Published in final edited form as:

Anticancer Drugs. 2015 October ; 26(9): 974–983. doi:10.1097/CAD.0000000000000274.

Bortezomib and Fenretinide Induce Synergistic Cytotoxicity in Mantle Cell Lymphoma Via Apoptosis, Cell Cycle Dysregulation, and I κ B α Kinase Down-regulation

Andrew J. Cowan¹, Shani L. Frayo¹, Oliver W. Press^{1,2}, Maria C. Palanca-Wessels³, John M. Pagel^{1,2}, Damian J. Green^{1,2}, and Ajay K. Gopal^{1,2}

¹Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle WA, USA

²Division of Medical Oncology, Department of Medicine, University of Washington, Seattle WA, USA

³Seattle Genetics, Inc, Seattle WA, USA

Abstract

Background—Mantle cell lymphoma (MCL) remains incurable for most patients and proteasome inhibitors like bortezomib induce responses in a minority of patients with relapsed disease. Fenretinide is a retinoid that has shown preclinical activity in B-cell lymphomas. We hypothesized that these agents could yield augmented anti-tumor activity.

Methods—Mantle cell lymphoma lines (Granta-519, Jeko-1, Rec-1) were treated with escalating concentrations of bortezomib and fenretinide singly and in combination. Cytotoxicity was assessed using the MTT assay. Flow cytometric methods assessed apoptosis and necrosis with annexin V-FITC/propidium iodide and G1 and G2 cell cycle changes with DAPI staining. Changes in Cyclin D1, Cyclin B, I κ B α , and IKK α expression were quantified by Western blot.

Results—Cytotoxicity was mediated via apoptosis; both agents showed observed vs expected cytotoxicity in Granta-519 of 92.2% vs 55.1%, in Jeko-1 of 87.6% vs 36.3%, and in Rec-1 of 63.2% vs 29.8%. Isobolographic analysis confirmed synergy in Jeko-1 and Rec-1. Bortezomib induced G2 phase arrest with a 1.7 fold-increase over control, and fenretinide resulted in G1 phase arrest, with an increase of 1.3 fold over control. In combination G2 phase arrest predominated, with a 1.4 fold-increase compared to control, and reduced expression of Cyclin D1 to 24%, Cyclin B to 52% and 64%, Cyclin D3 to 25% and 43%, I κ B α to 23% and 46%, and I κ B α kinase to 34% and 44%.

Corresponding author: Ajay Gopal M.D., Seattle Cancer Care Alliance Mailstop G3-200, 825 Eastlake Avenue E, Seattle WA 98109, agopal@u.washington.edu.

Competing interests: The authors declare that they have no competing interests.

Authors' contributions: AC participated in the design of the study and wrote the manuscript. SF conducted the cytotoxicity and apoptosis assays, synergy analyses, and Western blotting, and also aided in drafting the manuscript. OPW, CPW, JP, DJG and AKG conceived the study, participated in its design and coordination, and edited the manuscript. All authors read and approved the final manuscript.

Conclusions—Bortezomib and fenretinide exhibit synergistic cytotoxicity against MCL cell lines. This activity is mediated by I κ B α kinase modulation, decreased cyclin expression, cell cycle dysregulation, and apoptotic cell death.

Keywords

bortezomib; mantle cell lymphoma; non-Hodgkin lymphoma; fenretinide; retinoic acid derivatives

Background

Mantle cell lymphoma (MCL) is an aggressive disease, representing approximately 6% of all lymphomas in the US, with a median survival of greater than 5 years [1, 2]. The majority of patients with MCL are male and present at a median age of 68 [2, 3]. The t(11;14) (q13;q32) chromosomal translocation is characteristic of MCL and juxtaposes the *BCL1* gene with the immunoglobulin heavy chain gene locus, resulting in overexpression of cyclin D1 [4]. While some patients have a clinically indolent disease, MCL is generally aggressive with most patients demonstrating Stage III or IV disease at diagnosis [2]. Historically, MCL has been associated with a poorer prognosis than many other aggressive lymphomas [2]. Over the last 3 decades, there has been a dramatic improvement in the management of patients with MCL – with the advent of advances in transplantation, targeted novel therapies – and driven by an improved understanding of the molecular biology of MCL. Typically, front-line management of MCL takes a risk-adapted strategy, reserving intensive high-dose therapy followed by autologous stem cell transplantation for younger, fitter patients [2, 5]. The standard approach for elderly patients (defined as greater than 65 years old), is immunochemotherapy with bendamustine and rituximab, or rituximab and Cyclophosphamide, Hydroxydaunorubicin (Doxorubicin), Oncovin (Vincristine), Prednisone (CHOP), followed by maintenance rituximab [2]. Other options include rituximab in combination with bendamustine, chlorambucil, or Cyclophosphamide, Vincristine, Prednisone (CVP) [5]. Despite the efficacy of these regimens, MCL remains an incurable disease. Novel, improved treatments that maximize therapeutic benefits and minimize toxicities are needed.

