

Adjuvants of Potential Value in Multiple Myeloma Treatment

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Although survival in multiple myeloma (MM) has lengthened notably in recent years owing to novel chemotherapy regimens involving agents such as liposomal doxorubicin, thalidomide, lenalidomide, and proteasome inhibitors, in the great majority of cases chemoresistance eventually develops to these agents, and long term prognosis remains grim. There is however a growing research literature suggesting that certain adjuvant strategies, involving drugs that are currently available, may have the ability to potentiate response of MM to chemotherapy. Drugs or nutraceuticals which inhibit the NF-kappaB or hedgehog signaling pathways, class I histone deacetylase inhibitors, AMPK activators, inhibitors of autophagy, and the HIV protease inhibitor nelfinavir, appear to have potential in this regard, as borne out by the research abstracts collected here.

NF-kappaB Inhibitors

Both canonical and non-canonical NF-kappaB activity is constitutively elevated in a high proportion of multiple myelomas, and the favorable impact of the interaction of MM cells with bone marrow stromal cells on growth and survival of MM is mediated in large part by inducible NF-kappaB activity. Conversely, a large number of studies show that a variety of agents with inhibit NF-kappaB signaling tend to slow proliferation and up-regulate apoptosis in MM cells, and boost their to chemotherapies. Remarkably, there is recent evidence, that, contrary to expectation, bortezomib and certain other proteasome inhibitors (carfilzomib was not tested), while suppressing non-canonical NF-kappaB activity, actually can enhance canonical activation of NF-kappaB by suppressing levels of IkappaB; this may reflect activation of RIP2, and upstream activator of IKappaB kinase. Concurrent suppression of the canonical pathway with an inhibitor of IKK-beta enhances the cytotoxic impact of bortezomib on MM cell lines. Hence, clinically feasible agents which suppress the canonical pathway of NF-kappaB activation, via IKK-beta inhibition or other mechanisms, have the potential to boost responsiveness to bortezomib and potentially other proteasome inhibitors.

Although many chemical agents that can inhibit IKK-beta show utility pre-clinically in MM, none of these agents have so far achieved drug approval. However, salicylic acid is a clinically available inhibitor of IKK-beta that is now being employed for this purpose in type 2 diabetes, and is now being studied clinically in acute myelogenous leukemia as an NF-kappaB antagonist. Sadly, no studies to date have studied salicylate in MM – but it is reasonable to suspect that, in doses useful for treating rheumatoid arthritis (typically 1.5-2.25 g b.i.d.), it could directly retard MM growth and potentiate response to other chemotherapies for this cancer. Unlike NSAIDS or aspirin, salicylic acid (conveniently administered as the dimer salsalate) produces only a weak and reversible inhibition of COX enzymes that is not clinically significant; hence, salicylate therapy does not induce ulcers or renal damage. Its dose-limiting toxicity is ototoxicity – mild hearing loss, tinnitus – that is fully reversible if the dose is reduced. Most patients tolerate 3 g daily well.

Ni H, Ergin M, Huang Q et al. Analysis of expression of nuclear factor kappa B (NF-kappa B) in multiple myeloma: downregulation of NF-kappa B induces apoptosis. *Br J Haematol*

2001 November;115(2):279-86.

Abstract: Nuclear factor-kappa B (NF-kappa B) is an important transcription factor that regulates survival in many cells. Activated NF-kappa B has been shown to protect some haematopoietic neoplastic cells from apoptosis. In the present study, we analysed NF-kappa B status in 13 primary samples from patients with multiple myeloma (MM) and in four myeloma cell lines including U266, RPMI 8226, HS-Sultan and K620. Constitutive activation of NF-kappa B was evaluated by either immunohistochemistry or immunofluorescence using a monoclonal mouse anti-human p65 (Rel A) antibody, which recognizes the unbound, active form of p65 (Rel A). Constitutively active NF-kappa B was present in all MM patient samples as well as in all four myeloma cell lines. Inhibition of constitutively active NF-kappa B, by either proteasome inhibitors (MG132, gliotoxin) or inhibitors of I kappa B phosphorylation (Bay117082, and Bay117085), induced apoptosis as demonstrated by both flow cytometric analysis and light microscopic morphological evaluation. This chemically induced apoptosis was associated with decreased DNA binding of nuclear NF-kappa B as determined by the electrophoretic mobility shift assay. In addition, adenovirus vector with dominant negative I kappa B alpha (Ad5I kappa B) was used for inhibition of NF-kappa B in the U266 cell line. Compared with wild-type, super-repressor-treated cells showed an increased level of apoptosis. These results suggest that constitutive expression of NF-kappa B plays an important role in plasma cell survival in MM

Hideshima T, Chauhan D, Richardson P et al. NF-kappa B as a therapeutic target in multiple myeloma. *J Biol Chem* 2002 May 10;277(19):16639-47.

