

Research Article

Degrasyn Potentiates the Antitumor Effects of Bortezomib in Mantle Cell Lymphoma Cells *In vitro* and *In vivo*: Therapeutic ImplicationsLan V. Pham¹, Archito T. Tamayo¹, Changping Li¹, William Bornmann², Waldemar Priebe², and Richard J. Ford¹**Abstract**

Mantle cell lymphoma (MCL) is an aggressive histotype of B-cell non-Hodgkin lymphoma that has increased in incidence over the past few decades and is incurable, usually poorly responsive to standard chemotherapy combinations, and associated with poor prognoses. Discovering new therapeutic agents with low toxicity that produce better outcomes in patients with MCL is an ongoing challenge. Recent studies showed that degrasyn, a novel small-molecule inhibitor of the Janus kinase/signal transducer and activation of transcription (JAK/STAT) pathway, exerts antitumor activity in lymphoid tumors by inhibiting key growth and survival signaling (JAK/STAT) pathways. In the present study, we found that treatment of both typical and blastoid-variant MCL cells with degrasyn in combination with bortezomib resulted in synergistic growth inhibition and apoptosis induction *in vitro*. The apoptosis in these cells was correlated with the down-regulation of constitutive NF- κ B and phosphorylated STAT3 activation, leading to the inhibition of c-Myc, cyclin D1, and bcl-2 protein expression and the upregulation of bax protein expression. *In vivo*, degrasyn and bortezomib interacted to synergistically prevent tumor development and prolong survival durations in a xenotransplant severe combined immunodeficient mouse model of MCL. These findings suggest that agents such as degrasyn that can pharmacologically target constitutively expressed NF- κ B and STAT3 in MCL cells may be useful therapeutic agents for MCL when administered together with bortezomib. *Mol Cancer Ther*; 9(7): 2026–36. ©2010 AACR.

Introduction

Mantle cell lymphoma (MCL) is an important histotype of aggressive B-cell non-Hodgkin lymphoma (NHL-B) with the poorest long-term outcome among all NHL-B subtypes, the incidence of which has increased over recent decades in the United States and worldwide (1). MCL is characterized by cell cycle abnormalities, including ATM, p53-mediated cell cycle anomalies, DNA repair, apoptosis, in blastoid-variant MCL (BV-MCL), and decreased expression of cyclin-dependent kinase inhibitors such as p16, p21, and p27^{kip1} (2). Overexpression of the cyclin D1 oncogene and protein are associated with the characteristic nonrandom chromosomal translocation t(11:14)(q13;q32) in MCL as a result of enforced expression by the IgH enhancer on chromosome 14. MCL cases

are classified pathologically into at least two subtypes: (a) typical (classic), the less aggressive, small- to medium-cell histotype, and (b) blastoid (BV-MCL), the more aggressive, large-cell histotype (3, 4). The most common clinical course of MCL consists of continual relapses with a median survival duration in most studies of 3 to 4 years (5). Both subtypes of MCL are currently incurable, but some cases show sensitivity and partial or even complete response to dose-intensified regimens of combination chemotherapy (e.g., hyper-CVAD, R-CHOP-CVAD) with occasional extended periods of remission. Overall, however, both MCL and BV-MCL are associated with poor prognoses and relatively short survival (6, 7). Recent approaches to MCL therapy have included the use of humanized monoclonal antibodies (rituximab), various strategies involving allogeneic and autologous bone marrow transplantation (8, 9), and molecular therapeutic targeting with proteasome inhibitors [bortezomib (Velcade; refs. 10, 11), mammalian target of rapamycin antagonists (12, 13), and newer immunomodulatory agents (lenalidomide; Revlimid; refs. 14, 15)]. However, these therapeutic approaches have failed to produce durable remissions in most patients with MCL. Therefore, the need for additional novel approaches to MCL therapy is clearly needed.

Bortezomib (also known as Velcade; PS-341) is one of the first therapeutic proteasome inhibitors demonstrated to be effective in treating hematologic disorders (16, 17).

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It is approved in the United States for treating relapsed multiple myeloma and MCL (10). Unfortunately, MCL frequently fails to respond to treatment with bortezomib or subsequently becomes refractory to bortezomib (11). Because single-agent therapy is rarely effective against diseases such as MCL, investigating whether the efficacy of bortezomib can be increased by combining the proteasome inhibitor with other novel type of pharmacologic agents may be necessary. Recent studies, both laboratory and clinical, have indicated that the addition of numerous agents, including thalidomide, lenalidomide, arsenic trioxide, and histone deacetylase inhibitors, to bortezomib may be beneficial in treating MCL (18–20).

Recently, WP1066, a novel small-molecular-weight compound, was designed and synthesized by W. Priebe and his coworkers at UT M.D. Anderson. The design of WP1066 and the novel class of Janus kinase/signal transducer and activation of transcription (JAK/STAT) inhibitors to which WP1066 belongs was inspired by the scaffold of the natural product caffeic acid benzyl ester (CABE) and its low potency analog AG490 (originally synthesized as described in ref. 21). WP1066 abilities to suppress the JAK/STAT pathway were documented by suppressing cytokine (e.g., interleukin-6, interleukin-3) activation of STAT3 through downregulating the expression of JAKs upstream from STAT, particularly JAK2 (22–27). Follow-up of design lead optimization of WP1066 led to WP1130, an even more potent inhibitor of both JAK2 and STAT3, possessing additional activities that targeted the degradation of the oncoprotein c-Myc (28). Specifically, degrasyn induces rapid degradation of c-Myc, an oncoprotein found highly expressed in many tumor types that leads to tumor growth inhibition in multiple myeloma and melanoma cells *in vitro* and *in vivo*. Degrasyn was also shown to target Bcr/Abl protein expression that leads to the induction of cell death in chronic myelogenous leukemia cells through inhibition of the transcription factor phosphorylated Stat5 and the src kinase Hck (29).

The effect of the novel small-molecule inhibitor degrasyn and its molecular mechanism(s) targeting MCL has not been described. Because degrasyn and bortezomib target multiple pathways in different cancer cells, we hypothesized that the combination of bortezomib and degrasyn was likely to be an effective therapeutic strategy in treating MCL. In the present study, we evaluated the *in vitro* and *in vivo* activity of the novel small-molecule inhibitor degrasyn in combination with bortezomib in typical MCL and BV-MCL tumor cells.

Materials and Methods

MCL cell lines, primary MCL cells, and normal lymphocytes

The human typical MCL cell lines Mino, DBsp53, Jeko, and Granta and BV-MCL cell lines Z-138 and JMP-1 were previously described (30–34) and maintained in RPMI (Life Technologies) containing 15% FCS (Hyclone). Fresh biopsy- or pheresis-derived MCL cells were obtained

from patient samples stored in the Tissue Procurement and Banking Facility at The University of Texas M. D. Anderson Cancer Center. MCL cells were enriched using sheep RBC resetting followed by the RosetteSep (StemCell Technologies) and contained 98% CD20+ and <1% CD3+ T cells according to flow cytometry. These cells were also cultured in RPMI (Life Technologies) containing 15% FCS (Hyclone). Normal human B lymphocytes were purified from the donors' buffy coats using a human B-cell enrichment cocktail (StemCell Technologies). Purified B cells were activated through incubation for 48 hours with an anti-IgM antibody (3.5 μ g/mL; ICN) or with recombinant human CD154 (1 μ g/mL; Alexis). Peripheral blood mononuclear cells (PBMC) were purified from the donors' buffy coats by Ficoll gradient.

This study was conducted in accordance with the Helsinki protocol and approved by the M. D. Anderson Cancer Center Institutional Review Board. Informed consent was obtained from all patients whose tumor samples were used.

Antibodies, reagents, and materials

The primary antibodies used in our study included STAT3 and phosphorylated STAT3 (pSTAT3; BD Biosciences Pharmingen), bcl-2, bax, cyclin D1, c-Myc, and Oct-1 (Santa Cruz Biotechnology) and an anti- β -actin antibody (Sigma). The secondary antibodies used were peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies (Jackson ImmunoResearch Laboratories). WP1066, WP1129, and degrasyn (WP1130) were synthesized at M. D. Anderson; these compounds were solubilized in DMSO (100 mmol/L) that was further diluted in injectable saline for animal studies. Bortezomib was provided by Millennium.