Proteasome inhibitors (PI) were developed and studied in a wide variety of solid tumors and hematologic malignancies before clinical efficacy was demonstrated in multiple myeloma and mantle cell lymphoma [5]. The proteasome is an important cellular component responsible for degradation of proteins involved with apoptosis and cell cycle regulation [6]. The initial Phase II studies of single-agent bortezomib in MCL documented response rates between 38 and 55 percent, and a median time to progression of 6.2 months [7, 8]. The results from the phase II PINNACLE study were later updated to report an overall response rate of 33%, and a median time to progression of 6.2 months – indicating that bortezomib-induced responses are generally not durable. [9]. Bortezomib - similar to other PIs - inhibits the 20S proteasome, resulting in accumulation of BH3-only proteins, which act to induce apoptosis in cancer cells [10]. Proposed mechanisms by which PIs cause cytotoxicity include production of reactive oxygen species (ROS), upregulation of NOXA, and reduction of autocrine signaling by IL6 and IL10, among others [11-13]. Bortezomib and other proteasome inhibitors, paradoxically, also induce a calpain-mediated degradation of I κ B α ,

resulting in increased nuclear factor- κ B (NF- κ B) activation and diminishing apoptosis [14]. Clinically, bortezomib is active at plasma concentrations up to 0.5 μ mol/L at typical doses [15-17].

Retinoids are analogues of Vitamin A and represent both synthetic and natural compounds which, have been examined extensively in the treatment of human malignancies. The Retinoic acid receptor (RAR) and Retinoid X receptor (RXR) are two classes of receptors that the retinoid compounds are thought to act through – though retinoids also function in the absence of an identified receptor [18, 19]. Following dimerization, they act as ligand-dependent transcription factors, acting on various target genes. One such retinoid compound, N-(4-hydroxyphenyl) retinamide, also known as fenretinide, has been shown to be both anti-proliferative and pro-apoptotic in multiple pre-clinical studies employing both solid tumor and hematologic malignancy cell lines[20-25]. Although relatively weaker in binding to the RAR and RXR receptors compared with other compounds in this class, fenretinide also modulates apoptosis through down regulation of I κ B α kinase (IKK) and NF- κ B gene products[26], modulation of Bcl-2 [27, 28], and caspase activation [29]. Fenretinide has also been studied in Phase I and II human clinical trials in multiple solid tumors [30-38]. In these early-phase studies, plasma concentrations at peak and steady state were documented at 13 μ mol/L and 0.9-10 μ mol/L, respectively [33, 39].

We hypothesized that fenretinide could potentiate the anti-tumor activity of clinically attainable bortezomib concentrations in MCL lines based on a common mechanism of anti-tumor activity – induction of apoptosis – and a shared pathway involving regulation of I κ B α . We demonstrated that the combination of bortezomib and fenretinide revealed synergistic cytotoxic activity in all tested mantle cell lymphoma lines that appears to be mediated by modulation of I κ B α kinase, cell cycle dysregulation through G2 phase arrest, decreased expression of cyclins D1, D3, B, and apoptotic cell death.

Methods

Reagents and antibodies

Bortezomib was obtained from commercial sources (Millennium Pharmaceuticals, Cambridge, MA). Fenretinide was provided by CTEP-NCI. Antibodies raised against IKK α , IKK β , pIKK α / β , NF- κ B p65, pNF- κ B p65, I κ B α , pI κ B α , Cyclin A, Cyclin B1, Cyclin D1, Cyclin D2, Cyclin D3, Cyclin E, Cyclin E2, Cyclin H and β -actin were purchased from Cell Signaling Technology (Danvers, MA).

Cell lines

The human lymphoma cell lines Granta 519, Jeko-1 and Rec-1 were obtained from American Type Culture Collection (Rockville, MD) as part of the Lymphoma Research Foundation Mantle Cell Lymphoma Cell Bank. Granta and Rec-1 were cultured in RPMI 1640 supplemented with 10% FBS and were incubated at 37°C with 5% CO₂. Jeko-1 was cultured in RPMI 1640 supplemented with 20% FBS and were incubated in standard culture conditions.

In vitro studies—Cells were incubated under standard culture conditions for 24 hours at a concentration of 0.5×10^6 cells/mL in complete media containing either diluent, bortezomib, fenretinide, or the combination. Following incubation period cells were transferred to a 96 well plate or centrifuge tubes, and prepared for cytotoxicity, apoptosis, and protein expression. Data presented represent three independent experiments.

Cytotoxicity

Cytotoxicity was determined using the MTT assay as described previously[40]. Briefly: Treated cells were seeded at a concentration of 5×10^5 per mL into a flat bottomed 96 well plate. Thiazolyl Blue Tetrazolium Bromide (Sigma Aldrich, St. Louis, MO) was added to each well to a final concentration of 1mg/mL. After a 2 hour incubation, (37°C, 5% CO₂) 100µl lysis buffer containing 50% DMF, 20% SDS, 2.5% 1N HCl, and 2.5% 80% Acetic acid at pH 4.7 was added. The wells were mixed to dissolve the formazin crystals. The plate was read in a BioTek powerwave XS plate reader at 570nm using Gen5 software.

Synergy analyses

5×10^4 cells were plated in 96 well suspension culture plate. Bortezomib was added at concentrations ranging from 0-10nM. Fenretinide concentrations ranged from 0-15µM. Plates were incubated for 24 hours. The MTT assay performed, percent cytotoxic curves calculated, and Biosoft software used to determine synergy (Biosoft, Cambridge, United Kingdom).

Apoptosis—The FITC Annexin V Apoptosis kit was used from BD Pharmingen according to the directions provided with the kit (BD, San Jose CA, United States). Briefly, 500µL cells were put into 1.5mL centrifuge tubes. The cells were washed twice with PBS and re-suspended in 100µL 1× binding buffer containing 5% annexin V-FITC and 5% propidium iodide. After a 15 minute incubation, 250µL 1× binding buffer was added and the samples were analyzed on the Guava (Hayward, California, USA) minicyte flow cytometer.