Abstract: We have shown that thalidomide (Thal) and its immunomodulatory derivatives (IMiDs), proteasome inhibitor PS-341, and As(2)O(3) act directly on multiple myeloma (MM) cells and in the bone marrow (BM) milieu to overcome drug resistance. Although Thal/IMiDs, PS-341, and As(2)O(3) inhibit nuclear factor (NF)-kappaB activation, they also have multiple and varied other actions. In this study, we therefore specifically address the role of NF-kappaB blockade in mediating anti-MM activity. To characterize the effect of specific NF-kappaB blockade on MM cell growth and survival in vitro, we used an IkappaB kinase (IKK) inhibitor (PS-1145). Our studies demonstrate that PS-1145 and PS-341 block TNFalpha-induced NF-kappaB activation in a dose- and time-dependent fashion in MM cells through inhibition of IkappaBalpha phosphorylation and degradation of IkappaBalpha, respectively. Dexamethasone (Dex), which up-regulates IkappaBalpha protein, enhances blockade of NF-kappaB activation by PS-1145. Moreover, PS-1145 blocks the protective effect of IL-6 against Dex-induced apoptosis. TNFalpha-induced intracellular adhesion molecule (ICAM)-1 expression on both RPMI8226 and MM.1S cells is also inhibited by PS-1145. Moreover, PS-1145 inhibits both IL-6 secretion from BMSCs triggered by MM cell adhesion and proliferation of MM cells adherent to BMSCs. However, in contrast to PS-341, PS-1145 only partially (20-50%) inhibits MM cell proliferation, suggesting that NF-kappaB blockade cannot account for all of the anti-MM activity of PS-341. Importantly, however, TNFalpha induces MM cell toxicity in the presence of PS-1145. These studies demonstrate that specific targeting of NF-kappaB can overcome the growth and survival advantage conferred both by tumor cell binding to BMSCs and cytokine secretion in the BM milieu. Furthermore, they provide the framework for clinical evaluation of novel MM therapies based upon targeting NF-kappaB

Mitsiades N, Mitsiades CS, Poulaki V et al. Biologic sequelae of nuclear factor-kappaB blockade in multiple myeloma: therapeutic applications. *Blood* 2002 June 1;99(11):4079-86.

Abstract: The transcription factor nuclear factor-kappaB (NF-kappaB) confers significant survival potential in a variety of tumors. Several established or novel anti-multiple myeloma (anti-MM) agents, such as dexamethasone, thalidomide, and proteasome inhibitors (PS-341), inhibit NF-kappaB activity as part of their diverse actions. However, studies to date have not delineated the effects of specific inhibition of NF-kappaB activity in MM. We therefore investigated the effect of SN50, a cell-permeable specific inhibitor of NF-kappaB nuclear translocation and activity, on MM cells. SN50 induced apoptosis in MM cell lines and patient cells; down-regulated expression of Bcl-2, A1, X-chromosome-linked inhibitor-of-apoptosis protein (XIAP), cellular inhibitor-of-apoptosis protein 1 (cIAP-1), cIAP-2, and survivin; up-regulated Bax; increased mitochondrial cytochrome c release into the cytoplasm; and activated caspase-9 and caspase-3, but not caspase-8. We have previously demonstrated that tumor necrosis factor-alpha (TNF-alpha) is present locally in the bone marrow microenvironment and induces NF-kappaB-dependent up-regulation of adhesion molecules on both MM cells and bone marrow stromal cells, with resultant increased adhesion. In this study, TNF-alpha alone induced NF-kappaB nuclear translocation, cIAP-1 and cIAP-2 up-regulation, and MM cell proliferation; in contrast, SN50 pretreatment sensitized MM cells to TNF-alpha-induced apoptosis and cleavage of caspase-8 and caspase-3, similar to our previous finding of SN50-induced sensitization to apoptosis induced by the TNF-alpha family member TNF-related apoptosis-inducing ligand (TRAIL)/Apo2L. Moreover, SN50 inhibited TNF-alpha-induced expression of another NF-kappaB target gene, intercellular adhesion molecule-1. Although the p38 inhibitor PD169316 did not directly kill MM cells, it potentiated the apoptotic effect of SN50, suggesting an interaction between the p38 and NF-kappaB pathways. Our results therefore demonstrate that NF-kappaB activity in MM cells promotes tumor-cell survival and protects against apoptotic stimuli. These studies provide the framework for targeting NF-kappaB activity in novel biologically based therapies for MM

Mitsiades CS, Mitsiades N, Poulaki V et al. Activation of NF-kappaB and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. *Oncogene* 2002 August 22;21(37):5673-83.

Abstract: Interleukin-6 (IL-6) and insulin-like growth factor-1 (IGF-1) promote the proliferation of multiple myeloma (MM) cells and protect them against dexamethasone (Dex)-induced apoptosis. We have previously shown that Apo2 ligand/TNF-Related apoptosis inducing ligand (Apo2L/TRAIL) induces apoptosis of MM cells, including cells either sensitive or resistant to Dex and cytotoxic drugs, and overcomes the growth and survival effect of IL-6; conversely, NF-kappaB transcriptional activity attenuates their Apo2L/TRAIL-sensitivity. In the current study, we demonstrate that IGF-1 stimulates sustained activation of NF-kappaB and Akt; induces phosphorylation of the FKHRL-1 Forkhead transcription factor; upregulates a series of intracellular anti-apoptotic proteins including FLIP, survivin, cIAP-2, A1/Bfl-1, and XIAP; and decreases Apo2L/TRAIL-sensitivity of MM cells. In contrast, IL-6 does not cause sustained NF-kappaB activation, induces less pronounced Akt activation and FKHRL-1 phosphorylation than IGF-1, and increases the expression of only survivin. Forced overexpression of constitutively active Akt in MM-1S cells reduced their sensitivity to Apo2L/TRAIL and to doxorubicin (Doxo). In contrast, the Akt inhibitor IL-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate induced cell death of both Dex- and Doxo-sensitive and -resistant cells; opposed the protective effect of constitutive Akt activity against Apo2L/TRAIL; and abrogated the NF-kappaB activation, increase of anti-apoptotic proteins and protection against Apo2L/TRAIL induced by IGF-1.