Gel shift assays and DNA-binding ELISA

Electrophoretic mobility shift assays for nuclear factor NF- κ B DNA binding were done according to procedures previously described (35). The DNA binding activity of STAT3 subunits was analyzed using an ELISA according to the manufacturer's instructions (TransAM STAT Family Transcription Factor Assay kit; Active Motif). Briefly, nuclear extracts were placed in the wells of a 96-well plate that contained an immobilized oligonucleotide carrying a STAT consensus DNA binding site. STAT3 proteins bound to this immobilized oligonucleotide were detected by incubating nuclear extracts with a primary antibody recognizing active STAT3 followed by a horseradish peroxidase-conjugated secondary antibody and were quantified using spectrophotometry at 450 nm with a reference wavelength of 650 nm.

NF- κ B reporter plasmid transfection and luciferase assays

Mino cells were transiently transfected with 5 μ g of the 6 \times NF- κ B-luc reporter plasmid according to a nucleofector protocol from Amaxa Biosystems as previously described (31). After transfection, cells were pooled and

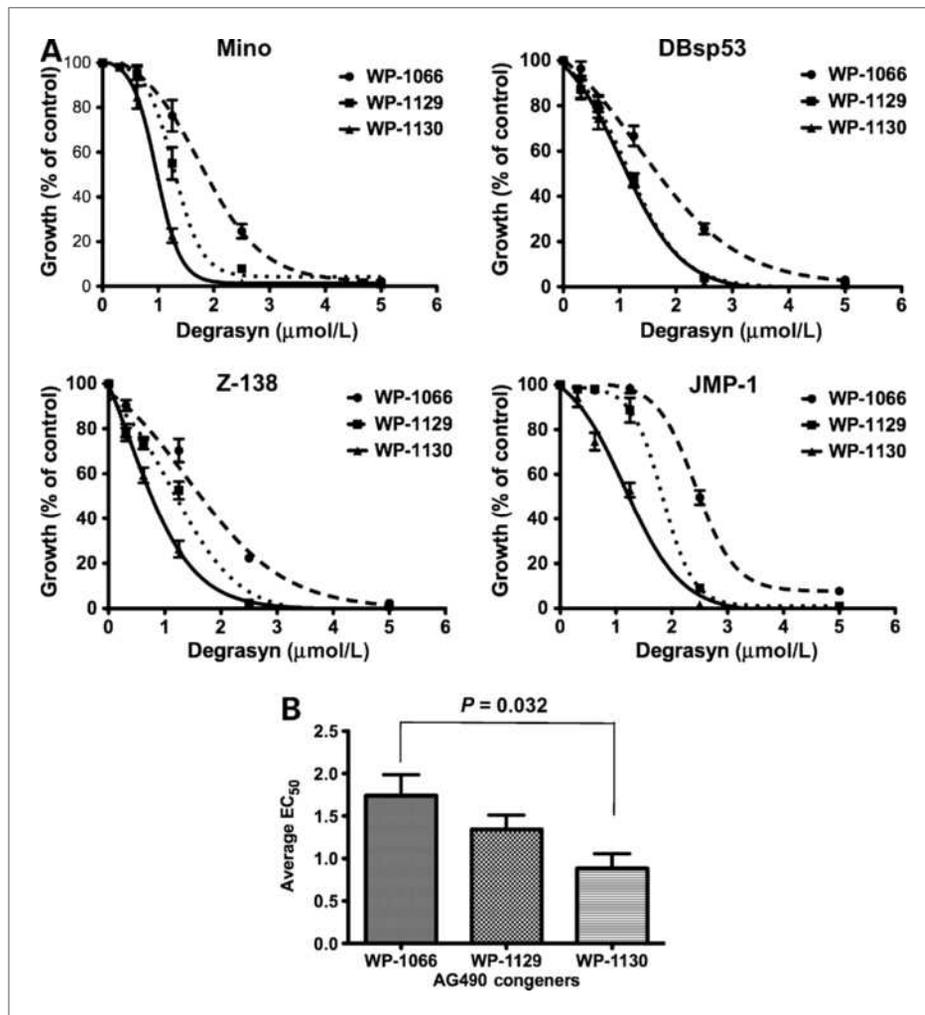


Figure 1. Effect of treatment with AG490 congeners on MCL cell proliferation. Mino, DB, Z-138, and JMP-1 cells were treated with WP1066, WP1129, or degrasyn (WP1130) in a dose-dependent manner for 48 h and then analyzed for cell proliferation using thymidine incorporation assays. Points, means and ranges of triplicate cultures of three independent experiments; bars, SD. B, the EC_{50} of each compound was determined using the GraphPad Prism software program for all four cell lines, and the average EC_{50} for each compound was calculated. Statistical analysis was determined using the Student's *t* test.

separated equally into a 12-well plate. Cells were then treated with specified drug concentrations for 6 and 24 hours. At appropriate time point, cells were harvested and lysed. Whole-cell lysates were used for luciferase assays using the BD Monolight Enhanced Luciferase Assay kit (BD Biosciences) that was normalized according to β -gal activity.

Cell proliferation assays and synergy calculation

In vitro thymidine incorporation proliferation assays were done for cell growth as previously described (25). Briefly, cells were plated (in triplicate) at 4×10^4 cells per well in 200 μ L of RPMI 1640 with 10% FCS and the indicated reagents in a 96-well plate, and incubated in 5% CO_2 at 37°C. After 24 hours, each well was pulsed with 0.5 μ Ci/10 μ L [3H]thymidine (Amersham) for 16 hours. Cells were harvested, and the radioactivity was measured. The CalcuSyn software program (Biosoft) was used to analyze the result of nonconstant ratio drug combination synergy studies. The combination index and isobologram plots for degrasyn and bortezomib were created using the Chou-Talalay method.

Immunoblot analysis

Whole-cell extracts were solubilized in 1% SDS sample buffer and electrophoresed on a 4% to 15% SDS-PAGE gel (Bio-Rad). Proteins were transferred from the gel onto a polyvinylidene difluoride membrane and probed with various specific primary antibodies and appropriate horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized using enhanced chemiluminescence (Amersham).

Apoptosis and caspase-3 assays

MCL cells were washed and stained with Annexin V-FITC and propidium iodide in accordance with the manufacturer's protocol recommendation (BD Pharmingen). Apoptosis in the cells was quantified using a fluorescence-activated cell sorter and the CellQuest software program (BD Biosciences). Caspase activity assays were done using a colorimetric substrate, and a protocol and materials supplied by the manufacturer (EMD Chemicals). In brief, cells were lysed, and 50 μ g of the resulting cell lysates was added to an assay buffer for a total

volume of 90 μL and incubated at 37°C for 10 minutes. A colorimetric substrate for caspase-3 (final concentration, 200 $\mu\text{mol/L}$) was added to the mixture. A_{405} values were recorded for each sample after incubation at 37°C for 2 hours.

RNA isolation and real-time PCR

Total RNA isolation was done by using Trizol LS Reagent (Invitrogen) according to manufactures instructions. Reverse transcription of RNA was carried out with the cDNA archive kit (ABI). Synthesized cDNA was subjected to real-time PCR for the detection of related genes transcripts (CYCLIN D1, C-MYC, 1-OCT, and GAPDH). In brief, 2.5 μL of cDNA was placed in 25 μL of reaction volume containing 12.5 μL of Taqman Universal PCR Master Mix, No AmpErase UNG, 8.75 μL water, and 1.25 μL of primers and probe sets. The primers and probes were purchased from Applied Biosystem (ABI; CYCLIN D1: HS9999904, C-MYC: HS9999903, 1-OCT: HS00427552). Primers and probes were designed to span exon-exon boundaries. Amplification was done in an ABI Fast 7500

Real-time PCR system (Applied Biosciences) using the cycling program: 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds. All samples were analyzed in triplicates. DNA contamination was evaluated by performing PCR on the nonreverse-transcribed control of each sample. The relative expression levels of the genes of interest were normalized to endogenous reference glyceraldehyde-3-phosphate dehydrogenase and relative to control sample as a calibrator using formula: $2^{-\Delta\Delta\text{CT}}$. The threshold cycle reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold.