Western Blotting—Western blot analyses were performed as previously described [41], with the expression of β-actin used as a loading control. Briefly, cells were washed 2 times in cold PBS and lysed for 30 minutes on ice in a buffer containing 50nM Tris HCl (pH 7.4), 1% NP40, 0.25% Sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride, 1mM sodium fluoride, 1% protease inhibitor cocktail (Sigma Aldrich, St Louis, MO), and 1% Halt Phosphatase (Thermo Scientific, Waltham, MA). At the end of the incubation the cells were centrifuged for 10 minutes at 20,000g, 4°C and the supernatant collected and stored at -80°C until the assay was performed. The supernatant was assayed for total protein using the BCA assay. 35-50µg protein was loaded onto a 12% SDS gel and run for 2 hours at 100v. The gels were transferred to nitrocellulose and proteins were detected using antibodies listed above. The blots were immersed in SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA) and exposed CL-Xposure film (Thermo Scientific) which was processed on a Kodak X-OMAT (Rochester, New York, USA) 2000 Processor. The data were analyzed with ImageJ.

Cell Cycle

Following treatment, culture cells were centrifuged for 5 minutes. The supernatant was removed and 200uL of a 10ug/mL DAPI (Sigma, St. Louis, MO) solution was added and cells were triturated using a 26g needle attached to a 1mL syringe. The cell solution was analyzed using a BD LSR2 with a UV laser. The fraction of the cell population in G1/G0, S, and G2 phases were quantified by applying the Dean-Jett-Fox model from FlowJo (Ashland, Oregon, USA), while the Sub-G1 fraction was determined from the total event count.

Statistical Analysis

All *in vitro* data were collected from a minimum of three independent experiments. An ANOVA was used to determine the statistical significance of differential findings between experimental and control groups in Graph-Pad Prism 3.0 software (Graph-Pad Software, Inc.). The significance level was set at a two-tailed P value of <0.05. Isobologram analyses were done with Biosoft software (Biosoft).

Results

Fenretinide demonstrated cytotoxicity in all tested mantle cell lymphoma lines *in vitro*

We performed an initial set of experiments to determine the degree of cytotoxic activity of fenretinide in Granta MCL cells. Fenretinide concentrations between 1 and 4 μ M were evaluated, and cytotoxicity measured after 24, 48, and 72 hours. Cytotoxicity by MTT assay demonstrated dose and time-dependent cell killing. Fenretinide at 1 μ M demonstrated cytotoxicity of 5.9%, 19.7%, and 43.6%; at 2 μ M cytotoxicity was 7.5%, 25.1%, and 49.0%; at 3 μ M, fenretinide cytotoxicity was 9.7%, 41.4%, and 67.8%; and at 4 μ M, fenretinide showed cytotoxicity of 17.2%, 41.9%, and 71.2%, at 24, 48, and 72 hours, respectively (Figure 1).

Fenretinide and bortezomib demonstrated synergistic cytotoxic activity *in vitro*

In view of the data demonstrating single agent cytotoxic activity, we tested the combination to evaluate potential synergy between fenretinide and bortezomib in MCL cell lines. Fenretinide concentrations were varied between 1 and 15 μ M in combination with bortezomib at concentrations ranging from 3 nM (Granta), 5 nM (Jeko-1), and 10 nM (Rec-1) – based on prior data documenting physiologic, clinically active concentrations of both bortezomib and fenretinide, and reflecting relative resistance of each cell line to bortezomib [15-17, 33, 39]. These data were used to generate standard isobolograms and calculate combination indices to confirm synergy (Table 1 and Figure 2). Combination indices (CIs) in general fell to the left of the line of additivity indicating synergistic anti-tumor effects in all lines ranging from the best CI in the Granta line of 0.81, to the most modest CI of 0.98 in the Jeko-1 cell line. Since CI values less than 1 indicate synergism (with lower ratios indicating a stronger degree of synergism), fenretinide and bortezomib are additive in Rec-1 and Jeko-1 lines and synergistic in Granta (see Figure 2D).

Fenretinide and bortezomib demonstrated cytotoxic activity and induced apoptosis and necrosis in all tested mantle cell lymphoma lines *in vitro*

Subsequent experiments examining the relative contributions of apoptosis and necrosis to the cell killing induced by the combination of bortezomib and fenretinide were designed using the optimal concentrations of the drugs identified in the isobolographic analysis. By MTT assay, bortezomib and fenretinide induced cytotoxic activity as single agents in Granta 519, Jeko-1, and Rec-1 MCL lines *in vitro*. Bortezomib alone at 3 nM (Granta), 5 nM (Jeko-1), and 10 nM (Rec-1) concentrations induced cytotoxicity, with values of 12.8%, 21.5%, and 42.9%, for Granta, Jeko-1, and Rec-1 cells, respectively (Figure 3). Single agent fenretinide yielded increased cytotoxicity in this model at a concentration of 5 μ M (Granta and Rec-1) and 7.5 μ M (Jeko-1) with target cell death rates of 55.1%, 36.3%, and 29.8% for the respective cell lines. We demonstrated supra-additive activity between these agents when co-incubated at the above conditions with combined cytotoxicity of 92.2%, 87.6%, and 63.2% for the respective cell lines (Figure 3).

In view of the marked cytotoxicity seen with both single agent and combination bortezomib and fenretinide, we next evaluated the relative contributions of apoptosis and necrosis to the cell killing observed in the MCL lines, using the Annexin V assay. Apoptosis after treatment with bortezomib was demonstrated in 34.1%, 22.8%, and 27.2% for Granta, Jeko-1, and Rec-1 cells, respectively (Figure 3). Treatment with fenretinide showed a similar trend, with apoptosis occurring in 62.3%, 12.6%, and 19.2% of cell lines, respectively. The combination of fenretinide and bortezomib yielded similar results to single agent treatment, with apoptosis occurring in 72.5%, 25.7%, and 14.3% of cell lines, respectively.