These findings therefore define an important role of the Akt pathway in modulating tumor cell responsiveness to Apo2L/TRAIL, delineate molecular mechanisms for the survival effects of IGF-1, and characterize differential pathophysiologic sequelae of IGF-1 vs IL-6 on MM cells. Importantly, they provide the basis for future clinical trials in MM combining conventional or novel agents with strategies designed to neutralize IGF-1

Dai Y, Pei XY, Rahmani M, Conrad DH, Dent P, Grant S. Interruption of the NF-kappaB pathway by Bay 11-7082 promotes UCN-01-mediated mitochondrial dysfunction and apoptosis in human multiple myeloma cells. *Blood* 2004 April 1;103(7):2761-70.

Abstract: Interactions between pharmacologic NF-kappaB inhibitors (eg, Bay 11-7082, SN-50) and the checkpoint abrogator UCN-01 have been examined in human multiple myeloma (MM) cells. Exposure of U266 cells to Bay 11-7082 (Bay) in combination with UCN-01 resulted in the abrogation of NF-kappaB/DNA binding activity and the synergistic induction of apoptosis. Comparable synergism was observed in other MM cell lines and patient-derived CD138+ cells and between an inhibitory peptide of NF-kappaB (SN50) and UCN-01. Bay/UCN-01-mediated lethality involved mitochondrial dysfunction, caspase cleavage, and poly adenosine diphosphate-ribose polymerase (PARP) degradation. Although Bay modestly blocked UCN-01-induced extracellular signal-regulated kinase (ERK) phosphorylation, coadministration activated c-Jun N-terminal kinase (JNK) and cdc2/cdk1 and down-regulated Mcl-1, XIAP, and Bcl-xL. Transfection with a constitutively activated mitogen-activated protein kinase kinase (MEK1)/green fluorescent protein (GFP) construct failed to block apoptosis induced by Bay/UCN-01 but significantly attenuated MEK inhibitor (U0126)/UCN-01-induced lethality. Inhibiting JNK activation with SP600125 or D-JNKI1 peptide markedly reduced Bay/UCN-01-mediated mitochondrial dysfunction and apoptosis and the down-regulation of Mcl-1, XIAP, and Bcl-xL but not of cdc2/cdk1 activation. Stable transfection of cells with dominant-negative caspase-9 dramatically diminished Bay/UCN-01 lethality without altering JNK or cdc2/cdk1 activation. Neither interleukin-6 (IL-6)- nor fibronectin-mediated adherence conferred resistance to Bay/UCN-01-induced apoptosis. Together, these findings suggest that a strategy combining UCN-01 with disruption of the IkappaB kinase (IKK)/IkappaB/NF-kappaB pathway warrants attention in MM

Watanabe M, Dewan MZ, Okamura T et al. A novel NF-kappaB inhibitor DHMEQ selectively targets constitutive NF-kappaB activity and induces apoptosis of multiple myeloma cells in vitro and in vivo. *Int J Cancer* 2005 March 10;114(1):32-8.

Abstract: Multiple myeloma (MM) is a fatal lymphoid malignancy that is incurable with conventional modalities of chemotherapy. Strong and constitutive activation of nuclear factor kappa B (NF-kappaB) is a common characteristic of MM cells. In our study we successfully target NF-kappaB with a novel NF-kappaB inhibitor dehydroxymethylepoxyquinomycin (DHMEQ). DHMEQ completely abrogates constitutive NF-kappaB activity and induces apoptosis of MM cells, whereas control peripheral blood mononuclear cells (PBMC) are resistant to NF-kappaB inhibition and apoptosis by DHMEQ treatment. DHMEQ inhibition of NF-kappaB triggers activation of caspases 8 and 9, as well as G0/G1 cell cycle arrest accompanied by downregulation of antiapoptotic genes Bcl-XL and c-FLIP and cell cycle progression gene cyclins D1 and D2. DHMEQ-mediated inhibition of vascular endothelial growth factor (VEGF) production in MM cells raises the possibility that DHMEQ abrogates the autocrine VEGF loop and enhances its antitumor effects by inhibiting neovascularization in the bone marrow. Using an in vivo NOD/SCID/gammac(null) (NOG) mice model, we show that DHMEQ has a potent inhibitory effect on the growth of MM cells. Compared to other compounds having the potential to inhibit NF-kappaB, DHMEQ is a unique compound

that blocks the translocation of NF-kappaB p65 into the nucleus and selectively targets NF-kappaB activated in tumor cells. Therefore, our study presents a new molecular target therapy in MM

Chiang MY, Stadtmauer EA. NF-kappaB, IL-6 and myeloma cell growth: making the connection. *Cancer Biol Ther* 2004 October;3(10):1018-20.

Sanda T, Iida S, Ogura H et al. Growth inhibition of multiple myeloma cells by a novel IkappaB kinase inhibitor. *Clin Cancer Res* 2005 March 1;11(5):1974-82.