Animal studies

Three-week-old female severe combined immunodeficient (SCID) mice were purchased from Taconic. The mice were housed five per cage and maintained under specific pathogen-free conditions at the SCID Mouse Barrier Facility at M. D. Anderson. The experimental protocol was reviewed and approved by the M. D. Anderson Institutional Animal Care and Use Committee. Mino cells

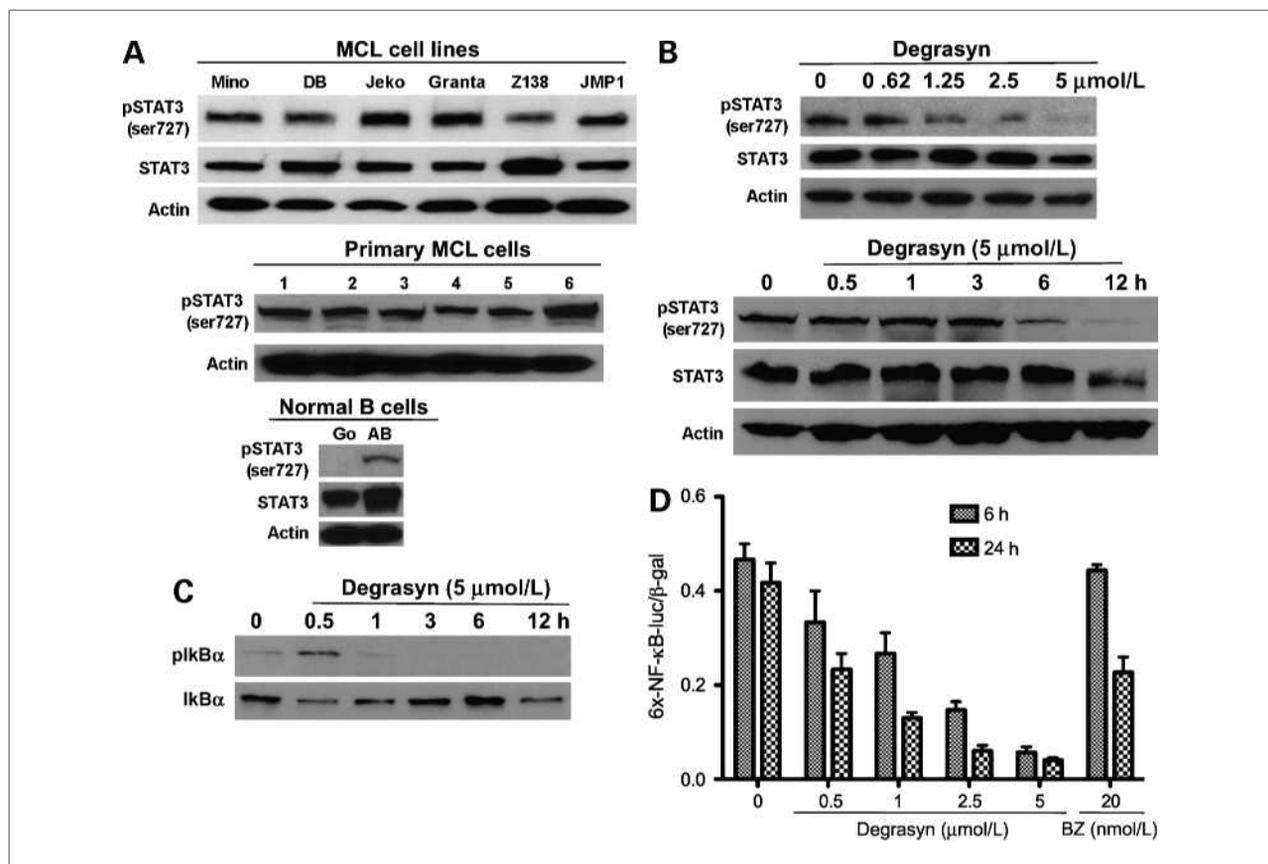


Figure 2. Degrasyn inhibits constitutively activated pSTAT3 and NF- κ B in MCL cells. A, cell extracts purified from six MCL cell lines (top), six primary MCL cells (middle), and control normal (unstimulated) and activated B cells (CD154 plus IgM; bottom) were subjected to Western blotting for STAT3, pSTAT3 (ser-727), and actin (loading control). B, mino MCL cells were treated with dose dependently (0–5 $\mu\text{mol/L}$) with degreasyn for 24 h (top) or time-dependent (0–12 h) with 5 $\mu\text{mol/L}$ of degreasyn (bottom). Cells extracts from the cells were purified and analyzed by Western blot for pSTAT3, STAT3, and Actin. C, Western blots from B were stripped and reblotted for pI κ B α and I κ B α . D, mino cells were cotransfected with the 6xNF- κ B-luc reporter plasmid and β -gal reporter plasmid, and then treated with degreasyn or bortezomib (BZ), as a control, for the indicated times. Luciferase activity was measured and normalized according to β -gal activity. Columns, mean of triplicate samples of three independent experiments; bars, SD.