Necrosis after treatment with bortezomib was induced in 10.2%, 21.2%, and 43.3% of Granta, Jeko-1, and Rec-1 cells, respectively (Figure 3). Treatment with fenretinide showed similar levels of necrosis, occurring in 16.6%, 19.4%, and 46.8% of cell lines, respectively. However, the combination of bortezomib and fenretinide showed a marked increase in necrosis in all cell lines except Granta. Necrosis from the combination occurred in 23%, 68.5%, and 71.8% of cell lines, respectively.

The combination of fenretinide and bortezomib decreases expression of cyclin B, cyclin D1, and cyclin D3

Prior data indicate that fenretinide can inhibit tumor necrosis factor-induced cyclin D1 expression [26]. Whether the combination of fenretinide and bortezomib can decrease levels of cyclin D1 further and whether other cell cycle regulators is are also impacted was investigated; the results are depicted in Figure 4, along with images of the Western blot assay used to perform the experiments. Concentrations of bortezomib used to address this question were 3 nM (Granta), 5 nM (Jeko-1), and 10 nM (Rec-1), while fenretinide concentrations were 5 μ M (Granta and Rec-1), and 7.5 μ M (Jeko-1). Bortezomib incubation alone did not change cyclin B1 levels; however, fenretinide reduced the fraction of cyclin B1 to 45% (Granta) and 70% (Jeko-1), and the combination of fenretinide and bortezomib reduction in cyclin B1 fraction to 52% (Granta) and 64% (Jeko-1). Similarly, while bortezomib did not induce a notable change in cyclin D3 levels, fenretinide caused a drop in fraction of cyclin D3 levels to 72% in both Granta and Jeko-1 lines, and the combination

yielded reduction in cyclin D3 levels to 25% (Granta) and 43% (Jeko-1). In the Jeko-1 line, bortezomib reduced cyclin D1 fraction to 59%, fenretinide fraction to 77%, and the combination reduced cyclin D1 fraction to 25%. No notable changes were observed in expression of cyclin A or cyclin H after treatment with single agents or combinations. These results show that the combination of fenretinide and bortezomib decreased the expression of cyclin D1, cyclin D3, and cyclin B1.

The combination of fenretinide and bortezomib decrease expression of I κ B α and IKK *in vitro*

We hypothesized that the combination of bortezomib and fenretinide would modulate both I κ B α and IKK *in vitro*, consistent with previous findings [26]. The concentrations of bortezomib used to test this hypothesis were 3 nM (Granta), 5 nM (Jeko-1), and 10 nM (Rec-1), while fenretinide concentrations were 5 μ M (Granta and Rec-1), and 7.5 μ M (Jeko-1). Bortezomib resulted in a reduction to 50% and 75% of I κ B α in Granta and Jeko-1 cell lines, respectively. The combination of fenretinide and bortezomib resulted in a reduction of I κ B α to 23% in Granta, and a reduction to 46% in Jeko-1. Reductions in IKK α were also seen; the combination of bortezomib and fenretinide reduced the fraction of IKK α to 34% in Granta and 44% in Jeko-1 cell line. We found that the combination resulted in decreases of both I κ B α and IKK (Figure 4).

The combination of fenretinide and bortezomib induced G2 phase arrest and increased proportion of sub-G1 cells

Proceeding from data showing changes in levels of expression of cell cycle modulators described previously, we hypothesized that the combination of both drugs could induce changes in the proportions of cells in different parts of the cell cycle. We tested Jeko-1 cells at 24 and 48 hours with control, bortezomib 5 nM, fenretinide 7.5 μ M, and combination of fenretinide 7.5 μ M and bortezomib 5 nM and analyzed with flow cytometry and DAPI. We verified that fenretinide increased the percentage of cells in G1 phase of the cell cycle, while bortezomib caused G2 arrest. Together, the combination of agents induced predominantly G2 phase arrest. We observed that Jeko-1 cells showed an increase in G1 arrest by 1.3 fold over control when treated with fenretinide, G2 phase cells increased by 1.7 fold when cells were incubated with bortezomib, and the combination of fenretinide and bortezomib resulted in a 1.4 fold increase in cells in G2 phase, but no change in G1 phase cells (Figure 5).

We also showed that the combination of fenretinide and bortezomib increased the fraction of cells in sub-G1 phase at both 24 and 48 hours (Figure 5). At 24 and 48 hours, respectively, control resulted in 5.6% and 5.8% of cells in sub-G1; bortezomib alone resulted in 22.9% and 17.1% of cells in sub-G1; fenretinide alone resulted in 24.8% and 24.68% of cells in sub-G1; the combination of fenretinide and bortezomib resulted in 28.88% and 43% of cells in sub-G1 phase.