Abstract: Involvement of nuclear factor-kappaB (NF-kappaB) in cell survival and proliferation of multiple myeloma has been well established. In this study we observed that NF-kappaB is constitutively activated in all human myeloma cell lines, thus confirming the previous studies. In addition, we found the phosphorylation of p65 subunit of NF-kappaB in addition to the phosphorylation of IkappaBalpha and the activation of NF-kappaB DNA binding and that various target genes of NF-kappaB including bcl-x(L), XIAP, c-IAP1, cyclin D1, and IL-6 are up-regulated. We then examined the effect of a novel IkappaB kinase inhibitor, 2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-yl nicotinonitrile (AHP). When myeloma cells were treated with AHP, the cell growth was efficiently inhibited with IC(50) values ranging from 18 to 35 mumol/L concomitantly with inhibition of the phosphorylation of IkappaBalpha/p65 and NF-kappaB DNA-binding, down-regulation of the NF-kappaB target genes, and induction of apoptosis. In addition, we observed the treatment of AHP augmented the cytotoxic effects of vincristine and melphalan (1-phenylalanine mustard), conventional antimyeloma drugs. These findings indicate that IkappaB kinase inhibitors such as AHP can sensitize myeloma cells to the cytotoxic effects of chemotherapeutic agents by blocking the antiapoptotic nature of myeloma cells endowed by the constitutive activation of NF-kappaB

Feinman R, Siegel DS, Berenson J. Regulation of NF-kB in multiple myeloma: therapeutic implications. *Clin Adv Hematol Oncol* 2004 March;2(3):162-6.

Abstract: The nuclear factor kappa B (NF-kappaB) family of transcription factors plays a major role in inflammation, immune and stress responses, oncogenesis, cell migration, and angiogenesis. Aberrant activation of NF-kappaB has also been shown to contribute to intrinsic and inducible drug resistance in numerous cancers, including multiple myeloma. The expression of NF-kappaB-responsive targets will vary depending on the cellular context and type of inducer. The regulation of NF-kappaB activity occurs at multiple levels involving the IkappaB kinase (IKK) complex, members of the IkappaB family, recruitment of heterologous transcription factors and coactivators by NF-kappaB, and post-translational modifications of p65. This article highlights regulatory mechanisms responsible for constitutive NF-kappaB activation and provides justification for target-based therapy for NF-kappaB in multiple myeloma

Hideshima T, Neri P, Tassone P et al. MLN120B, a novel IkappaB kinase beta inhibitor, blocks multiple myeloma cell growth in vitro and in vivo. *Clin Cancer Res* 2006 October 1;12(19):5887-94.

Abstract: PURPOSE: The purpose of this study is to delineate the biological significance of IkappaB kinase (IKK) beta inhibition in multiple myeloma cells in the context of bone marrow stromal cells (BMSC) using a novel IKKbeta inhibitor MLN120B.

EXPERIMENTAL DESIGN: Growth-inhibitory effect of MLN120B in multiple myeloma cells in the presence of cytokines [interleukin-6 (IL-6) and insulin-like growth factor-I (IGF-1)], conventional agents (dexamethasone, melphalan, and doxorubicin), or BMSC was assessed in vitro. In vivo anti-multiple myeloma activity of MLN120B was evaluated in severe combined immunodeficient (SCID)-hu model. **RESULTS:** MLN120B inhibits both baseline and tumor necrosis factor-alpha-induced nuclear factor-kappaB activation, associated with down-regulation of IkappaBalpha and p65 nuclear factor-kappaB phosphorylation. MLN120B triggers 25% to 90% growth inhibition in a dose-dependent fashion in multiple myeloma cell lines and significantly augments tumor necrosis factor-alpha-induced cytotoxicity in MM.1S cells. MLN120B augments growth inhibition triggered by doxorubicin and melphalan in both RPMI 8226 and IL-6-dependent INA6 cell lines. Neither IL-6 nor IGF-1 overcomes the growth-inhibitory effect of MLN120B. MLN120B inhibits constitutive IL-6 secretion by BMSCs by 70% to 80% without affecting viability. Importantly, MLN120B almost completely blocks stimulation of MM.1S, U266, and INA6 cell growth, as well as IL-6 secretion from BMSCs, induced by multiple myeloma cell adherence to BMSCs. MLN120B overcomes the protective effect of BMSCs against conventional (dexamethasone) therapy. **CONCLUSIONS:** Our data show that the novel IKKbeta inhibitor MLN120B induces growth inhibition of multiple myeloma cells in SCID-hu mouse model. These studies provide the framework for clinical evaluation of MLN120B, alone and in combined therapies, trials of these novel agents to improve patient outcome in multiple myeloma

Jourdan M, Moreaux J, Vos JD et al. Targeting NF-kappaB pathway with an IKK2 inhibitor induces inhibition of multiple myeloma cell growth. *Br J Haematol* 2007 July;138(2):160-8.

Abstract: The pathophysiologic basis for multiple myeloma (MM) has been attributed to the dysregulation of various paracrine or autocrine growth factor loops and to perturbations in several signal transduction pathways including IkappaB kinase/nuclear factor-kappaB (IKK/NF-kappaB). The present study aimed at investigating the effect of a pharmaceutical IKK2 inhibitor, the anilinopyrimidine derivative AS602868, on the in vitro growth of 14 human MM cell lines (HMCL) and primary cells from 13 patients. AS602868 induced a clear dose-dependent inhibition of MM cell growth on both HMCL and primary MM cells, which was the result of a simultaneous induction of apoptosis and inhibition of the cell cycle progression. Combination of AS602868 with suboptimal doses of melphalan or Velcade showed an additive effect in growth inhibition of HMCL. AS602868 also induced apoptosis of primary myeloma cells. Importantly, AS602868 did not alter the survival of other bone marrow mononuclear cells (CD138(-)) co-cultured with primary MM (CD138(+)) cells, except for CD34(+) haematopoietic stem cells. The results demonstrate the important role of NF-kappaB in maintaining the survival of MM cells and suggest that a pharmacological inhibition of the NF-kappaB pathway by the IKK2 inhibitor AS602868 can efficiently kill HMCL and primary myeloma cells and therefore might represent an innovative approach for treating MM patients

Gilmore TD. Multiple myeloma: lusting for NF-kappaB. *Cancer Cell* 2007 August;12(2):95-7.