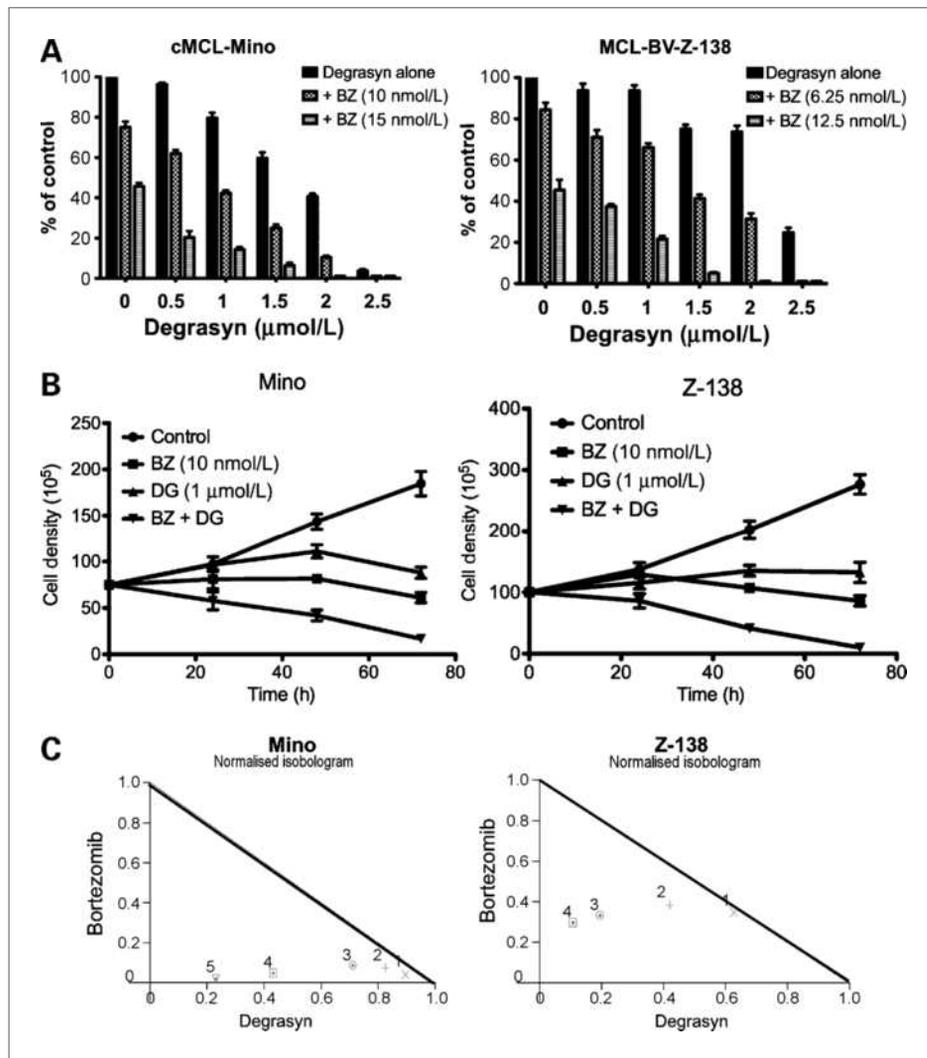


Figure 3. Degrasyn interacts with bortezomib to synergize growth inhibition in MCL cells. **A**, typical MCL (Mino) and BV-MCL (Z-138) cells were treated with a combination of bortezomib and degrassyn in a dose-dependent manner as indicated and were analyzed for cell proliferation using thymidine incorporation assays. A significant cell growth inhibition was observed in degrassyn plus bortezomib treatment compared with single agent alone in both cell lines, Mino [degrassyn alone versus BZ (15 nmol/L); $P = 0.015$] and Z-138 [degrassyn alone versus BZ (12.5 nmol/L); $P = 0.0018$]. Columns, mean of triplicate cultures of three independent experiments; bars, SD. Statistical analysis was determined using the Student's t test. **B**, typical MCL (Mino) and BV-MCL (Z-138) cells were left nontreated or treated with a low dose of degrassyn (DG, 1 μmol/L), bortezomib (BZ, 10 nmol/L), or the combination of both. Cell number was determined by trypan blue exclusion. Columns, mean of triplicate cultures of two independent experiments; bars, SD. **C**, representative normalized isobolograms showing the synergy between bortezomib and degrassyn in typical MCL and BV-MCL cells.

(50×10^6) were injected i.p. into the mice using a 27-gauge needle. One week after inoculation, mice were pooled and randomized into four treatment groups ($n = 10$ /group). Group I received a vehicle control (DMSO) in injection saline (100 μL, twice weekly, i.p.); group II received bortezomib alone (0.25 mg/kg, 100 μL, twice weekly, i.p.); group III received degrassyn alone (20 mg/kg, 100 μL, twice weekly, i.p.); and group IV received a combination of bortezomib (0.25 mg/kg) and degrassyn (20 mg/kg; 100 μL, twice weekly, i.p.). Two additional groups ($n = 5$ /group) that were not inoculated with the tumor cells but received treatment similar to that in groups III and IV were included. Treatment was continued for up to 8 weeks, at which time one mouse in each group was selected at random, euthanized, and subjected to necropsy. The remaining mice were monitored closely and euthanized based on the recommendation of the veterinarians. Three different technicians did the treatment for these mice on different schedules.

Statistical analysis

The IC_{50} s were calculated by using the sigmoidal dose-response curves. Confidence intervals (CI) are shown in between parenthesis. Data from the *in vitro* assays were analyzed using a t test with a robust variance estimate. All significance testing was done at the $P < 0.05$ level. For the animal study, Kaplan-Meier survival curves were created for the mice using the GraphPad Prism software program (version 5.0b; GraphPad Software), and pairwise comparison of survival curves and determination of P values were done using the Log-rank (Mantel-Cox) Test.

Results

Growth inhibition of MCL cell lines with the AG490 congeners

Previous studies in nonlymphoid cells showed that treatment with WP1066, a derivative of the classic JAK2-inhibiting compound AG490, inhibited both JAK2 and STAT3 activation, leading to the inhibition of proliferation

and induction of apoptosis in malignant glioma and melanoma cells (25, 26). Upon further screening and synthesis, we discovered that two additional compounds, WP1129 and degrasyn (WP1130), had superior inhibitory activity

Table 1. Combination index

Bortezomib (nmol/L)	Degrasyn ($\mu\text{mol/L}$)		
	1	1.5	2
Mino			
2	1.17	0.94	0.81
4	1.19	0.91	0.73
6	1.12	0.79	0.61
8	0.91	0.48	0.32
10	0.68	0.25	0.32
Z-138			
2	1.28	1.07	0.59
4	1.11	0.91	0.49
6	1.09	0.74	0.44
8	1.01	0.62	0.41
10	0.95	0.61	0.33
DB			
2	1.21	1.09	0.76
4	1.18	1.03	0.68
6	1.19	0.94	0.63
8	1.11	0.97	0.51
10	0.91	0.71	0.28
JMP-1			
2	1.21	1.07	0.97
4	1.18	1.05	0.86
6	1.15	0.91	0.78
8	1.06	0.89	0.56
10	0.98	0.75	0.45
Combination index	Synergism		
<0.2	very strong		
0.2–0.3	strong		
0.3–0.7	moderate		
0.7–0.85	slight		
0.85–1.0	synergy		
>1.0	no synergy		

NOTE: Combination index <0.2, synergism; 0.2–0.3 very strong; 0.3–0.7, moderate; 0.7–0.85 slight; 0.85–1 synergy; >1.0 no synergy. The drug combination index for bortezomib and degrasyn was calculated by using the CalcuSyn software program. A combination index value of 1 indicated an additive effect of the two drugs. Combination index values of <1 indicated synergy; the lower the value, the stronger the synergy. In contrast, combination index values of >1 indicated antagonism.

Abbreviation: CI, combination index.

in the JAK/STAT pathway when compared with the compound WP1066. We examined the antiproliferative effect of these compounds in MCL cell lines (two typical MCL and two BV-MCL lines). We exposed the four cell lines to each compound at increasing concentrations (0–5 $\mu\text{mol/L}$) and then analyzed them for cell proliferation using thymidine incorporation assays. These studies showed that cell growth suppression was seen in all three compounds for all four cell lines (Fig. 1A), and the half maximal effective concentration (EC_{50}) of degrasyn was lower than that of the other two compounds in all four MCL cell lines (Fig. 1B), suggesting that degrasyn is a more effective anti-tumor agent in MCL cells.

Degrasyn inhibits constitutively activated pSTAT3 and NF- κB in MCL cells

To determine the mechanism of growth suppression by degrasyn, we tested whether degrasyn can target STAT3 and NF- κB activation in MCL cells, as previous studies had shown that both STAT3 and NF- κB were constitutively activated in these cells (31, 36). First, we evaluated the expression of pSTAT3 in MCL cell lines and patient MCL cells by Western blotting and showed that pSTAT3 (serine 727) was constitutively expressed in all of the cell lines and patient MCL cells (Fig. 2A). Constitutive pSTAT3 expression in MCL cells can be downregulated by treatment with degrasyn in a dose- and time-dependent manner in Mino MCL cells (Fig. 2B). Interestingly, degrasyn can also downregulate constitutive cytoplasmic pI $\kappa\text{B}\alpha$ protein level in MCL cells within 3 hours of treatment, suggesting that degrasyn targets upstream of the NF- κB pathway (Fig. 2C). Expression of transcription factor NF- κB was also assayed using a reporter gene assay, in which nuclear expression of NF- κB directed the induction of firefly luciferase activity. Degrasyn treatments resulted in the reduced expression of luciferase activity in a dose- and time-dependent manner (Fig. 2D). These results suggest that degrasyn can directly or indirectly target both STAT3 and NF- κB pathways in MCL cells.

Degrasyn interacts with bortezomib to synergize growth inhibition and apoptosis induction in MCL cells

Bortezomib is a proteasome inhibitor approved for the conventional treatment of MCL. To examine the feasibility of combining degrasyn with bortezomib to treat MCL, we performed *in vitro* and *in vivo* experiments assessing the tolerability and antitumor efficacy of these agents in MCL. First, we evaluated the effects of degrasyn in combination with bortezomib in MCL cells *in vitro*. Figure 3A shows the combined effect of degrasyn and bortezomib in the representative MCL and BV-MCL cell lines Mino and Z-138 on cell growth inhibition. Figure 3B shows that a significant decrease in the cell growth of Mino and Z-138 cell lines was observed in response to treatment with combined low doses of degrasyn (1 $\mu\text{mol/L}$) and bortezomib (10 nmol/L) than with either agent alone, in a time-dependent manner up to 72 hours. We then