Discussion

This study was performed to investigate the impact of fenretinide, bortezomib, and the combination of the two agents on cytotoxicity (apoptosis and necrosis), cell cycle regulation,

and the NF- κ B activation pathway. We found that single agent bortezomib and fenretinide each trigger cytotoxicity *in vitro*, as previously reported, and demonstrate that the combination exhibits synergism in MCL cell lines. This finding was confirmed by isobolographic analysis, performed at concentrations of fenretinide that are physiologically attainable in humans [36]. We confirmed that the cytotoxicity was partially mediated by cell cycle dysregulation, from inhibition of cyclin D1, cyclin D3, and cyclin B1. The degree of cyclin D1 inhibition seen with the combination was of particular interest, in view of the defining role of the t(11;14) translocation in the pathogenesis of MCL. Both necrosis and apoptosis played a role in fenretinide and bortezomib mediated cytotoxicity. Similar to prior studies, bortezomib induced predominantly G2/M phase arrest, while fenretinide produced G1 phase arrest [42-44]. In combination, the two drugs yielded primarily G2 phase arrest, consistent with previous publications [43].

Resistance to cell death, or evasion of apoptosis, is proposed as one of the central mechanisms underlying cancer development [45]. Bortezomib and fenretinide independently influence this pathway. Here, we have demonstrated that in combination, there is impressive synergism in cytotoxicity. One potential explanation for the synergistic effect is shared modulation of the NF- κ B pathway. Bortezomib is thought to act through inhibition of the 26S proteasome, preventing degradation of I κ B α , resulting in decreased activation of NF- κ B. In contrast, fenretinide is thought to act through multiple different mechanisms, including binding to the RAR and RXR receptors (albeit weakly), but also acts on the NF- κ B pathway through suppression of NF- κ B dependent gene expression, inhibition of NF- κ B activation, and inhibition of IKK [26]. Although bortezomib and fenretinide act in concert on the NF- κ B pathway, fenretinide may also potentiate bortezomib through mitochondrial depolarization, which is known to induce apoptosis [18].

Our findings confirm prior publications looking at *in vitro* effects of fenretinide and bortezomib. Here, we sought to further characterize the impact of this combination in MCL, a setting where bortezomib as a single agent is relevant, whereas prior research has used metastatic melanoma and neuroblastoma cell lines [46, 47]. Our finding of synergy between these agents in MCL has been suggested previously through a report that physiologically attainable doses of fenretinide could be used with bortezomib to obtain improved suppression of TNF-induced NF- κ B activation [26]. While the capacity of fenretinide to inhibit tumor necrosis factor-dependent I κ B α degradation has also been demonstrated, we have shown decreased expression of both I κ B α and IKK with combination bortezomib and fenretinide. This is in contrast to prior studies that have suggested the inhibition of I κ B α degradation is partially responsible for the mechanism of fenretinide. One potential explanation for this paradoxical finding is that bortezomib has been shown to induce calpain-mediated degradation of I κ B α , and facilitate p65 nuclear translocation. We have shown that fenretinide reduces expression of IKK, which may to some degree abrogate the effect that bortezomib has on calpain-mediated degradation of I κ B α . Moreover, fenretinide has been shown to inhibit phosphorylation and nuclear translocation of p65, which may be responsible for mitigation of p65 activation seen with bortezomib, and subsequent improvement of its cytotoxic effect [26].

The combination of fenretinide and bortezomib induces impressive synergistic toxicity in MCL lines that is related to different pathways that both agents act on to induce apoptosis in cancer cells. Although the therapeutic arena for non-Hodgkin lymphoma, and in particular, MCL, has expanded rapidly in recent years, this combination appears to have marked activity in MCL and warrants further investigation to provide new strategies for future treatments.

Conclusions

The combination of bortezomib and fenretinide is synergistically cytotoxic against MCL lines. This appears to be mediated by modulation of IKK and $I\kappa B\alpha$, cell cycle dysregulation, and apoptotic cell death. These findings, along with the moderate toxicity profile of bortezomib and fenretinide, support the clinical evaluation of such combinations for the treatment of MCL. The combination of these two agents is promising and warrants further evaluation *in vivo* and in clinical trials.

Acknowledgments

This work was supported by NIH T32 training grant T32CA009515. NIH P01CA044991, K08CA151682 and K24CA184039; Hutchinson Center/University of Washington Cancer Consortium Cancer Center Support Grant P30 CA015704; Lymphoma Research Foundation Mantle Cell Lymphoma Research Initiative, Clinical Scholar Award by the Leukemia and Lymphoma Society to A.K.G. CTEP, CLL Topics; the Mary Wright Memorial Fund; the David and Patricia Giuliani Family Foundation; and philanthropic gifts from Frank and Betty Vandermeer and Don and Debbie Hunkins.

References

1. A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's Lymphoma Classification Project. *Blood*. 1997; 89(11):3909–18. [PubMed: 9166827]
2. Vose JM. Mantle cell lymphoma: 2013 Update on diagnosis, risk-stratification, and clinical management. *American journal of hematology*. 2013; 88(12):1082–8. [PubMed: 24273091]
3. Zhou Y, Wang H, Fang W, Romaguer JE, Zhang Y, Delasalle KB, et al. Incidence trends of mantle cell lymphoma in the United States between 1992 and 2004. *Cancer*. 2008; 113(4):791–8. [PubMed: 18615506]
4. Sola B, Roue G, Duquesne F, Avet-Loiseau H, Macro M, Salaun V, et al. Expression of cyclins D-type in B-chronic lymphoproliferative disorders. *Leukemia*. 2000; 14(7):1318–9. [PubMed: 10914560]
5. Dreyling M, Ferrero S, Hermine O. How I manage mantle cell lymphoma. *Leukemia*. 2014
6. Zavrski I, Naujokat C, Niemoller K, Jakob C, Heider U, Langelotz C, et al. Proteasome inhibitors induce growth inhibition and apoptosis in myeloma cell lines and in human bone marrow myeloma cells irrespective of chromosome 13 deletion. *Journal of cancer research and clinical oncology*. 2003; 129(7):383–91. [PubMed: 12851815]
7. Goy A, Bernstein SH, Kahl BS, Djulbegovic B, Robertson MJ, de Vos S, et al. Bortezomib in patients with relapsed or refractory mantle cell lymphoma: updated time-to-event analyses of the multicenter phase 2 PINNACLE study. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*. 2009; 20(3):520–5. [PubMed: 19074748]
8. O'Connor OA, Wright J, Moskowitz C, Muzzy J, MacGregor-Cortelli B, Stubblefield M, et al. Phase II clinical experience with the novel proteasome inhibitor bortezomib in patients with indolent non-Hodgkin's lymphoma and mantle cell lymphoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2005; 23(4):676–84. [PubMed: 15613699]