Abstract: Multiple myeloma (MM) is a late-stage B cell malignancy that has received much attention recently because of its therapeutic susceptibility to proteasome inhibitors. Two papers in this issue of *Cancer Cell* show that primary MM samples and MM cell lines frequently have mutations in genes encoding regulators and effectors of NF-kappaB signaling, and that these mutations lead to chronic NF-kappaB target gene expression, which

is required for the viability of these MM tumor cells. These results reveal the molecular basis for constitutive NF-kappaB activity in many MMs and further validate the NF-kappaB signaling pathway as an appropriate target for MM therapy

Annunziata CM, Davis RE, Demchenko Y et al. Frequent engagement of the classical and alternative NF-kappaB pathways by diverse genetic abnormalities in multiple myeloma. *Cancer Cell* 2007 August;12(2):115-30.

Abstract: Mechanisms of constitutive NF-kappaB signaling in multiple myeloma are unknown. An inhibitor of IkappaB kinase beta (IKKbeta) targeting the classical NF-kappaB pathway was lethal to many myeloma cell lines. Several cell lines had elevated expression of NIK due to genomic alterations or protein stabilization, while others had inactivating mutations of TRAF3; both kinds of abnormality triggered the classical and alternative NF-kappaB pathways. A majority of primary myeloma patient samples and cell lines had elevated NF-kappaB target gene expression, often associated with genetic or epigenetic alteration of NIK, TRAF3, CYLD, BIRC2/BIRC3, CD40, NFKB1, or NFKB2. These data demonstrate that addiction to the NF-kappaB pathway is frequent in myeloma and suggest that IKKbeta inhibitors hold promise for the treatment of this disease

Keats JJ, Fonseca R, Chesi M et al. Promiscuous mutations activate the noncanonical NF-kappaB pathway in multiple myeloma. *Cancer Cell* 2007 August;12(2):131-44.

Abstract: Activation of NF-kappaB has been noted in many tumor types, however only rarely has this been linked to an underlying genetic mutation. An integrated analysis of high-density oligonucleotide array CGH and gene expression profiling data from 155 multiple myeloma samples identified a promiscuous array of abnormalities contributing to the dysregulation of NF-kappaB in approximately 20% of patients. We report mutations in ten genes causing the inactivation of TRAF2, TRAF3, CYLD, cIAP1/cIAP2 and activation of NFKB1, NFKB2, CD40, LTBR, TACI, and NIK that result primarily in constitutive activation of the noncanonical NF-kappaB pathway, with the single most common abnormality being inactivation of TRAF3. These results highlight the critical importance of the NF-kappaB pathway in the pathogenesis of multiple myeloma

Romagnoli M, Desplanques G, Maiga S et al. Canonical nuclear factor kappaB pathway inhibition blocks myeloma cell growth and induces apoptosis in strong synergy with TRAIL. *Clin Cancer Res* 2007 October 15;13(20):6010-8.

Abstract: PURPOSE: Intrinsic activation of nuclear factor kappaB (NF-kappaB) characterizes various hematologic malignancies. In this study, we specifically address the role of NF-kappaB blockade in mediated antimyeloma activity using the IkappaB kinase-2 pharmacologic inhibitor, AS602868. EXPERIMENTAL DESIGN: Human myeloma cell lines (n = 16) and primary myeloma cells (n = 10) were tested for their sensitivity to AS602868 in terms of proliferation and apoptosis. Both in vitro and in vivo experiments were conducted. Functional mechanisms regarding the apoptotic pathways triggered by AS602868 were studied. The potential proapoptotic synergy between AS602868 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was also evaluated. RESULTS: Our results show that AS602868 efficiently targeted the canonical NF-kappaB pathway in myeloma cells and potently inhibited their growth in inducing apoptosis through Bax and caspase-3 activation. AS602868 also induced apoptosis in primary myeloma cells even in the presence of bone marrow mononuclear cells. Moreover, the IkappaB kinase-2 inhibitor targeted the paracrine effect on the bone marrow environment. Indeed, it decreased the intrinsic and

myeloma-induced secretion of interleukin-6 from bone marrow stromal cells. In addition, AS602868 inhibited myeloma cell growth in the MM.1S xenograft myeloma model. Of particular interest, AS602868 strongly increased myeloma sensitivity to TRAIL in blocking TRAIL-induced NF-kappaB activation and in decreasing the expression of antiapoptotic proteins such as cFLIP and cIAP-1/2. CONCLUSIONS: Taken together, our data point out the interest to inhibit the canonical NF-kappaB pathway in myeloma and clearly encourage clinical evaluation of novel therapies based on targeting NF-kappaB, especially in combination with TRAIL

Li ZW, Chen H, Campbell RA, Bonavida B, Berenson JR. NF-kappaB in the pathogenesis and treatment of multiple myeloma. *Curr Opin Hematol* 2008 July;15(4):391-9.