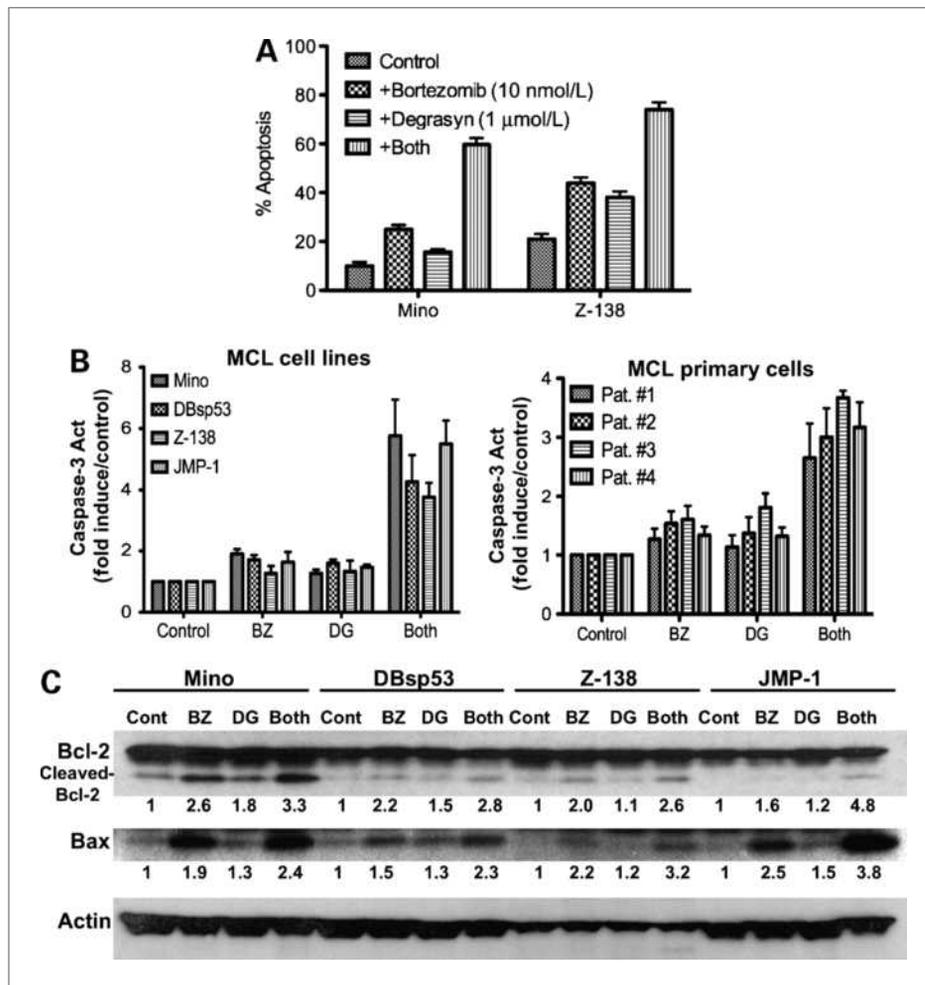


Figure 4. Degrasyn interacts with bortezomib to synergize apoptosis induction. **A**, typical MCL (Mino) and BV-MCL (Z-138) cells were treated with bortezomib alone (10 nmol/L), degrasyn alone (1 μmol/L), or a combination of the two for 48 h and then analyzed for apoptosis using Annexin V assays. Columns, mean of three independent experiments; bars, SD. **B**, MCL cell lines and primary MCL cells were treated with bortezomib (BZ; 10 nmol/L) alone, degrasyn (DG) alone, or a combination of the two for 48 h. Cell extracts were purified and subjected to caspase-3 assays. Columns, mean of three independent experiments; bars, SD. **C**, MCL cells were treated with bortezomib (BZ; 10 nmol/L) alone, degrasyn (DG; 1 μmol/L) alone, or a combination of the two. After 48 h, cell extracts were analyzed for bcl-2 and bax protein expression using Western blotting. Actin was used as a loading control. Cont, control. Numbers below each panel, the relative intensity of each protein band compared with the control sample for each cell line.

evaluated the synergistic effect of the two compounds in four representative MCL cell lines. The concentrations of bortezomib and degrasyn that produced the synergistic effects on cell growth inhibition ranged from 2 to 12 nmol/L and from 0.5 to 2.0 μmol/L, respectively, as shown in a combination index for four representative MCL cell lines (Table 1). Figure 3C shows representative isobologram analysis confirming the synergistic effect of degrasyn and bortezomib in Mino and Z-138 cells. These data show synergistic anti-MCL activity of degrasyn plus bortezomib.

Next, we examined whether degrasyn and bortezomib can interact to synergize apoptosis induction using an Annexin V–based method of early apoptosis detection. At a concentration of 10 nmol/L, bortezomib induced apoptosis in ~15% of Mino cells after 48 hours of treatment, whereas at a concentration of 1 μmol/L, degrasyn induced apoptosis in 5% of Mino cells over the same period. However, the combination of bortezomib and degrasyn administered at similar concentrations induced apoptosis in >50% of MCL of Mino and Z-138 cells (Fig. 4A). These results were similar for both typical MCL and BV-MCL cell lines. In addition, we measured caspase-3 activation,

a signature event during apoptosis, in MCL cell lines and primary MCL tumor cells after 48 hours of treatment with degrasyn and bortezomib alone or in combination. The results showed that degrasyn interacted with bortezomib to activate caspase-3 in all four MCL cell lines (both typical MCL and BV-MCL) and four primary MCL tumor cells [two typical MCL (pat # 1 and 2) and two BV-MCL (pat # 3 and 4); Fig. 4B]. Moreover, the apoptotic effect of degrasyn plus bortezomib was evidenced by the induction of bcl-2 protein cleavage and bax protein expression (Fig. 4C). These results suggested the existence of potential synergy between degrasyn and bortezomib in targeting MCL cells through cell growth inhibition and apoptosis induction.

Degrasyn and bortezomib synergy is associated with NF-κB and pSTAT3 inhibition, concurrent with cyclin D1 and c-Myc downregulation in MCL cells

Next, we sought to determine the molecular basis for the mechanism of degrasyn and bortezomib synergy in MCL cells by examining the expression of key growth and survival genes in MCL cells. In these experiments,

we treated Mino cells with degrasyn and bortezomib alone or in combination for 24 and 48 hours and then examined the STAT3 and NF- κ B activity in the cells. The results showed that the combination of degrasyn and

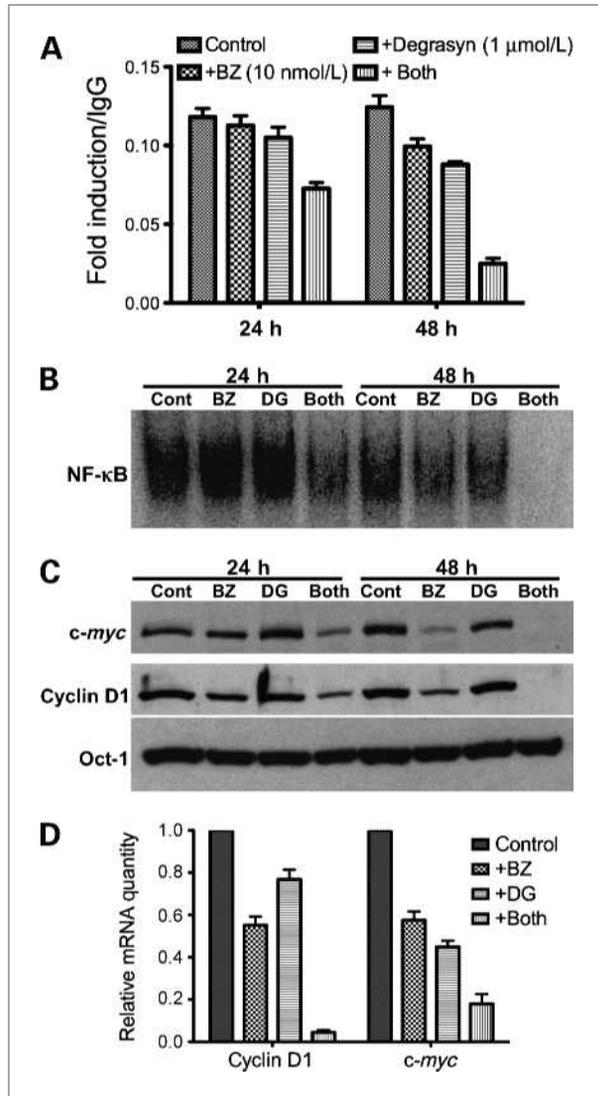


Figure 5. Degrasyn and bortezomib synergy is associated with NF- κ B and STAT3 inhibition, concurrent with the downregulation of the cell growth and survival genes *cyclin D1* and *c-Myc* in MCL cells. A, Mino cells were treated with bortezomib (BZ; 10 nmol/L) alone, degrasyn (1 μ mol/L) alone, or a combination of the two. After 24 and 48 h, nuclear extracts from the cells were purified and subjected to STAT DNA-binding ELISA. Columns, mean of triplicate samples of three independent experiments; bars, SD. B, nuclear extracts from A were also examined by electrophoretic mobility shift assay for NF- κ B DNA binding. Cont, control; BZ, bortezomib; DG, degrasyn. C, nuclear extracts from A were Western blotted for c-Myc, cyclin D1, and Oct-1 (loading control) protein expression. D, Mino cells were treated with bortezomib (10 nmol/L) alone, degrasyn (1 μ mol/L) alone, or a combination of the two. After 24 h, RNA from cells was purified and real-time PCR was performed for *c-myc* and *cyclin D1*. Relative mRNA expressions were normalized with glyceraldehyde-3-phosphate dehydrogenase and Oct-1. Columns, mean of triplicate samples of two independent experiments; bars, SD.