9. Fisher RI, Bernstein SH, Kahl BS, Djulbegovic B, Robertson MJ, de Vos S, et al. Multicenter phase II study of bortezomib in patients with relapsed or refractory mantle cell lymphoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2006; 24(30): 4867–74. [PubMed: 17001068]
10. Pham LV, Tamayo AT, Yoshimura LC, Lo P, Ford RJ. Inhibition of constitutive NF-kappa B activation in mantle cell lymphoma B cells leads to induction of cell cycle arrest and apoptosis. *Journal of immunology (Baltimore, Md : 1950)*. 2003; 171(1):88–95.
11. Baran-Marszak F, Boukhiar M, Harel S, Laguillier C, Roger C, Gressin R, et al. Constitutive and B-cell receptor-induced activation of STAT3 are important signaling pathways targeted by bortezomib in leukemic mantle cell lymphoma. *Haematologica*. 2010; 95(11):1865–72. [PubMed: 20663948]
12. Rizzatti EG, Mora-Jensen H, Weniger MA, Gibellini F, Lee E, Daibata M, et al. Noxa mediates bortezomib induced apoptosis in both sensitive and intrinsically resistant mantle cell lymphoma cells and this effect is independent of constitutive activity of the AKT and NF-kappaB pathways. *Leukemia & lymphoma*. 2008; 49(4):798–808. [PubMed: 18398749]
13. Weniger MA, Rizzatti EG, Perez-Galan P, Liu D, Wang Q, Munson PJ, et al. Treatment-induced oxidative stress and cellular antioxidant capacity determine response to bortezomib in mantle cell lymphoma. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2011; 17(15):5101–12. [PubMed: 21712452]
14. Li C, Chen S, Yue P, Deng X, Lonial S, Khuri FR, et al. Proteasome inhibitor PS-341 (bortezomib) induces calpain-dependent IkkappaB(alpha) degradation. *The Journal of biological chemistry*. 2010; 285(21):16096–104. [PubMed: 20335171]
15. Attar EC, De Angelo DJ, Supko JG, D'Amato F, Zahrieh D, Sirulnik A, et al. Phase I and pharmacokinetic study of bortezomib in combination with idarubicin and cytarabine in patients with acute myelogenous leukemia. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2008; 14(5):1446–54. [PubMed: 18316568]
16. Horton TM, Pati D, Plon SE, Thompson PA, Bomgaars LR, Adamson PC, et al. A phase 1 study of the proteasome inhibitor bortezomib in pediatric patients with refractory leukemia: a Children's Oncology Group study. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2007; 13(5):1516–22. [PubMed: 17332297]
17. Ogawa Y, Tobinai K, Ogura M, Ando K, Tsuchiya T, Kobayashi Y, et al. Phase I and II pharmacokinetic and pharmacodynamic study of the proteasome inhibitor bortezomib in Japanese patients with relapsed or refractory multiple myeloma. *Cancer science*. 2008; 99(1):140–4. [PubMed: 17970782]
18. Barna G, Sebestyen A, Weischede S, Petak I, Mihalik R, Formelli F, et al. Different ways to induce apoptosis by fenretinide and all-trans-retinoic acid in human B lymphoma cells. *Anticancer research*. 2005; 25(6b):4179–85. [PubMed: 16309214]
19. Chambon P. A decade of molecular biology of retinoic acid receptors. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 1996; 10(9):940–54. [PubMed: 8801176]
20. Faderl S, Lotan R, Kantarjian HM, Harris D, Van Q, Estrov Z. N-(4-Hydroxyphenyl) retinamide (fenretinide, 4-HPR), a retinoid compound with antileukemic and proapoptotic activity in acute lymphoblastic leukemia (ALL). *Leukemia research*. 2003; 27(3):259–66. [PubMed: 12537979]
21. Formelli F, Cleris L. Synthetic retinoid fenretinide is effective against a human ovarian carcinoma xenograft and potentiates cisplatin activity. *Cancer research*. 1993; 53(22):5374–6. [PubMed: 8221674]
22. Lovat PE, Ranalli M, Annichiarico-Petruzzelli M, Bernassola F, Piacentini M, Malcolm AJ, et al. Effector mechanisms of fenretinide-induced apoptosis in neuroblastoma. *Experimental cell research*. 2000; 260(1):50–60. [PubMed: 11010810]
23. Pergolizzi R, Appierto V, Crosti M, Cavadini E, Cleris L, Guffanti A, et al. Role of retinoic acid receptor overexpression in sensitivity to fenretinide and tumorigenicity of human ovarian carcinoma cells. *International journal of cancer Journal international du cancer*. 1999; 81(5):829–34. [PubMed: 10328240]
24. Shan D, Gopal AK, Press OW. Synergistic effects of the fenretinide (4-HPR) and anti-CD20 monoclonal antibodies on apoptosis induction of malignant human B cells. *Clinical cancer*