Abstract: PURPOSE OF REVIEW: This review aims to summarize recent advances in the mechanisms through which the activation of the transcription factor NF-kappaB contributes to the pathogenesis of multiple myeloma. RECENT FINDINGS: This transcription factor regulates expression of numerous genes involved in multiple myeloma pathogenesis, including growth, survival, immortalization, angiogenesis and metastasis. Recently, mutations of NF-kappaB signaling molecules have been identified in multiple myeloma cells. In addition, interactions between multiple myeloma cells and the bone marrow environment play critical roles in NF-kappaB activation as well as in multiple myeloma pathogenesis. Moreover, several drugs that are effective against multiple myeloma, including bortezomib, thalidomide, lenalidomide and arsenic trioxide, have been found to block activation of NF-kappaB. The combination of conventional chemotherapeutic drugs and those that block NF-kappaB activation has now proven to be effective in the treatment of multiple myeloma. SUMMARY: Recent studies further underscore the critical role of NF-kappaB in multiple myeloma pathogenesis and have provided the rationale for multiple myeloma therapy with NF-kappaB-specific inhibitors combined with conventional chemotherapeutic drugs

Markovina S, Callander NS, O'Connor SL et al. Bortezomib-resistant nuclear factor-kappaB activity in multiple myeloma cells. *Mol Cancer Res* 2008 August;6(8):1356-64.

Abstract: Bortezomib (Velcade/PS341), a proteasome inhibitor used in the treatment of multiple myeloma (MM), can inhibit activation of nuclear factor-kappaB (NF-kappaB), a family of transcription factors often deregulated and constitutively activated in primary MM cells. NF-kappaB can be activated via several distinct mechanisms, including the proteasome inhibitor-resistant (PIR) pathway. It remains unknown what fraction of primary MM cells harbor constitutive NF-kappaB activity maintained by proteasome-dependent mechanisms. Here, we report an unexpected finding that constitutive NF-kappaB activity in 10 of 14 primary MM samples analyzed is refractory to inhibition by bortezomib. Moreover, when MM cells were cocultured with MM patient-derived bone marrow stromal cells (BMSC), microenvironment components critical for MM growth and survival, further increases in NF-kappaB activity were observed that were also refractory to bortezomib. Similarly, MM-BMSCs caused PIR NF-kappaB activation in the RPMI8226 MM cell line, leading to increased NF-kappaB-dependent transcription and resistance to bortezomib-induced apoptosis. Our findings show that primary MM cells frequently harbor PIR NF-kappaB activity that is further enhanced by the presence of patient-derived BMSCs. They also suggest that this activity is likely relevant to the drug resistance development in some patients. Further elucidation of the mechanism of PIR NF-kappaB regulation could lead to the identification of novel diagnostic biomarkers and/or therapeutic targets for MM treatment

Hideshima T, Chauhan D, Kiziltepe T et al. Biologic sequelae of I{kappa}B kinase (IKK) inhibition in multiple myeloma: therapeutic implications. *Blood* 2009 May 21;113(21):5228-36.

Abstract: Nuclear factor-kappaB (NF-kappaB) has an important role in multiple myeloma (MM) cell pathogenesis in the context of the bone marrow (BM) microenvironment. In NF-kappaB signaling cascades, IkappaB kinase alpha (IKKalpha) and IKKbeta are key molecules that predominantly mediate noncanonical and canonical pathways, respectively. In this study, we examined the biologic sequelae of the inhibition of IKKalpha versus IKKbeta in MM cell lines. All MM cell lines have constitutive canonical NF-kappaB activity, and a subset of MM cell lines shows noncanonical NF-kappaB activity. Adhesion to BM stromal cells further activates both canonical and noncanonical NF-kappaB activity. IKKbeta inhibitor MLN120B blocks canonical pathway and growth of MM cell lines but does not inhibit the noncanonical NF-kappaB pathway. Although IKKalpha knockdown induces significant growth inhibition in the cell lines with both canonical and noncanonical pathways, it does not inhibit NF-kappaB activation. Importantly, IKKalpha down-regulation decreases expression of beta-catenin and aurora-A, which are known to mediate MM cell growth and survival. Finally, IKKbeta inhibitor enhances the growth inhibition triggered by IKKalpha down-regulation in MM cells with both canonical and noncanonical NF-kappaB activity. Combination therapy targeting these kinases therefore represents a promising treatment strategy in MM

Sung B, Kunnumakkara AB, Sethi G, Anand P, Guha S, Aggarwal BB. Curcumin circumvents chemoresistance in vitro and potentiates the effect of thalidomide and bortezomib against human multiple myeloma in nude mice model. *Mol Cancer Ther* 2009 April;8(4):959-70.