bortezomib effectively downregulated constitutive STAT3 and NF- κ B DNA binding activity after 24 hours; the combination significantly inhibited constitutive STAT3 (Fig. 5A) and NF- κ B (Fig. 5B) DNA binding activity after 48 hours of treatment. We observed the downregulation of the protein expression (Fig. 5C) as well as mRNA expression (Fig. 5D) of *c-Myc* and *cyclin D1*, target genes of STAT3 and NF- κ B, in a similar manner. These results suggested that the combination of degrasyn and bortezomib can target multiple growth and survival signaling pathways as well as targeting genes downstream from these pathways in MCL cells.

Effects of *in vitro* treatment of normal human lymphocytes with degrasyn and bortezomib

To better understand the effects of degrasyn and bortezomib in the normal human B-cell lineage, we also evaluated the cytotoxicity of degrasyn alone and in combination with bortezomib in human PBMCs and activated normal B cells *in vitro*. We exposed purified PBMCs to degrasyn in dose-response studies at increasing concentrations (0–2.5 μ mol/L) alone and with 10 or 15 nmol/L bortezomib. Degrasyn alone had a minimal cytotoxic effect on PBMCs; however, with exposure to the combination of degrasyn and bortezomib, the cytotoxicity of degrasyn and bortezomib in PBMCs increased as the concentration of degrasyn reached >1 μ mol/L (Supplementary Fig. S1A). We activated purified human B cells with recombinant CD40L, anti-IgM antibody, and interleukin-4 for 24 hours and then exposed the proliferating B-cell blasts to degrasyn alone or in combination with bortezomib in a manner similar to that used with PBMCs. Unlike PBMCs, the majority of the activated B cells responded to treatment with degrasyn preferentially alone as well as in combination with bortezomib (Supplementary Fig. S1B), suggesting that degrasyn targets highly proliferative lymphocytes.

Antitumor effects of degrasyn in combination with bortezomib in a xenotransplant SCID mouse model of MCL

To determine whether degrasyn also enhances the antitumor effects of bortezomib in MCL tumor cells *in vivo*, we used a xenotransplant SCID mouse model of MCL. We i.p. injected young SCID mice with Mino MCL cells and randomized the animals into four treatment groups as indicated in Fig. 6A. One week after tumor-cell inoculation, we began administering a low-dose combination of degrasyn (20 mg/kg) and bortezomib (0.25 mg/kg) treatment to the mice (i.p.) twice weekly for ~8 weeks. The dose and schedule for both drugs were well tolerated and effective in other tumor models (29, 37). On day 65, when tumors began to appear, we randomly selected one mouse from each treatment group for sacrifice and necropsy. As shown in Fig. 6B, both bortezomib and degrasyn alone had some individual therapeutic effects in MCL tumor inhibition, whereas treatment with a combination of both drugs resulted in complete disappearance

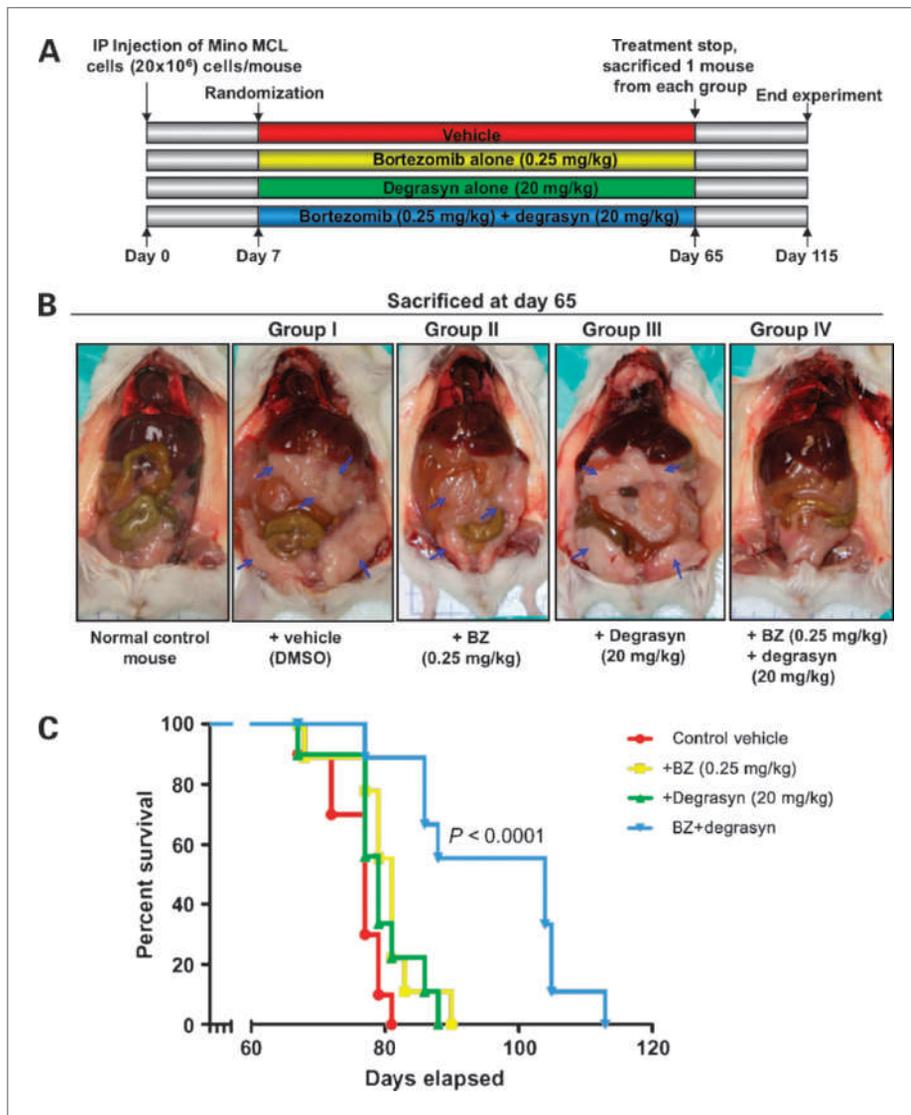


Figure 6. Antitumor effect(s) of degrasyn in combination with bortezomib in a MCL xenotransplant SCID mouse model. A, schematic representation of the experimental protocol for the animal study described in Materials and Methods. Mice received a vehicle control (DMSO; group I), bortezomib alone (0.25 mg/kg; group II), degrasyn alone (20 mg/kg; group III), or bortezomib plus degrasyn (group IV). B, images of representative mice in each group at necropsy (day 65). Arrows, tumors. BZ, bortezomib. C, Kaplan-Meier survival curve showing survival for mice treated with bortezomib, degrasyn, or bortezomib plus degrasyn at the indicated concentrations in the xenotransplant SCID mouse model of MCL ($n = 10$). Bortezomib plus degrasyn-treated mice show significantly increased survival ($P < 0.0001$) compared with the nontreated control group. The mean overall survival was 79 d (95% CI, 65–90) in the nontreated or single agent-treated cohorts versus 104 d (95% CI, 90–120) in the group treated with combination of bortezomib and degrasyn.

of tumors on gross pathologic inspection. We closely monitored the remaining mice and sacrificed them at the development of high tumor burden/morbidity. We created a survival curve showing that mice given both degrasyn and bortezomib survived longer than did mice given either agent alone ($P < 0.0001$; Fig. 6C). Bortezomib or degrasyn alone showed modest prolongation of survival compared with vehicle alone ($P < 0.0213$ and $P < 0.105$, respectively; Fig. 6C). In addition, normal control mice ($n = 5$) given treatment with degrasyn alone or in combination with bortezomib remained alive and showed no signs of body weight loss (data not shown) or organ (bone marrow, lungs, kidneys, and liver) toxicity 6 months later (Supplementary Fig. S2). These results suggested that the combination of degrasyn and bortezomib has potential therapeutic efficacy against MCL and warrants further experimental and clinical investigation.