- research : an official journal of the American Association for Cancer Research. 2001; 7(8):2490–5. [PubMed: 11489831]
25. Simeone AM, Ekmekcioglu S, Broemeling LD, Grimm EA, Tari AM. A novel mechanism by which N-(4-hydroxyphenyl) retinamide inhibits breast cancer cell growth: the production of nitric oxide. *Molecular cancer therapeutics*. 2002; 1(12):1009–17. [PubMed: 12481423]
 26. Shishodia S, Gutierrez AM, Lotan R, Aggarwal BB. N-(4-hydroxyphenyl) retinamide inhibits invasion, suppresses osteoclastogenesis, and potentiates apoptosis through down-regulation of I(kappa)B(alpha) kinase and nuclear factor-kappaB-regulated gene products. *Cancer research*. 2005; 65(20):9555–65. [PubMed: 16230421]
 27. Boya P, Morales MC, Gonzalez-Polo RA, Andreau K, Gourdier I, Perfettini JL, et al. The chemopreventive agent N-(4-hydroxyphenyl) retinamide induces apoptosis through a mitochondrial pathway regulated by proteins from the Bcl-2 family. *Oncogene*. 2003; 22(40):6220–30. [PubMed: 13679861]
 28. Delia D, Aiello A, Formelli F, Fontanella E, Costa A, Miyashita T, et al. Regulation of apoptosis induced by the retinoid N-(4-hydroxyphenyl) retinamide and effect of deregulated bcl-2. *Blood*. 1995; 85(2):359–67. [PubMed: 7811993]
 29. Gopal AK, Pagel JM, Hedin N, Press OW. Fenretinide enhances rituximab-induced cytotoxicity against B-cell lymphoma xenografts through a caspase-dependent mechanism. *Blood*. 2004; 103(9):3516–20. [PubMed: 14695237]
 30. Cheung E, Pinski J, Dorff T, Groshen S, Quinn DI, Reynolds CP, et al. Oral fenretinide in biochemically recurrent prostate cancer: a California cancer consortium phase II trial. *Clinical genitourinary cancer*. 2009; 7(1):43–50. [PubMed: 19213668]
 31. Kummar S, Gutierrez ME, Maurer BJ, Reynolds CP, Kang M, Singh H, et al. Phase I trial of fenretinide lym-x-sorb oral powder in adults with solid tumors and lymphomas. *Anticancer research*. 2011; 31(3):961–6. [PubMed: 21498721]
 32. Moore MM, Stockler M, Lim R, Mok TS, Millward M, Boyer MJ. A phase II study of fenretinide in patients with hormone refractory prostate cancer: a trial of the Cancer Therapeutics Research Group. *Cancer chemotherapy and pharmacology*. 2010; 66(5):845–50. [PubMed: 20082080]
 33. Otterson GA, Lavelle J, Villalona-Calero MA, Shah M, Wei X, Chan KK, et al. A phase I clinical and pharmacokinetic study of fenretinide combined with paclitaxel and cisplatin for refractory solid tumors. *Investigational new drugs*. 2005; 23(6):555–62. [PubMed: 16034523]
 34. Puduvalli VK, Yung WK, Hess KR, Kuhn JG, Groves MD, Levin VA, et al. Phase II study of fenretinide (NSC 374551) in adults with recurrent malignant gliomas: A North American Brain Tumor Consortium study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2004; 22(21):4282–9. [PubMed: 15514370]
 35. Schneider BJ, Worden FP, Gadgeel SM, Parchment RE, Hodges CM, Zwiebel J, et al. Phase II trial of fenretinide (NSC 374551) in patients with recurrent small cell lung cancer. *Investigational new drugs*. 2009; 27(6):571–8. [PubMed: 19225720]
 36. Vaishampayan U, Heilbrun LK, Parchment RE, Jain V, Zwiebel J, Boinpally RR, et al. Phase II trial of fenretinide in advanced renal carcinoma. *Investigational new drugs*. 2005; 23(2):179–85. [PubMed: 15744595]
 37. Villablanca JG, Krailo MD, Ames MM, Reid JM, Reaman GH, Reynolds CP. Phase I trial of oral fenretinide in children with high-risk solid tumors: a report from the Children's Oncology Group (CCG 09709). *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2006; 24(21):3423–30. [PubMed: 16849757]
 38. Villablanca JG, London WB, Naranjo A, McGrady P, Ames MM, Reid JM, et al. Phase II study of oral capsular 4-hydroxyphenylretinamide (4-HPR/fenretinide) in pediatric patients with refractory or recurrent neuroblastoma: a report from the Children's Oncology Group. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2011; 17(21):6858–66. [PubMed: 21908574]
 39. Garaventa A, Luksch R, Lo Piccolo MS, Cavadini E, Montaldo PG, Pizzitola MR, et al. Phase I trial and pharmacokinetics of fenretinide in children with neuroblastoma. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2003; 9(6):2032–9. [PubMed: 12796365]

40. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *Journal of immunological methods*. 1989; 119(2): 203–10. [PubMed: 2470825]
41. Menzies BE, Kenoyer A. Signal transduction and nuclear responses in *Staphylococcus aureus*-induced expression of human beta-defensin 3 in skin keratinocytes. *Infection and immunity*. 2006; 74(12):6847–54. [PubMed: 16954397]
42. Brignole C, Marimpietri D, Pastorino F, Nico B, Di Paolo D, Cioni M, et al. Effect of bortezomib on human neuroblastoma cell growth, apoptosis, and angiogenesis. *Journal of the National Cancer Institute*. 2006; 98(16):1142–57. [PubMed: 16912267]
43. Hill DS, Martin S, Armstrong JL, Flockhart R, Tonison JJ, Simpson DG, et al. Combining the endoplasmic reticulum stress-inducing agents bortezomib and fenretinide as a novel therapeutic strategy for metastatic melanoma. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2009; 15(4):1192–8. [PubMed: 19228725]
44. Yin D, Zhou H, Kumagai T, Liu G, Ong JM, Black KL, et al. Proteasome inhibitor PS-341 causes cell growth arrest and apoptosis in human glioblastoma multiforme (GBM). *Oncogene*. 2005; 24(3):344–54. [PubMed: 15531918]
45. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011; 144(5):646–74. [PubMed: 21376230]
46. Kim JH, Tanabe T, Chodak GW, Rukstalis DB. In vitro anti-invasive effects of N-(4-hydroxyphenyl)-retinamide on human prostatic adenocarcinoma. *Anticancer research*. 1995; 15(4): 1429–34. [PubMed: 7654032]
47. Pagnan G, Di Paolo D, Carosio R, Pastorino F, Marimpietri D, Brignole C, et al. The combined therapeutic effects of bortezomib and fenretinide on neuroblastoma cells involve endoplasmic reticulum stress response. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2009; 15(4):1199–209. [PubMed: 19228726]

Figure 1. Fenretinide induces a time and dose-dependent cytotoxic response in MCL cells. Granta-519 MCL cells were incubated with fenretinide at 1, 2, 3, and 4 μM ; cytotoxicity was measured via the MTT assay at 24, 48, and 72 hours.

Figure 2.

Fenretinide and bortezomib induce a synergistic cytotoxic response. A-C: Individual data for data used to generate the normalized isobologram analysis; Granta-519 (A), Jeko-1 (B), and Rec-1(C) MCL cell lines were incubated for 24 hours with 0 – 10 nM bortezomib or 0 – 15 μ M fenretinide. Cytotoxicity was measured using the MTT assay. D: Normalized Isobologram showing synergy of bortezomib and fenretinide. This represents 3nM (Granta), 5 nM (Jeko-1) bortezomib, 10nM (Rec-1) and 1-15 μ M fenretinide, X=1uM, +=2uM, ●=5uM, ■=7.5uM, ▼=10 uM, ▲=12.5uM, ►=15uM.

Figure 3.

Combination bortezomib and fenretinide induces cytotoxic response in MCL cells. A: Cytotoxicity was measured with the MTT assay after exposure to bortezomib at 3 nM (Granta), 5 nM (Jeko-1), 10 nM (Rec-1); fenretinide at 5 μ M (Granta and Rec-1), and 7.5 μ M (Jeko-1) B: Apoptosis, cytotoxicity and necrosis were measured with the Annexin V assay after exposure to bortezomib at 3 nM (Granta), 5 nM (Jeko-1), 10 nM (Rec-1); fenretinide at 5 μ M (Granta and Rec-1), and 7.5 μ M (Jeko-1); and the combination for 24 hours.

Figure 4.

Bortezomib and fenretinide reduce expression of cyclin B1, cyclin D1, cyclin D3, IκBα, and IKK. A – D: Cyclin B1, Cyclin D1, Cyclin D3, IκBα, and IKK were measured after exposure to bortezomib at 3 nM (Granta), 5 nM (Jeko-1), 10 nM (Rec-1); fenretinide at 5 μM (Granta and Rec-1), and 7.5 μM (Jeko-1); and the combination for 24 hours.

Figure 5.

Bortezomib and fenretinide induced predominantly G2 phase arrest *in vitro*. A – B: Cell cycle analysis at 24 hours (A) and 48 hours (B) of Jeko-1 MCL cells treated with (starting at upper left corner and going clockwise) either control, 5 nM bortezomib, combination bortezomib at 5 nM with fenretinide 7.5 μ M, and fenretinide alone at 7.5 μ M. Quantifications of proportion of cells in G1, S, and G2 phase are listed for each experiment. The percentage of cells in sub-G1 is depicted in each plot. All studies performed using DAPI staining for DNA content analysis.

Table 1
Combination index values of synergistic activity in mantle cell lymphoma cell lines

Granta			
Fenretinide (μM)	Bortezomib (nM)	Fa^a	Combination Index^b
1	3	0.5172	0.864
2	3	0.7496	0.812
5	3	0.908	0.819
7.5	3	0.9406	0.854
10	3	0.9608	0.849
12.5	3	0.9674	0.901
15	3	0.9723	0.945
Jeko-1			
Fenretinide (μM)	Bortezomib (nM)	Fa	Combination Index
1	5	0.0963	1.285
2	5	0.317	0.983
5	5	0.6007	0.9
7.5	5	0.7233	0.899
10	5	0.7618	0.962
12.5	5	0.7916	1.023
15	5	0.8266	1.062
Rec-1			
Fenretinide (μM)	Bortezomib (nM)	Fa	Combination Index
1	10	0.8041	0.675
2	10	0.317	0.983
5	10	0.6007	0.9
7.5	10	0.8823	0.94
10	10	0.8925	1.018
12.5	10	0.9099	1.008
15	10	0.5062	0.884

^a Fa is the fraction affected by the dose of bortezomib or fenretinide

^b Combination index (CI) values of less than 1 indicate synergy, equal to 1 indicate additive effect, and >1 indicating antagonism. CI values shown are the means.