Abstract: Curcumin (diferuloylmethane), a yellow pigment in turmeric, has been shown to inhibit the activation of nuclear factor-kappaB (NF-kappaB), a transcription factor closely linked to chemoresistance in multiple myeloma cells. Whether curcumin can overcome chemoresistance and enhance the activity of thalidomide and bortezomib, used to treat patients with multiple myeloma, was investigated in vitro and in xenograft model in nude mice. Our results show that curcumin inhibited the proliferation of human multiple myeloma cells regardless of their sensitivity to dexamethasone, doxorubicin, or melphalan. Curcumin also potentiated the apoptotic effects of thalidomide and bortezomib by down-regulating the constitutive activation of NF-kappaB and Akt, and this correlated with the suppression of NF-kappaB-regulated gene products, including cyclin D1, Bcl-xL, Bcl-2, TRAF1, cIAP-1, XIAP, survivin, and vascular endothelial growth factor. Furthermore, in a nude mice model, we found that curcumin potentiated the antitumor effects of bortezomib ($P < 0.001$, vehicle versus bortezomib+curcumin; $P < 0.001$, bortezomib versus bortezomib+curcumin), and this correlated with suppression of Ki-67 ($P < 0.001$ versus control), CD31 ($P < 0.001$ versus vehicle), and vascular endothelial growth factor ($P < 0.001$ versus vehicle) expression. Collectively, our results suggest that curcumin overcomes chemoresistance and sensitizes multiple myeloma cells to thalidomide and bortezomib by down-regulating NF-kappaB and NF-kappaB-regulated gene products

Hideshima T, Ikeda H, Chauhan D et al. Bortezomib induces canonical nuclear factor-kappaB activation in multiple myeloma cells. *Blood* 2009 July 30;114(5):1046-52.

Abstract: Bortezomib is a proteasome inhibitor with remarkable preclinical and clinical antitumor activity in multiple myeloma (MM) patients. The initial rationale for its use in MM

was inhibition of nuclear factor (NF)-kappaB activity by blocking proteasomal degradation of inhibitor of kappaBalpha (IkappaBalpha). Bortezomib inhibits inducible NF-kappaB activity; however, its impact on constitutive NF-kappaB activity in MM cells has not yet been defined. In this study, we demonstrate that bortezomib significantly down-regulated IkappaBalpha expression and triggered NF-kappaB activation in MM cell lines and primary tumor cells from MM patients. Importantly, no inhibition of p65 (RelA) nuclear translocation was recognized after bortezomib treatment in a murine xenograft model bearing human MM cells. Bortezomib-induced NF-kappaB activation was mediated via the canonical pathway. Moreover, other classes of proteasome inhibitors also induced IkappaBalpha down-regulation associated with NF-kappaB activation. Molecular mechanisms whereby bortezomib induced IkappaBalpha down-regulation were further examined. Bortezomib triggered phosphorylation of IkappaB kinase (IKKbeta) and its upstream receptor-interacting protein 2, whereas IKKbeta inhibitor MLN120B blocked bortezomib-induced IkappaBalpha down-regulation and NF-kappaB activation, indicating receptor-interacting protein 2/IKKbeta signaling plays crucial role in bortezomib-induced NF-kappaB activation. Moreover, IKKbeta inhibitors enhanced bortezomib-induced cytotoxicity. Our studies therefore suggest that bortezomib-induced cytotoxicity cannot be fully attributed to inhibition of canonical NF-kappaB activity in MM cells

Wang Q, Li J, Gu J et al. Potentiation of (-)-epigallocatechin-3-gallate-induced apoptosis by bortezomib in multiple myeloma cells. *Acta Biochim Biophys Sin (Shanghai)* 2009 December;41(12):1018-26.

Abstract: The green tea constituent, (-)-epigallocatechin-3-gallate (EGCG), has chemopreventive and anticancer effects. This is partially because of the selective ability of EGCG to induce apoptosis and death in cancer cells without affecting normal cells. In the present study, the activity of EGCG against the myeloma cell line, KM3, was examined. Our results demonstrated, for the first time, that the treatment of the KM3 cell line with EGCG inhibits cell proliferation and induces apoptosis, and there is a synergistic effect when EGCG and bortezomib are combined. Further experiments showed that this effect involves the NF-kappaB pathway. EGCG inhibits the expression of the P65 mRNA and P65/pP65 protein, meanwhile it downregulates pIkappaBalpha expression and upregulates IkappaBalpha expression. EGCG also activates caspase-3, -8, cleaved caspase-9, and poly-ADP-ribose polymerase (PARP) and subsequent apoptosis. These findings provided experimental evidence for efficacy of EGCG alone or in combination with bortezomib in multiple myeloma therapy

Liang Y, Xu RZ, Zhang L, Zhao XY. Berbamine, a novel nuclear factor kappaB inhibitor, inhibits growth and induces apoptosis in human myeloma cells. *Acta Pharmacol Sin* 2009 December;30(12):1659-65.

Abstract: AIM: We sought to investigate the effect of berbamine on the growth of human multiple myeloma cell line KM3 and elucidate the mechanism of its action. METHODS: MTT assay was used to determine the inhibitory effect of berbamine alone or combined with chemotherapeutic drugs. Flow cytometry was performed to characterize cell cycle profile in response to berbamine treatment. Western blot was used to measure the protein levels of p65, IkappaB Kinase alpha (IKKalpha), TNFAIP3 (A20), IkappaBalpha, p-IkappaBalpha, cyclinD1, Bcl-2, BAX, Bcl-x(L), Bid, and survivin. RESULTS: Berbamine inhibits the proliferation of KM3 cells in a dose- and time-dependent manner. Combination of berbamine with dexamethasone (Dex), doxorubicin (Dox) or arsenic trioxide (ATO) resulted in enhanced inhibition of cell growth. Flow cytometric analysis revealed that KM3 cells were