Discussion

Intracellular signaling pathways that control cellular growth and survival mechanisms are complex, interactive, and often cross-talk with each other in various types of human hematologic/lymphoid tumors (38, 39). Frequently, however, similar or identical pathways (NF- κ B, JAK/STAT, AKT/phosphatidylinositol 3-kinase, etc.) are used and constitutively activated in these tumors (31, 40, 41). Targeting these pathways with new therapeutic agents is likely to provide improved treatment outcomes if adequate agent targeting, molecular specificity, and drug delivery can be achieved. In the present study, we showed that the novel small-molecule compound degrasyn targets multiple signaling pathways in MCL cells, and that treating these cells with degrasyn in combination with the proteasome inhibitor

bortezomib not only synergizes MCL cell growth inhibition and apoptosis induction *in vitro* but also prolongs host survival in a SCID mouse model of MCL *in vivo*.

MCL, particularly BV-MCL, remains a poor responder to current combination chemotherapy among the various histotypes of NHL-B and represents a continuing therapeutic challenge (3). In some patients with MCL however, the disease does respond to treatment, sometimes for extended disease-free durations, with dose-intensification, most recently with combination chemotherapies such as hyper-CVAD and R-CHOP, which are often followed by autologous bone marrow transplantation (2, 7). Our goal in these studies was to continue to develop new, increasingly effective targeted approaches to MCL therapy, particularly for BV-MCL. These studies used experimental therapeutic methodologies, including *in vitro* techniques with representative MCL cell lines and patient tumor samples, as well as more translational *in vivo* studies with xenotransplant SCID mouse models of MCL.

Bortezomib is a boronic acid-derived effective reversible inhibitor of the 26S proteasome that, surprisingly, has had relatively minor toxic effects (e.g., minor platelet count decreases, neuropathy) in initial clinical trials for MCL (17). These studies suggested that bortezomib likely would be more effective in combination with conventional chemotherapy and possibly even more effective with molecularly targeted novel therapeutic agents, particularly small-molecule inhibitors such as degrasyn. Although degrasyn is structurally related to the classic JAK2 inhibitor AG490, when tested against aggressive NHL-B MCL cells *in vitro*, we observed that degrasyn was also an effective inhibitor of constitutive NF- κ B activation present in typical MCL and BV-MCL cells. The molecular mechanism of how degrasyn downregulates NF- κ B activity in MCL cells remains uncertain. However, recent studies have indicated that degrasyn directly targets c-Myc for proteasomal degradation within hours after treatment (28), suggesting that NF- κ B signaling may be a downstream target of c-Myc as previously shown in *Drosophila* flies (42). Another possible mechanism of NF- κ B inhibition exerted by degrasyn occurs through the JAK/STAT signaling pathway, as previous studies have shown that STAT3 maintains constitutive NF- κ B activity in tumor cells by prolonging NF- κ B nuclear retention through acetyltransferase p300-mediated RelA acetylation (41). Therefore, inhibition of STAT3 activity by degrasyn may have affected the status of NF- κ B activity in MCL cells, as seen in the present study. Constitutive STAT3 and NF- κ B signaling and interaction are highly displayed in murine and human cancers, and their roles are central to tumor cell growth and survival (39, 41, 43). Studies have shown that concurrently targeting both constitutive STAT3 and NF- κ B activity in lymphoid tumor cells is effective both *in vivo* and *in vitro* (38, 44). The synergy between degrasyn and bortezomib resulted

in the downregulation of STAT3, and NF- κ B activity in MCL cells may provide a molecular mechanism accounting for their effect in prolonging the survival of MCL xenotransplanted SCID mice treated with these compounds. However, other still unidentified factors may also contribute to the observed synergistic effects of degrasyn and bortezomib on MCL cell growth and survival.

In terms of cell type specificity, degrasyn is more effective in myeloid and lymphoid tumor cells than normal CD34+ hematopoietic precursor cells, dermal fibroblasts, and endothelial cells (29). The present study also showed that degrasyn has minimal cytotoxicity in normal peripheral blood mononuclear lymphocytes but maximal cytotoxicity in targeted highly proliferative B lymphocytes, suggesting that degrasyn is a potential therapeutic drug candidate for aggressive or relapsed/refractory B-cell lymphomas, which very much need effective new therapies. When combined with bortezomib, degrasyn has antitumor activity in SCID mice with MCL but is not toxic in normal healthy mice, making degrasyn a candidate combinatorial therapeutic agent together with bortezomib in MCL therapy.

In summary, we have shown that MCL cells, both typical MCL and BV-MCL, treated with degrasyn in conjunction with bortezomib resulted in synergistic growth inhibition and apoptosis induction *in vitro*. Apoptosis in these cells was correlated with the downregulation of constitutive NF- κ B and pSTAT3 activation, leading to the inhibition of c-Myc and cyclin D, and the upregulation of cleaved-bcl-2 and bax protein expression. *In vivo*, degrasyn and bortezomib combined to synergistically prevent MCL tumor development in a xenotransplant SCID mouse model. Agents such as degrasyn that can pharmacologically target constitutively expressed key growth/survival signaling pathways and cell cycle regulators, including NF- κ B, STAT3, c-Myc, and cyclin D1, in MCL cells may prove to be useful therapeutic agents for MCL when combined with effective proteasome inhibitors such as bortezomib.

Disclosure of Potential Conflicts of Interest

Dr. Waldemar Priebe is the lead inventor in the patent disclosing WP1130 and related analogs and has financial interest in the company that licensed this patent.

