marker) control or pBABE-puro encoding wild-type caspase-8. Following puromycin selection, comparisons were made between I9.2 reconstituted caspase-8 (I9.2 RC8), I9.2 puro

against the wild-type Jurkat, and I9.2 cell lines to assess caspase-8 expression, cleavage and TRAIL induced death via Western blot (Figures 6A,B) and MTT assay (Figure 6C,D). The results showed the I9.2 RC8 (behaved identically to wild-type cell line). In conclusion we were successful in identifying a physiologically relevant system in which to study future caspase-8 constructs.

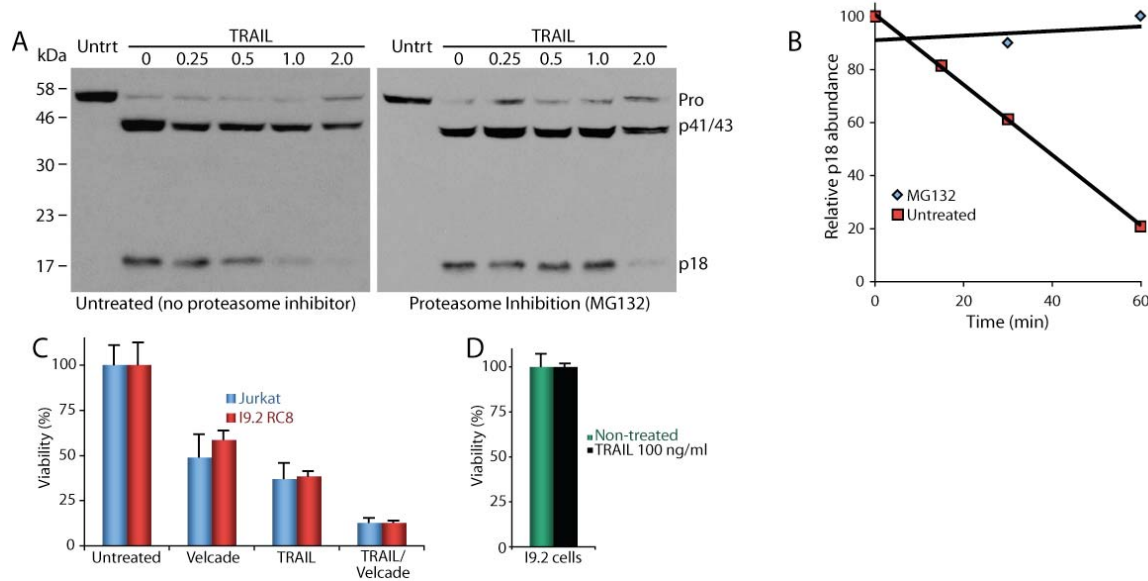


Figure 6. Development of a model for studying modified caspase-8 constructs. **(A)** Jurkat cells were assayed for the half-life of the caspase-8 p18 fragment. Cells were either left untreated or pre-treated with the proteasome inhibitor MG132 (10 μ M) for 9 hours. Cells were then treated with TRAIL for two hours to process caspase-8, after which TRAIL was removed and a caspase-8 inhibitor and cycloheximide was added for the indicated times. Caspase-8 was detected by Western blot analysis. **(B)** Quantification of the blots in shown in (A). In the absence of proteasome inhibition the p18 fragment has a 25 min half-life. **(C)** Caspase-8 was reconstituted in the caspase-8 deficient I9.2 cells (I9.2 RC8) and tested for their ability to undergo TRAIL mediated cell death. I9.2 RC8 cells underwent cell death in an identical fashion to wild-type jurkats. **(D)** Control experiment to show that I9.2 cells cannot undergo TRAIL-induced cell death.

KEY RESEARCH ACCOMPLISHMENTS:

- The GST-TRAIL combined with Velcade does not result in a decrease in body condition score or body weight.
- The half-life of GST-TRAIL in mice is 7 hours.
- TRAIL plus Velcade therapy prevents tumor growth in mouse models of prostate cancer.
- TRAIL plus Velcade therapy reverses the leaky vasculature associated with advanced cancer.
- TRAIL plus Velcade initiates caspase-8 activity and apoptosis in vivo.
- Caspase-8 activity can be measured in vivo by monitoring HDAC7 cleavage by Western blot analysis.
- Velcade achieves much of its anti-tumor activity in vivo by activating apoptosis through the death receptor pathway.
- The caspase-8 deficient I9.2 line is a physiologically relevant model in which to study the biochemical and biological functions of mutant caspase-8 constructs.

REPORTABLE OUTCOMES:

1. Peer-reviewed manuscript entitled: P.A. Christian, J.A. Thorpe, and S.R. Schwarze. 2009. Velcade sensitizes prostate cancer cells to TRAIL induced apoptosis and suppresses tumor growth in vivo. *Cancer Biol. Ther.* 8:69-76.
2. An R01 based on some of this work has been submitted to NIH. 1 R01 CA140466-01
3. The studies described above will be a large part of a graduate student's (Perry Christian) PhD thesis project.

CONCLUSION:

Targeting tumors with therapies to induce cell death through the extrinsic pathway initially provided much excitement and promise. Many companies have generated humanized antibodies to activate the TRAIL or Fas receptors to initiate cell death. However, these clinical studies were not successful as most tumors are at least partially resistant to death receptor signaling. Our study has demonstrated in vivo that proteasome inhibition, currently used in the clinic, can sensitize death receptor resistant tumors to the effects of TRAIL once again. This strategy could readily be employed in the clinic as Velcade is approved for use in patients and clinical studies using humanized TRAIL receptor activating antibodies are available.

Second, we have demonstrated for the first time that the efficacy observed in some tumors using Velcade can be at least in part due to signaling through the death receptor pathways. Therefore, the use of this agent alone may be most beneficial in tumors that contain high levels of death ligands such as TNF- α , TRAIL, and Fas ligand. In the future, it would be important to confirm this finding by blocking each of the death ligands in vivo and determining how this step affects the efficacy of Velcade. Of note, the use of the form of TRAIL used here is not amenable to use in the clinic as it has a large non-human protein (GST) attached to the N-terminus. This method allowed rapid purification and stabilization of the protein in vivo for the study to be conducted. In the future, either humanized TRAIL receptor antibodies need to be employed with Velcade in patients or other humanized strategies to generate a stable form of TRAIL need to be derived for this methodology to be applied directly to the clinic.