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References

- Fisher RI. Mantle-cell lymphoma: classification and therapeutic implications. *Ann Oncol* 1996;7 Suppl 6:S35–9.
- Obrador-Hevia A, Fernandez de Mattos S, Villalonga P, Rodriguez J. Molecular biology of mantle cell lymphoma: from profiling studies to new therapeutic strategies. *Blood Rev* 2009;23:205–16.
- Bernard M, Gressin R, Lefrere F, et al. Blastic variant of mantle cell lymphoma: a rare but highly aggressive subtype. *Leukemia* 2001;15:1785–91.
- Jaffe ES, Harris NL, Diebold J, Muller-Hermelink HK. World Health Organization Classification of lymphomas: a work in progress. *Ann Oncol* 1998;9 Suppl 5:S25–30.
- Bosch F, Lopez-Guillermo A, Campo E, et al. Mantle cell lymphoma: presenting features, response to therapy, and prognostic factors. *Cancer* 1998;82:567–75.
- Fisher RI. Mantle cell lymphoma: at last, some hope for successful innovative treatment strategies. *J Clin Oncol* 2005;23:657–8.
- Schmidt C, Dreyling M. Therapy of mantle cell lymphoma: current standards and future strategies. *Hematol Oncol Clin North Am* 2008;22:953–63, ix.
- Khouri IF, Lee MS, Saliba RM, et al. Nonablative allogeneic stem-cell transplantation for advanced/recurrent mantle-cell lymphoma. *J Clin Oncol* 2003;21:4407–12.
- Maris MB, Sandmaier BM, Storer BE, et al. Allogeneic hematopoietic cell transplantation after fludarabine and 2 Gy total body irradiation for relapsed and refractory mantle cell lymphoma. *Blood* 2004;104:3535–42.
- Fisher RI, Bernstein SH, Kahl BS, et al. Multicenter phase II study of bortezomib in patients with relapsed or refractory mantle cell lymphoma. *J Clin Oncol* 2006;24:4867–74.
- Goy A, Bernstein SH, Kahl BS, et al. Bortezomib in patients with relapsed or refractory mantle cell lymphoma: updated time-to-event analyses of the multicenter phase 2 PINNACLE study. *Ann Oncol* 2009;20:520–5.
- Rizzieri DA, Feldman E, Dipersio JF, et al. A phase 2 clinical trial of deforolimus (AP23573, MK-8669), a novel mammalian target of rapamycin inhibitor, in patients with relapsed or refractory hematologic malignancies. *Clin Cancer Res* 2008;14:2756–62.
- Witzig TE, Geyer SM, Ghobrial I, et al. Phase II trial of single-agent temsirolimus (CCI-779) for relapsed mantle cell lymphoma. *J Clin Oncol* 2005;23:5347–56.
- Habermann TM, Lossos IS, Justice G, et al. Lenalidomide oral monotherapy produces a high response rate in patients with relapsed or refractory mantle cell lymphoma. *Br J Haematol* 2009;145:344–9.
- Tempecul A, Ianotto JC, Morel F, Marion V, De Braekeleer M, Berthou C. Lenalidomide, as a single agent, induces complete remission in a refractory mantle cell lymphoma. *Ann Hematol* 2009;88:921–2.
- Goy A. New directions in the treatment of mantle cell lymphoma: an overview. *Clin Lymphoma Myeloma* 2006;7 Suppl 1:S24–32.
- Suh KS, Goy A. Bortezomib in mantle cell lymphoma. *Future Oncol* 2008;4:149–68.
- O'Connor OA, Czuczman MS. Novel approaches for the treatment of NHL: proteasome inhibition and immune modulation. *Leuk Lymphoma* 2008;49 Suppl 1:59–66.
- Pei XY, Dai Y, Grant S. Synergistic induction of oxidative injury and apoptosis in human multiple myeloma cells by the proteasome inhibitor bortezomib and histone deacetylase inhibitors. *Clin Cancer Res* 2004;10:3839–52.
- Wen J, Feng Y, Huang W, et al. Enhanced antimyeloma cytotoxicity by the combination of arsenic trioxide and bortezomib is further potentiated by p38 MAPK inhibition. *Leuk Res* 2009;34:85–92.
- Levitzi A, Gazit A. Tyrosine kinase inhibition: an approach to drug development. *Science* 1995;267:1782–8.
- Ferrajoli A, Faderl S, Van Q, et al. WP1066 disrupts Janus kinase-2 and induces caspase-dependent apoptosis in acute myelogenous leukemia cells. *Cancer Res* 2007;67:11291–9.
- Hussain SF, Kong LY, Jordan J, et al. A novel small molecule inhibitor of signal transducers and activators of transcription 3 reverses immune tolerance in malignant glioma patients. *Cancer Res* 2007;67:9630–6.
- Iwamaru A, Szymanski S, Iwado E, et al. A novel inhibitor of the STAT3 pathway induces apoptosis in malignant glioma cells both *in vitro* and *in vivo*. *Oncogene* 2007;26:2435–44.
- Kong LY, Abou-Ghazal MK, Wei J, et al. A novel inhibitor of signal transducers and activators of transcription 3 activation is efficacious against established central nervous system melanoma and inhibits regulatory T cells. *Clin Cancer Res* 2008;14:5759–68.
- Kong LY, Wei J, Sharma AK, et al. A novel phosphorylated STAT3 inhibitor enhances T cell cytotoxicity against melanoma through inhibition of regulatory T cells. *Cancer Immunol Immunother* 2009;58:1023–32.
- Verstovsek S, Manshouri T, Quintas-Cardama A, et al. WP1066, a novel JAK2 inhibitor, suppresses proliferation and induces apoptosis in erythroid human cells carrying the JAK2 V617F mutation. *Clin Cancer Res* 2008;14:788–96.
- Bartholomeusz G, Talpaz M, Bornmann W, Kong LY, Donato NJ. Degrasyn activates proteasomal-dependent degradation of c-Myc. *Cancer Res* 2007;67:3912–8.
- Bartholomeusz G, Talpaz M, Kapuria V, et al. Activation of a novel Bcr/Abl destruction pathway by WP1130 induces apoptosis of chronic myelogenous leukemia cells. *Blood* 2007;109:3470–8.
- Fu L, Lin-Lee YC, Pham LV, Tamayo A, Yoshimura L, Ford RJ. Constitutive NF- κ B and NFAT activation leads to stimulation of the BlyS survival pathway in aggressive B-cell lymphomas. *Blood* 2006;107:4540–8.
- Pham LV, Tamayo AT, Yoshimura LC, Lo P, Ford RJ. Inhibition of constitutive NF- κ B activation in mantle cell lymphoma B cells leads to induction of cell cycle arrest and apoptosis. *J Immunol* 2003;171:88–95.
- Amin HM, McDonnell TJ, Medeiros LJ, et al. Characterization of 4 mantle cell lymphoma cell lines. *Arch Pathol Lab Med* 2003;127:424–31.
- Goy A, Remache YK, Gu J, et al. Establishment and characterization of a new mantle cell lymphoma cell line M-1. *Leuk Lymphoma* 2004;45:1255–60.
- Medeiros LJ, Estrov Z, Rassidakis GZ. Z-138 cell line was derived from a patient with blastoid variant mantle cell lymphoma. *Leuk Res* 2006;30:497–501.
- Pham LV, Tamayo AT, Yoshimura LC, et al. A CD40 Signalosome anchored in lipid rafts leads to constitutive activation of NF- κ B and autonomous cell growth in B cell lymphomas. *Immunity* 2002;16:37–50.
- Lai R, Rassidakis GZ, Medeiros LJ, Leventaki V, Keating M, McDonnell TJ. Expression of STAT3 and its phosphorylated forms in mantle cell lymphoma cell lines and tumours. *J Pathol* 2003;199:84–9.
- LeBlanc R, Catley LP, Hideshima T, et al. Proteasome inhibitor PS-341 inhibits human myeloma cell growth *in vivo* and prolongs survival in a murine model. *Cancer Res* 2002;62:4996–5000.
- Lam LT, Wright G, Davis RE, et al. Cooperative signaling through the signal transducer and activator of transcription 3 and nuclear factor- κ B pathways in subtypes of diffuse large B-cell lymphoma. *Blood* 2008;111:3701–13.
- Lee TL, Yeh J, Friedman J, et al. A signal network involving coactivated NF- κ B and STAT3 and altered p53 modulates BAX/BCL-XL expression and promotes cell survival of head and neck squamous cell carcinomas. *Int J Cancer* 2008;122:1987–98.
- Kawauchi K, Ogasawara T, Yasuyama M, Otsuka K, Yamada O. Regulation and Importance of the PI3K/Akt/mTOR Signaling Pathway in Hematologic Malignancies. *Anticancer Agents Med Chem* 2009;9:1024–38.
- Lee H, Hermann A, Deng JH, et al. Persistently activated Stat3 maintains constitutive NF- κ B activity in tumors. *Cancer Cell* 2009;15:283–93.
- Orian A, van Steensel B, Delrow J, et al. Genomic binding by the *Drosophila* Myc, Max, Mad/Mnt transcription factor network. *Genes Dev* 2003;17:1101–14.
- Bharti AC, Shishodia S, Reuben JM, et al. Nuclear factor- κ B and STAT3 are constitutively active in CD138+ cells derived from multiple myeloma patients, and suppression of these transcription factors leads to apoptosis. *Blood* 2004;103:3175–84.
- Mackenzie GG, Queisser N, Wolfson ML, Fraga CG, Adamo AM, Oteiza PI. Curcumin induces cell-arrest and apoptosis in association with the inhibition of constitutively active NF- κ B and STAT3 pathways in Hodgkin's lymphoma cells. *Int J Cancer* 2008;123:56–65.

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Degrasyn Potentiates the Antitumor Effects of Bortezomib in Mantle Cell Lymphoma Cells *In vitro* and *In vivo*: Therapeutic Implications

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