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APPENDICES:

An appendix follows which includes the manuscript published from this research.

Research Paper

Velcade sensitizes prostate cancer cells to TRAIL induced apoptosis and suppresses tumor growth in vivo

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Key words: velcade, TRAIL, apoptosis, prostate cancer, xenograft

Inducing apoptosis via the extrinsic death receptor pathway is an attractive anti-cancer treatment strategy, however, numerous cancer cells exhibit significant resistance to death ligand stimuli. Here, we investigated the anti-neoplastic capability of proteasome inhibition, through the administration of Velcade, to synergize with a death receptor agonist in vivo. The death ligand-resistant LNCaP prostate xenograft model was utilized. Tumors were established and mice were treated with Velcade, TRAIL (TNF-Related Apoptosis Inducing Ligand) or the combined regimen. Only mice treated with a combination of Velcade and TRAIL was tumor growth inhibited with a corresponding loss of the hemorrhagic phenotype, decreased tumor cell proliferation and increased tumor cell apoptosis. Next, to determine if the extrinsic pathway is critical for mediating the anti-tumor efficacy that can be achieved in some cell types with Velcade treatment alone, the death receptor sensitive PC-3 xenograft model was used. PC-3 tumors exhibited a 54% decrease in tumor volume in response to Velcade, while c-FLIP overexpressing PC-3 xenografts were resistant to the treatment. These findings suggest that the extrinsic apoptotic pathway can mediate the anti-tumor effects of Velcade and support the therapeutic use of proteasome inhibition in combination with a death receptor stimulus in the treatment of prostate cancer.

Introduction

The induction of apoptosis by chemotherapeutic agents is a central process to initiate tumor regression. Apoptosis can be induced by both cell-intrinsic and cell-extrinsic pathways.¹ The cell-intrinsic apoptotic pathway (mitochondrial pathway) induces cell death through mitochondrial outer-membrane permeabilization (MOMP). Once MOMP occurs, cytochrome-*c* is released and oligomerizes with Apaf-1 to form the apoptosome.² Procaspase-9 associates with the Apaf-1/cytochrome-*c* apoptosome where it is autoprocessed to form active caspase-9 with subsequent activation of effector caspases 3 and 7. A majority of chemotherapeutic agents work through the intrinsic apoptotic pathway.

The cell-extrinsic apoptotic pathway is initiated by the binding of extracellular death ligands to their corresponding transmembrane death receptors. Death ligands are members of the tumor necrosis factor (TNF) super family of molecules i.e., FasL, TNF α and TRAIL (TNF related apoptosis inducing ligand).³ The binding of a death ligand to its corresponding receptor results in clustering of the death receptors and signals the assembly of the Death Induced Signaling Complex (DISC) on the cytoplasmic side of the plasma membrane.⁴ Procaspase-8 associates with this complex and is autocatalytically cleaved at the DISC to form active caspase-8.⁵ Active caspase-8 can cleave downstream effector caspases and other death substrates fully inducing the apoptotic response. c-FLIP is a key member of the DISC complex and can function to inhibit procaspase-8 activation.⁶ The discovery of chemotherapeutic agents that act through the cell-extrinsic apoptotic pathway would provide an important alternative to those drugs that trigger the mitochondrial pathway.

Velcade (Bortezomib) is a potent, small molecule inhibitor of the 20S chymotrypsin-like activity of the 26S proteasome.⁷ The ability of Velcade to induce apoptosis and its potential as an anti-tumor agent has been widely studied.^{7,8} Velcade is currently approved for the clinical treatment of multiple myeloma. The efficacy of Velcade in the treatment of hormone-refractory prostate cancer has shown promise^{9,10} and is being studied in ongoing clinical trials. Although Velcade is approved for the treatment of neoplastic disease, the mechanism by which it mediates its anti-tumor effect in vivo is not fully understood. We have previously demonstrated that proteasome inhibition by Velcade retards active caspase-8 degradation, giving one possible explanation for its pro-apoptotic activity. This also suggests that in the tumor microenvironment, the cell-extrinsic pathway may play a role in the observed efficacy of this agent.¹¹

Numerous reports have shown that TRAIL can induce apoptosis in a variety of human tumor cell lines.^{3,12,13} However, a large number of prostate cancer cells exhibit a high level of resistance to the activity of death ligands. We, as well as others, have demonstrated that Velcade treatment can sensitize multiple death ligand-resistant prostate cancer cell lines to readily undergo TRAIL or FasL-mediated apoptosis.^{11,14} The ability of Velcade to synergize with a death ligand and induce apoptosis in death ligand-resistant cancer cells is very robust in vitro. Here, we examined the ability of Velcade to synergize with TRAIL in vivo using the death receptor resistant LNCaP tumor xenograft model. Our results show that Velcade is able to overcome this resistance to TRAIL-induced death and that the death receptor

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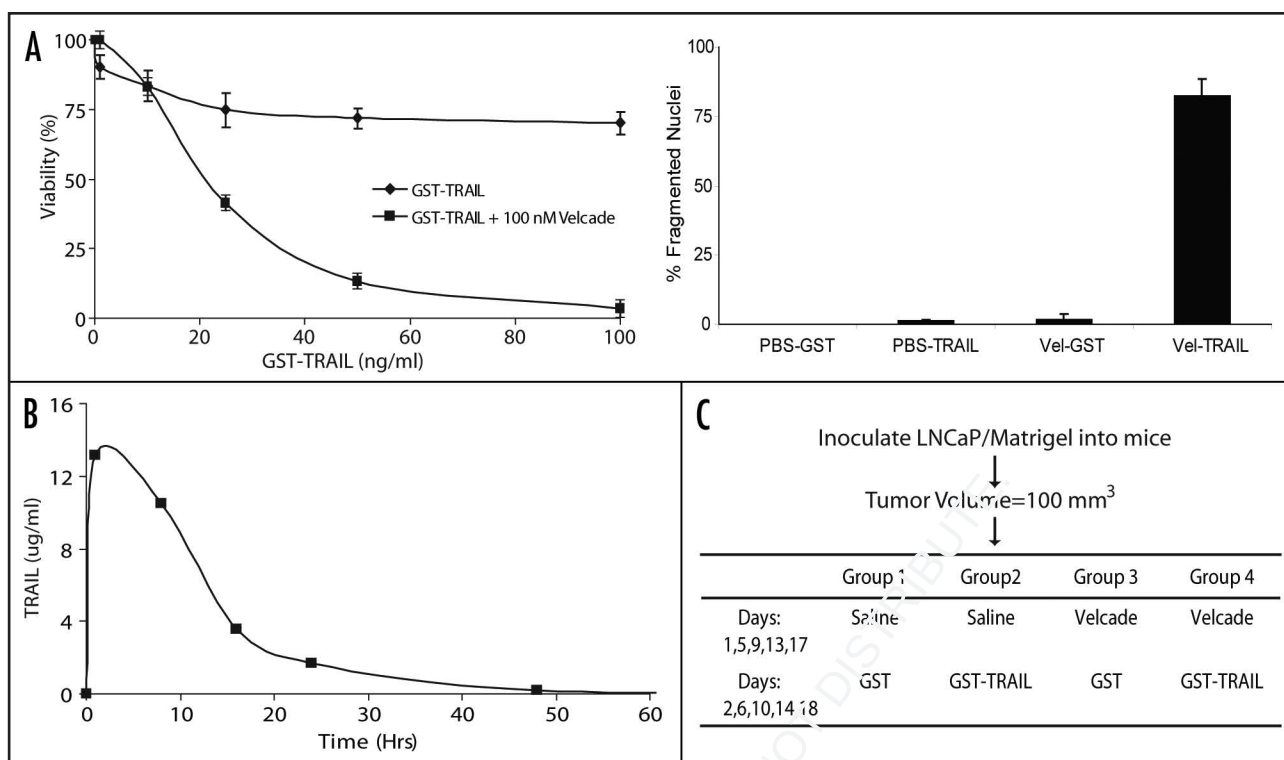


Figure 1. Biological activity of GST-TRAIL. (A) GST-TRAIL dose response. An MTT viability assay (left) was performed on LNCaP cells treated with increasing concentrations of GST-TRAIL in the presence or absence of Velcade (50 nM). Quantification of apoptotic nuclei (right) was performed on LNCaP cells treated with GST-TRAIL or Velcade alone as well as the combined treatment. (B) GST-TRAIL half-life in vivo. Mice were injected with 200 μ g of GST-TRAIL in PBS (500 μ l volume). Blood samples were taken throughout a 48-hour time period, serum was obtained and an ELISA assay was performed to detect and quantify recombinant TRAIL. (C) Design for the LNCaP xenograft experiment. LNCaP cells in an equal volume of Matrigel were sub-cutaneously injected into 28 nude mice. Once tumor volumes reached 100 mm³, mice were randomly selected into one of four groups. Mice were either injected (i.p.) with PBS or Velcade (1 mg/kg) followed by i.p. injections of GST or GST-TRAIL (200 μ g) 24 hours later. The injection schedule was continued for a total of five cycles.

pathway is critical to the anti-tumor efficacy of Velcade in vivo.

Results

GST-TRAIL generation and stability in vivo. A GST fusion system was established¹⁶ to generate the large amount of recombinant TRAIL necessary for this in vivo approach. To verify that the GST-TRAIL fusion protein was biologically active, LNCaP cells were treated in vitro with increasing concentrations of GST-TRAIL alone or in combination with Velcade (50 nM) for 16 hours. GST-TRAIL treatment alone resulted in a small decrease in the viable cell number, however, no rounded and floating cells were observed. In contrast, the addition of Velcade readily sensitized LNCaP cells to the killing effect of GST-TRAIL. The dose required to reduce cell number by 50% (LD₅₀) in the presence of Velcade was found to be 20 ng/mL (Fig. 1A, left). Quantification of fragmented nuclei was carried out to assess the apoptotic response. Consistent with our previous studies using commercially prepared TRAIL,¹¹ GST-TRAIL also led to a robust apoptotic response when combined with Velcade (Fig. 1A, right).

The activity of Velcade combined with TRAIL in vivo was tested through the use of the LNCaP xenograft model. Prior to initiating these studies, the ability of recombinant GST-TRAIL to enter the serum and its relative stability following injection was determined. Mice were injected (i.p.) once with GST-TRAIL and blood was drawn periodically up to 48 hours. Serum samples were analyzed for TRAIL using an immunoassay (Fig. 1B). TRAIL levels peaked two

hours post-injection and declined steadily with a half-life of seven hours. At 48 hours post-injection, TRAIL concentrations were 180 ng/mL, well above the concentration required to induce apoptosis in vitro in the presence of Velcade.

Velcade combined with TRAIL retards tumor growth. The anti-tumor efficacy of Velcade and TRAIL alone and the combination treatment were next assessed in vivo. Due to the short half-life of GST-TRAIL, mice were first treated with Velcade (terminal elimination half-life >40 Hrs¹⁹) and then administered GST-TRAIL the following day to comprise one treatment cycle. Once tumor volume reached 100 mm³, mice bearing LNCaP xenografts were randomly assigned to four groups and treated with PBS-GST, PBS-TRAIL, Velcade-GST and Velcade-GST-TRAIL for five total cycles (Fig. 1C). Regimens were well tolerated as determined by stable body weights (Suppl. Fig. 1A) and no change in body condition scores (data not shown). Tumor size was measured with a caliper and the tumor volume was calculated. Mice treated with the Velcade-GST or PBS-TRAIL regimens exhibited tumor growth rates indistinguishable from the control PBS-GST treatment. However, mice doubly treated with Velcade-TRAIL showed a significant inhibition of tumor growth beginning six days after the first treatment and lasting throughout the five cycle regimen (Fig. 2A).

After the final treatment cycle, tumors were excised and grossly examined. A surprising finding was the loss of the 'bloody' hemorrhagic phenotype in the combined Velcade and TRAIL treatment that is characteristically associated with LNCaP xenografts (Fig. 2B,

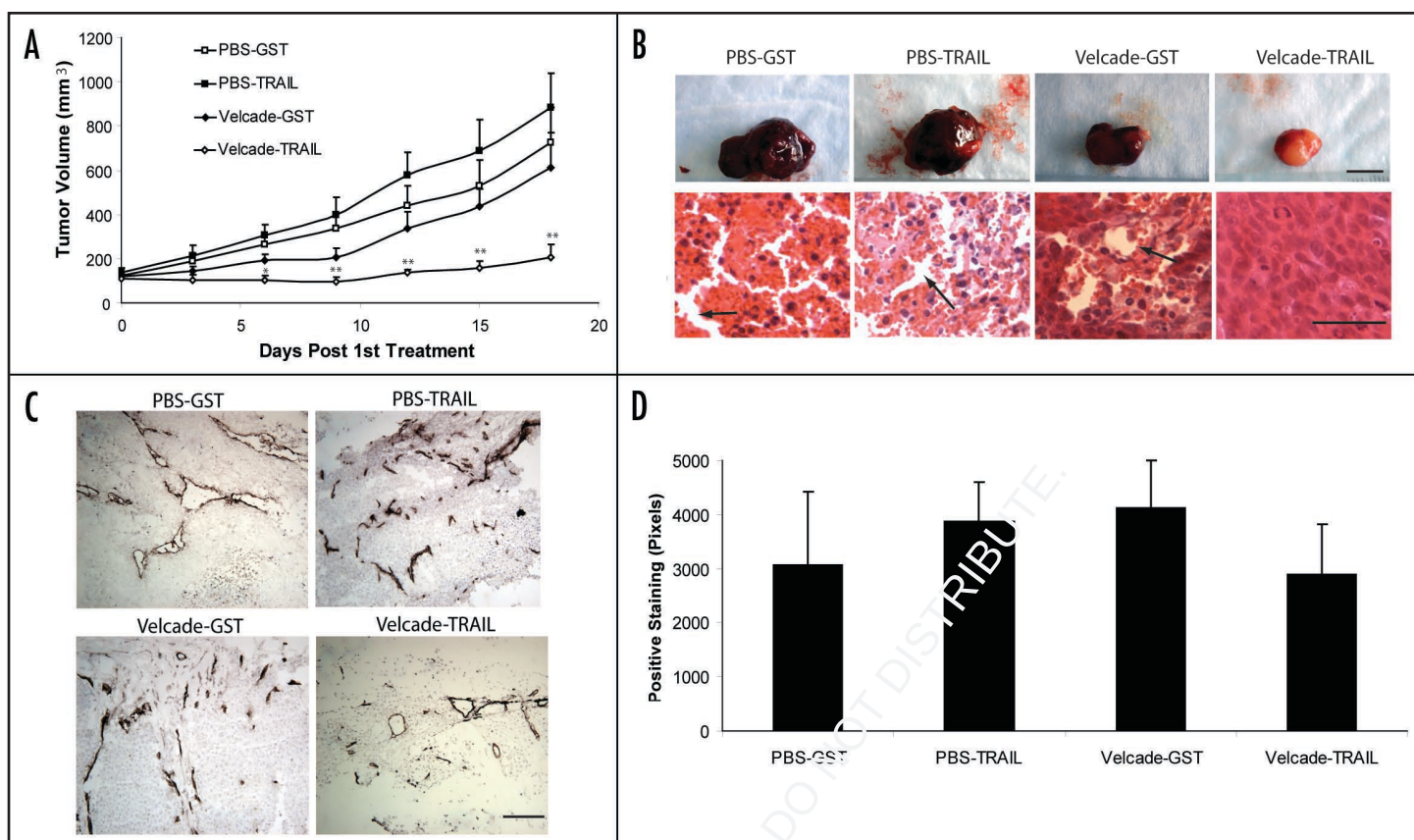


Figure 2. Velcade combined with TRAIL retards tumor growth. (A) Tumor growth curve for LNCaP xenografts in mice undergoing Velcade and TRAIL treatment regimens. * $p < 0.005$, ** $p < 0.001$. (B) LNCaP tumor analysis. (Top) Tumors were harvested 30 hours after the last treatment. Gross digital images were taken from mice treated for five cycles. Scale bar represents 1 cm. (Bottom) H & E staining of tumors was carried out. Arrows denote blood pools. Scale bar represents 100 μm. (C) CD31 immunohistochemistry. Tumors harvested as in (B) were analyzed by CD31 immunostaining. Scale bar represents 100 μm. (D) Quantification of CD31 staining was carried out by tracing the CD31 positive endothelial lumen and quantifying the number of pixels. The examination was repeated by two independent investigators.

top). Standard H & E staining of paraffin embedded tumor sections revealed that mice doubly treated with Velcade and TRAIL exhibited a compact, well-organized tumor cell arrangement. In contrast, tumors exposed to the individual Velcade and TRAIL treatments closely resembled the control tumors and contained large blood pools and loosely organized tumor cells as shown in Figure 2B, bottom. This change in tumor architecture with the Velcade-TRAIL regimen is consistent with the loss of the hemorrhagic appearance. We detected no difference in the amount of necrosis within the tumors. Tumor vasculature was observed and quantified by immunohistochemical analysis using CD31 as a marker for endothelial cells. Regardless of treatment regimen, a similar CD31 positive cell abundance and staining pattern was observed in all tumors (Fig. 2C and D).

Induction of apoptosis and reduced cell proliferation in the Velcade and TRAIL regimen. Tumor specimens were examined to investigate the basic mechanism behind the TRAIL and Velcade efficacy. First, tumor tissue was examined for apoptosis. TUNEL staining was employed to detect apoptotic cells after the fifth treatment cycle. Tumor specimens that were treated for five cycles contained TUNEL positive cells in a disperse, random fashion. No significant differences between the four treatment groups were observed after five treatment cycles (Fig. 3A, right). Tumors exposed only to the first treatment cycle were further examined to determine if apoptosis was being

induced at an earlier time point. For this study, an additional second set of mice bearing LNCaP xenograft tumors were administered the four treatment regimens for one cycle only. Tumors were excised 30 hours after the protein injection. TUNEL analysis on tumor tissue following one treatment cycle resulted in focused apoptotic nuclei staining in and immediately surrounding the blood pools. Tumor sections obtained from doubly treated Velcade and GST-TRAIL mice contained a higher percentage of TUNEL positive nuclei than those tissues treated with Velcade or GST-TRAIL only and the apoptotic cells had dispersed beyond the immediate blood pool boundaries (Fig. 3A, left). TUNEL analysis was also performed on prostate tissue from treated mice to test if the prostate tissue was affected by the treatment regimens. No observable apoptotic effect was found in any of the four treatment groups (data not shown).

The effect of the Velcade and TRAIL regimens on the proliferative state of the LNCaP xenograft tumors was also analyzed following the fifth treatment cycle. Two hours prior to euthanasia, BrdU was injected into mice to label nuclei in S-phase. BrdU was detected histologically from frozen tumor sections and the percent positive cells calculated. Mice treated with TRAIL or Velcade alone contained tumor cells with a comparable percentage of BrdU positive stained nuclei as the negative control group. In contrast, tumor cells from mice receiving the combined Velcade and GST-TRAIL treatment contained significantly fewer BrdU positive cells (Fig. 3B and C).

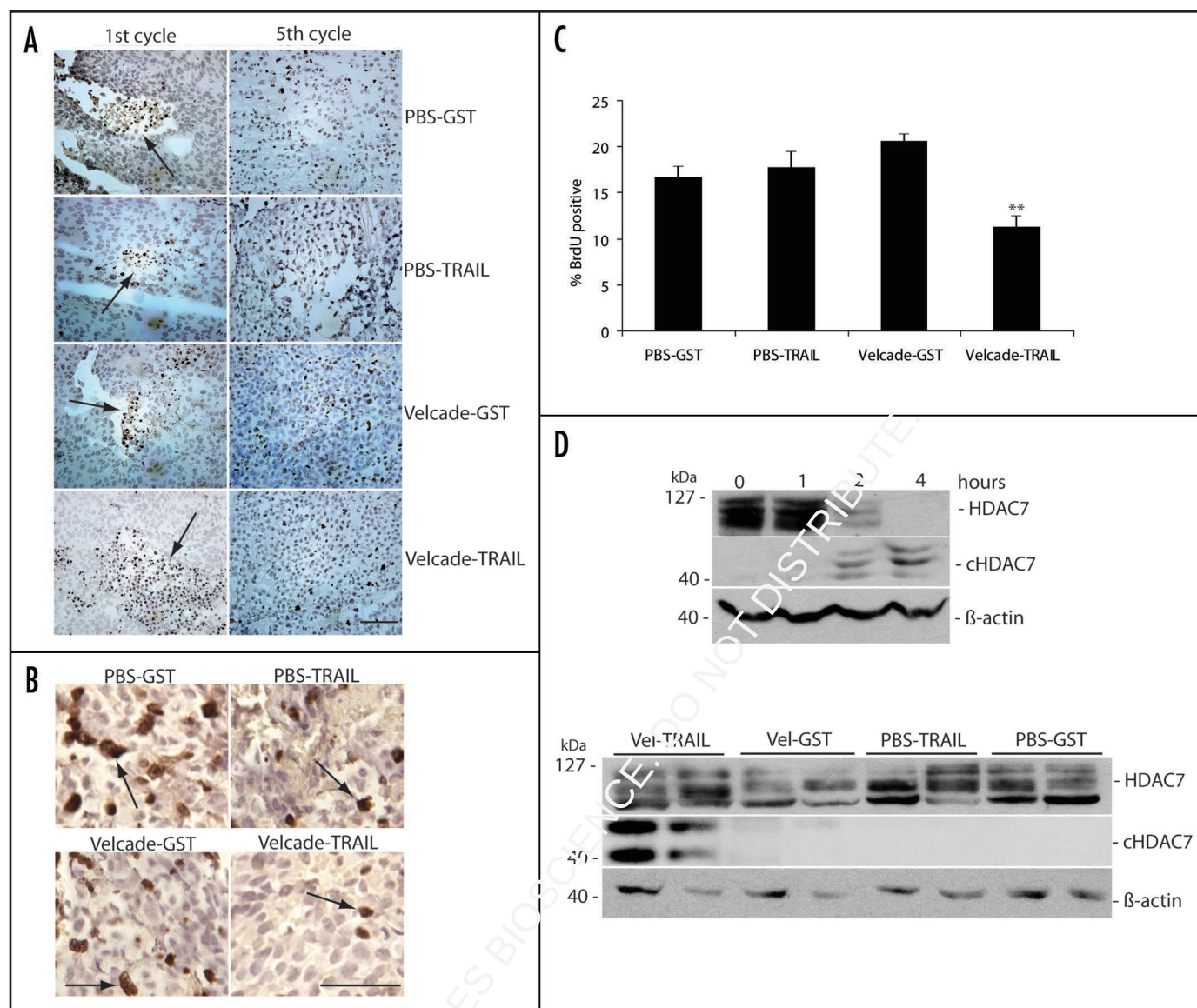


Figure 3. Velcade combined with TRAIL induces apoptosis and inhibits tumor cell proliferation. (A) TUNEL staining of LNCaP tumors derived from mice treated for one or five cycles. Arrows denote blood pools containing TUNEL positive tumor nuclei. Scale bar represents 100 µm. (B and C) Proliferation status following Velcade and TRAIL regimens. Tumors were excised after the fifth treatment cycle. Frozen tissue sections were prepared and immunostained for BrdU. Arrows indicate BrdU positive tumor cell nuclei. ** $p < 0.001$. (D) HDAC7 cleavage assay. (Left) LNCaP cells were treated with GST-TRAIL (1 µg/ml) in combination with 100 nM Velcade for the indicated times. Cell lysates were analyzed for HDAC7 cleavage by Western blot. (Right) Tumor cell extracts were prepared from tumors harvested 30 hours after the first treatment cycle. Extracts were examined for HDAC7 cleavage by Western blot analysis.

Together, these results demonstrate that the efficacy of Velcade combined with TRAIL initially results in the induction of apoptosis and then in cell growth suppression.

Velcade combined with TRAIL treatment induces caspase-8 activation in vivo. Next, whether the apoptosis induced by Velcade and TRAIL combined could be attributed to signaling through the death receptor pathway was examined. Caspase-8 activation is a hallmark of death ligand induced-apoptosis. However, the use of tumor homogenates to measure caspase-8 was problematic due to the large amount of non-specific cleavage of standard peptide-linked reporters. Therefore, we assayed the cleavage of HDAC7, a highly specific caspase-8 substrate.²⁰ HDAC7 processing was initially assessed by Western blot analysis in vitro. LNCaP cells were treated in culture with Velcade (100 nM) and GST-TRAIL (100 µg/mL) for up to four

hours. Two hours after treatment with Velcade and GST-TRAIL, intact HDAC7 diminished while a signal with a molecular weight consistent with the single HDAC7 cleavage (58 and 45 kDa) appeared (Fig. 3D, left).

Tumor homogenates derived from mice exposed to one treatment cycle were analyzed for HDAC7 processing. Full-length HDAC7 was observed in all tumors, however, only tumor extracts from the combined Velcade and TRAIL regimen showed HDAC7 cleavage (Fig. 3D right). Therefore, caspase-8 activity is associated with the apoptosis induction and is consistent with the Velcade and TRAIL combined regimens conferring activation of the extrinsic apoptotic pathway.

Efficacy of velcade as a single agent requires a functional death receptor pathway. The LNCaP xenograft study suggested that the

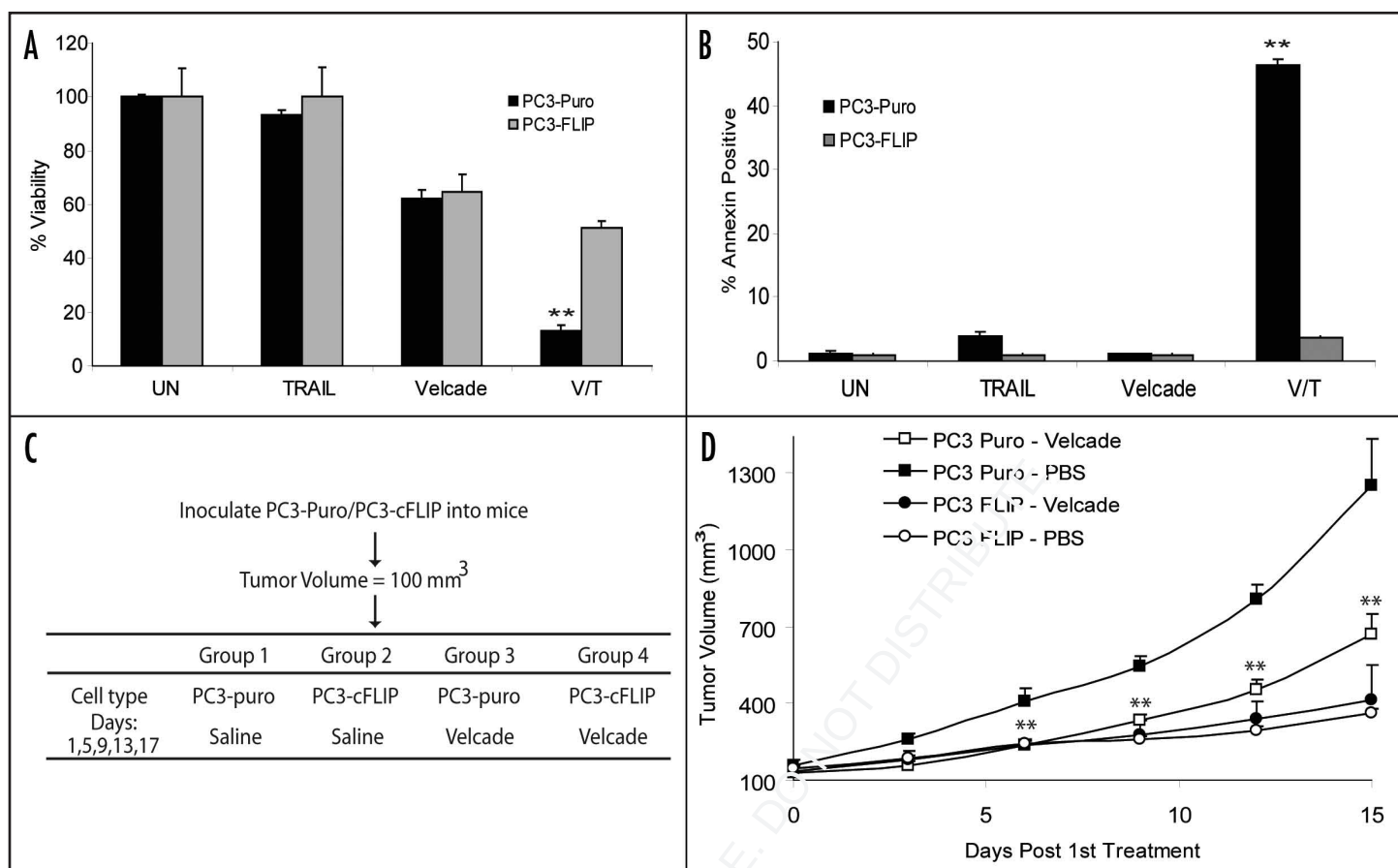


Figure 4. Velcade activity requires a functional death receptor pathway. (A) An MTT assay for cell viability was carried out on PC-3 cells expressing c-FLIP or empty vector, treated with GST-TRAIL alone (20 ng/ml), Velcade (100 nM) alone or combination of the two. ** $p < 0.001$. (B) Annexin staining for apoptosis induction in PC-3 cells expressing c-FLIP or Puro control. Cells were treated as in (A) and subjected to annexin V staining followed by analysis by flow cytometry. ** $p < 0.001$. (C) Study design for PC3-cFLIP tumor xenograft experiment. PC-3 cells harboring an empty vector (PC3-Puro) or stably expressing c-FLIP (PC3-cFLIP) were inoculated into nude mice and tumors were allowed to establish. Once tumor volume reached 100 mm³, mice were injected with PBS or Velcade (1 mg/kg) every four days for five total cycles. (D) Tumor growth curve for PC-3 xenografts. ** $p < 0.001$.

efficacy of Velcade in vivo may be dependent on functioning of the death receptor-mediated apoptotic pathway. We hypothesized that in tumor types in which Velcade alone has efficacy, the anti-tumor effects can be mediated through the extrinsic pathway. PC-3 cells are susceptible to apoptosis mediated by TRAIL and Fas ligand¹¹ and are sensitive to the effects of Velcade as a xenograft.²¹ As a tool to determine the specific contribution of the death receptor apoptotic pathway in the efficacy of Velcade, death receptor resistant PC-3 cells were generated by stable c-FLIP expression. The polyclonal c-FLIP overexpressing PC-3 population and the vector only (pBabe puro) PC-3 cells were tested for their sensitivity to TRAIL in vitro. Control PC3-Puro cells were highly sensitive to the combined Velcade-TRAIL treatment with a marked decrease in viability and apoptosis induction in approximately 50% of cells (Fig. 4A and B). However, PC3 cells overexpressing c-FLIP were resistant to the combined treatment with only 4% of the cell population being annexin V positive.

To determine the contribution of the death receptor pathway in vivo, mice harboring PC3-cFLIP and PC3 vector transduced tumor xenografts were generated and treated with and without Velcade as detailed in Figure 4C. A significant reduction in tumor growth was observed in Velcade treated PC3-Puro mice compared to the PBS treated PC3-Puro control ($p = 0.0003$ at day 12, $p = 0.007$

at day 15) (Fig. 4D). Unexpectedly, the PC3-cFLIP tumors grew more slowly than the vector only transduced cells. To address this, a different death ligand resistant cell line was generated by the overexpression of a dominant negative FADD (dnFADD) construct. PC3 cells stably overexpressing dnFADD showed an increase in viability in response to Velcade and TRAIL, similar to PC3-FLIP cells in vitro (Suppl. Fig. 1B). Next, tumor xenografts were established to assess the sensitivity of death ligand resistant PC3-dnFADD cell to Velcade in vivo. As seen with the PC3-FLIP tumors, PC3-dnFADD tumors grew at a slower rate than PC3-Puro control tumors (Suppl. Fig. 1C). Importantly, while the control PC-3 xenografts were responsive to Velcade, blocking death receptor signaling with either c-FLIP or dnFADD completely conferred resistance to the anti-tumor effects of the drug. Together, these findings suggest that a functional death receptor pathway is critical to the anti-tumor efficacy of Velcade.

Discussion

Here, we demonstrated the ability of Velcade, a proteasome inhibitor clinically used for the treatment of multiple myeloma, to sensitize established prostate tumor xenografts that are normally completely resistant to the activity of death ligands to the anti-tumor effects of TRAIL. Various biochemical and histologic assessments

of the tumors at early and late time points revealed that death receptor activation, apoptosis induction and growth arrest all play a role in the observed efficacy. Another remarkable finding was the loss of the bloody, hemorrhagic phenotype normally observed with LNCaP xenografts. CD31 immunostaining was performed to visualize the vascular morphology as a possible explanation. However, no significant change in CD31 positive cell distribution was noted. Surprisingly, in response to the Velcade and TRAIL regimen a reorganization of the tumor cell architecture from a loosely organized collection of cells, to a tightly compacted arrangement was observed. This arrangement largely excluded the bulk of the direct contact between the cells and blood. These data support a model in which the combined Velcade and TRAIL regimen in tumor xenografts initiates its activity by conferring death receptor activation with subsequent caspase-8 enzymatic induction. This initiator caspase leads to apoptosis within the tumor xenograft. During multiple treatment cycles, the tumor cells eventually reorganize in a manner that largely protects the cells from interacting with the majority of the GST-TRAIL within the serum. At the conclusion of the final treatment cycle, it is unclear if the reduction in cell proliferation is due to the presence of TRAIL and Velcade, or a response to the lack of stimulatory growth factors or other nutrients that no longer freely bathe the tumor. Regardless, it is clear that combining Velcade with TRAIL has a marked anti-tumor response in this xenograft model.

The precise molecular mechanism by which proteasome inhibition acts to elicit its anti-cancer apoptotic activity has been difficult to resolve and is likely multi-factorial. This is largely due to the vast number of cellular pathways regulated by the 26S proteasome. Proteasome inhibition has been reported to involve a wide spectrum of cellular effects, including NF κ B activation,^{7,8} an increase in DR5 expression,^{22,23} decreased c-FLIP expression,²⁴ accumulation of the pro-apoptotic Bik,²⁵ and caspase-3 activation through the intrinsic apoptotic pathway.²⁶ By utilizing a PC-3 cell line which is defective in its death receptor apoptotic pathway, we were able to test the contribution of the extrinsic apoptotic pathway on the efficacy of Velcade in vivo. Based on the findings that PC3-cFLIP and PC3-dnFADD tumors did not respond to Velcade treatment, a functional extrinsic pathway appears to play a critical role in promoting the therapeutic response to proteasome inhibition. Surprisingly, blocking DISC formation slowed tumor growth in vivo, but not in vitro. It remains possible that a decrease in the tumor growth rate affects to some extent the response to Velcade. In the future, using Velcade responsive cell lines that do not exhibit a growth inhibition upon DISC inhibition may resolve this issue. In the tumor microenvironment, the ability of TNF ligands to switch from functional tumor promoters to apoptotic death ligands may provide the necessary death stimulus responsible for the efficacy of Velcade.²⁷

Determining the level of active caspase-8 in vivo has proven to be technically challenging. Here, by detecting the downstream cleavage of a specific caspase-8 substrate, HDAC7,²⁰ it was possible to reveal the activation status of this enzyme in the LNCaP xenograft model. This methodology to assess caspase-8 is advantageous over reporter-linked peptides, which notoriously have non-specific activity associated with their use, especially in tissue extracts.

Although it was possible to successfully demonstrate the utility of TRAIL as a bio-active agent, the use of this recombinant protein

strategy has several shortcomings which have slowed its development as an anti-cancer therapy.²⁸ First, pharmacokinetic studies of recombinant human TRAIL in both primates and mice have shown limited TRAIL half-lives of 30 and four minutes, respectively.^{29,30} A second concern is the antigenicity and potential for targeting the recombinant protein by the immune system. These limitations may be overcome with the recent development of agonistic antibodies for the TRAIL receptors, DR4 and DR5. Activating antibodies for these receptors induce caspase-8 dependent apoptosis in multiple cell lines.³¹ The next logical step in the development of this therapeutic strategy will be determining the efficacy of combining Velcade with DR4 and DR5 activating antibodies. To this end, Shander et al., recently demonstrated that combining Velcade with a TRAIL DR5 activating antibody reduced the metastatic potential of renal and mammary tumor xenografts.³² One advantage of using recombinant TRAIL is that it activates both TRAIL receptors in a biologically relevant manner. It remains to be determined if DR5 activating antibodies used alone or combined with DR4 will reproduce the full physiologic apoptotic potential of TRAIL. These findings suggest that proteasome inhibition may allow therapeutic strategies that activate death receptor signaling in the treatment of several cancer types to reach their full potential.

Materials and Methods

Cell culture. All prostate cancer cell lines were obtained from ATCC (Manassas, VA) and grown in DMEM containing 5% FBS. Velcade (Millenium Pharmaceuticals, Cambridge, MA) was donated by the University of Kentucky, Markey Cancer Center Pharmacy.

Cell viability. Cell viability was measured using a colorimetric MTT assay. Media was removed from the cells and a 1 mg/ml solution of thiazolyl blue tetrazolium bromide (MTT) (Alfa Aesar, Ward Hill, MA) in 1x PBS was added. The samples were read on a spectrophotometer at 570 nm minus 690 nm. All assays were conducted in replicates of four to six.

Western blot analysis. Cell lysates were prepared and Western blot analysis was carried out as previously described.¹⁵ Tumor lysates were prepared by homogenizing excised tumor tissue in extraction buffer (0.1% CHAPS, 25 mM HEPES, 5 mM EDTA, 2 mM DTT). Extracts were flash frozen in liquid nitrogen and stored at -70°C. Antibodies were obtained from the following sources: HDAC7 (Santa Cruz, H-273) and β -actin (Sigma).

Recombinant TRAIL production. The C-terminal region of human TRAIL (NM_003810, amino acids 95–281) was cloned into a prokaryotic GST expression vector pGex4T-3 (Amersham Biosciences, Piscataway, NJ) and verified by sequence analysis. The GST-TRAIL fusion protein was purified by affinity chromatography as described.¹⁶ A PD10 G25 Sephadex gel filtration column (Amersham Biosciences) was used to desalt the protein into 1x PBS. Biological activity of the GST-TRAIL fusion protein was evaluated by combining it with Velcade.

c-FLIP cloning and gene expression. Human c-FLIP (NM_003879) and a dominant negative FADD (AA 117–208; NM_003824) were cloned into a retroviral pBabe puro (Addgene, Cambridge, MA) expression vector and verified by sequence analysis. Retrovirus was generated as previously described¹⁷ and was added to PC-3 cells for 24 hours. PC-3 cells were selected by treatment with 2 μ g/mL of Puromycin (EMD Biosciences, San Diego, CA). Selection

was carried out for one week.

Quantification of fragmented nuclei. LNCaP cells were treated with 100 ng/mL GST-TRAIL, 100 nM Velcade, or the combination for 24 hours. Cells were harvested and fixed in 4% PBS buffered paraformaldehyde. Cells were then stained with Hoechst 33342 dye (20 µg/mL) in 0.5% NP-40.

GST-TRAIL serum concentration determination. Blood samples were taken from mice by saphenous vein puncture¹⁸ over a 48-hour time period. Samples were centrifuged (16,000 xg for 10 min), supernatant was collected and serial dilutions were made. GST-TRAIL concentration was determined using the Quantikine Immunoassay (R&D Systems, Minneapolis, MN). Purified recombinant TRAIL (Chemicon) was used as a standard.

Tumor xenografts. LNCaP cells were trypsinized, washed and resuspended in DMEM. 2×10^6 LNCaP cells in an equal volume of Matrigel (Becton Dickinson) (total volume = 0.2 mL) were subcutaneously injected into the inguinal fat pad of 28 athymic nude mice (7 per group) (Harlan, Indianapolis, IN). Tumor size was measured bi-weekly using a caliper and the volume calculated by the formula $(L \times W^2)/2$. When tumor volume reached 100 mm³, mice were randomly selected into one of four groups and administered PBS, GST (200 µg), GST-TRAIL (200 µg) or Velcade (1 mg/kg) by intraperitoneal (i.p.) injections once every four days for a total of five cycles. Tumor volumes were measured every three days once treatment cycles began. Once tumors reached a volume of 1000 mm³, mice were euthanized and tumors were excised.

PC-3 cells expressing pBabe puro or stably expressing pBabe puro containing c-FLIP were trypsinized, washed and resuspended in DMEM. 12 Athymic nude mice (3 per group) were challenged with 2×10^6 PC-3 cells in 0.2 mL total volume subcutaneously injected into the left flank. Tumor size was measured as described above. When tumor volume reached 100 mm³, i.p. injections of 1x PBS or Velcade (1 mg/kg) were administered once every four days for four total cycles. Tumor volumes were measured every three days once treatment cycles began.

Hematoxylin and eosin staining. Tumors were fixed in neutral buffered formalin and paraffin embedded. Sections were cut (6 µm), mounted on Superfrost Plus slides and dried overnight at room temperature. Re-hydrated sections were stained with Harris' Hematoxylin (EMD) for five min followed by Eosin Y (EMD) (1%) staining for one min. Sections were dehydrated in ethanol followed by a xylene rinse and mounted with permount.

Immunohistochemistry. Excised tumor tissue was embedded in tissue freezing medium (Triangle Biomedical Sciences) and flash frozen in liquid nitrogen. Cryostat sections (6 µm) were mounted on Superfrost Plus slides and stored at -70°C. For bromodeoxyuridine (BrdU) staining, BrdU (100 mg/kg) was i.p. injected into mice two hours prior to euthanasia. Sections were fixed in neutral buffered formalin for 10 min. Slides were washed with 1x PBS and blocked for endogenous peroxidase for 15 min in 1% H₂O₂. Anti-BrdU antibody (Neomarkers) was diluted 1:50 in DAKO antibody diluent (DAKO Corporation) and added to sections for one hour. Slides were washed in TBST and probed with a 1:100 dilution of biotinylated anti-mouse antibody for 30 min. Slides were washed in TBST and enzyme labeled with neutravidin-HRP (1:500) for 15 min followed by TBST wash. DAB (Pierce) was used for detection. Sections were counterstained with hematoxylin, blued in 37 mM ammonia,

dehydrated and mounted in permount. Four representative areas on each slide were imaged and percent BrdU positive nuclei calculated. For TUNEL staining, the TACS apoptosis detection kit was used (Trevigen, Gaithersburg, MD). Briefly, frozen sections were formalin fixed, incubated with Proteinase K for 30 min followed by treatment with 1% H₂O₂ for 5 min. Sections were labeled with TdT enzyme, probed with an anti-BrdU antibody, neutravidin-HRP and detection was performed by DAB. Slides were counterstained, blued, dehydrated and mounted in permount.

Statistical analysis. Differences in BrdU incorporation, cell viability and annexin staining were measured by two-tailed student's t-test. Differences in tumor volume between groups were analyzed by one-way ANOVA.

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/ChristianCBT8-1-Sup.pdf

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