arrested at G(1) phase and apoptotic cells increased from 0.54% to 51.83% for 36 h. Morphological changes of cells undergoing apoptosis were observed under light microscope. Berbamine treatment led to increased expression of A20, down-regulation of IKK α , p-IkappaB α , and followed by inhibition of p65 nuclear localization. As a result, NF-kappaB downstream targets such as cyclinD1, Bcl-x(L), Bid and survivin were down-regulated. CONCLUSION: Berbamine inhibits the growth of KM3 cells by inducing G(1) arrest as well as apoptosis. Berbamine blocks NF-kappaB signaling pathway through up-regulating A20, down-regulating IKK α , p-IkappaB α , and then inhibiting p65 nuclear translocation, and resulting in decreased expression of the downstream targets of NF-kappaB. Our results suggest that berbamine is a novel inhibitor of NF-kappaB activity with remarkable anti-myeloma efficacy

Demchenko YN, Glebov OK, Zingone A, Keats JJ, Bergsagel PL, Kuehl WM. Classical and/or alternative NF-kappaB pathway activation in multiple myeloma. *Blood* 2010 April 29;115(17):3541-52.

Abstract: Mutations involving the nuclear factor-kappaB (NF-kappaB) pathway are present in at least 17% of multiple myeloma (MM) tumors and 40% of MM cell lines (MMCLs). These mutations, which are apparent progression events, enable MM tumors to become less dependent on bone marrow signals that activate NF-kappaB. Studies on a panel of 51 MMCLs provide some clarification of the mechanisms through which these mutations act and the significance of classical versus alternative activation of NF-kappaB. First, only one mutation (NFKB2) selectively activates the alternative pathway, whereas several mutations (CYLD, NFKB1, and TACI) selectively activate the classical pathway. However, most mutations affecting NF-kappaB-inducing kinase (NIK) levels (NIK, TRAF2, TRAF3, cIAP1&2, and CD40) activate the alternative but often both pathways. Second, we confirm the critical role of TRAF2 in regulating NIK degradation, whereas TRAF3 enhances but is not essential for cIAP1/2-mediated proteasomal degradation of NIK in MM. Third, using transfection to selectively activate the classical or alternative NF-kappaB pathways, we show virtually identical changes in gene expression in one MMCL, whereas the changes are similar albeit nonidentical in a second MMCL. Our results suggest that MM tumors can achieve increased autonomy from the bone marrow microenvironment by mutations that activate either NF-kappaB pathway

Meinel FG, Mandl-Weber S, Baumann P, Leban J, Schmidmaier R. The novel, proteasome-independent NF-kappaB inhibitor V1810 induces apoptosis and cell cycle arrest in multiple myeloma and overcomes NF-kappaB-mediated drug resistance. *Mol Cancer Ther* 2010 February;9(2):300-10.

Abstract: Evidence is increasing that aberrant NF-kappaB activation is crucial for multiple myeloma pathophysiology and a promising target for new antimyeloma therapies. In this study, we assessed the in vitro antimyeloma activity of the novel NF-kappaB inhibitor V1810. Pharmacokinetics and toxicity were studied in vivo. In mice, V1810 plasma concentrations of 10 micromol/L can be reached without relevant toxicity. At this concentration, V1810 potently induces apoptosis in all four multiple myeloma cell lines assessed (IC(50) = 5-12 micromol/L) as well as in primary multiple myeloma cells (IC(50) = 5-40 micromol/L). Apoptosis induced by V1810 is associated with proteasome-independent inhibition of NF-kappaB signaling (41% relative reduction), downregulation of Mcl-1, and caspase 3 cleavage. In OPM2, U266, and RPMI-8226 cells, induction of apoptosis is accompanied by cell cycle arrest. Western blots revealed downregulation of Cdk4 as well as cyclin D1 (U266) or cyclin D2 (OPM2, NCI-H929, RPMI-8226), but not cyclin D3.

Consistently, retinoblastoma protein was found to be hypophosphorylated. Furthermore, V1810 reverses NF-kappaB activation induced by the genotoxic drugs melphalan and doxorubicin. V1810 and melphalan synergistically decrease multiple myeloma cell viability. Taken together, the novel, proteasome-independent NF-kappaB inhibitor V1810 induces apoptosis and cell cycle arrest in multiple myeloma cells at a concentration range that can be achieved in vivo. Moreover, V1810 reverses NF-kappaB activation by alkylating drugs and overcomes NF-kappaB-mediated resistance to melphalan

Walsby EJ, Pratt G, Hewamana S et al. The NF-kappaB inhibitor LC-1 has single agent activity in multiple myeloma cells and synergizes with bortezomib. *Mol Cancer Ther* 2010 June;9(6):1574-82.

Abstract: Multiple myeloma remains incurable with conventional therapeutics. Thus, new treatments for this condition are clearly required. In this study we evaluated the novel NF-kappaB inhibitor LC-1 in multiple myeloma cell lines and plasma cells derived from multiple myeloma patients. LC-1 was cytotoxic to multiple myeloma cell lines H929, U266, and JJN3, and induced apoptosis in a dose-dependent manner with an overall LD(50) of 3.6 micromol/L (+/-1.8) after 48 hours in culture. Primary multiple myeloma cells, identified by CD38 and CD138 positivity, had a mean LD(50) for LC-1 of 4.9 micromol/L (+/-1.6); normal bone marrow cells were significantly less sensitive to the cytotoxic effects of LC-1 (P = 0.0002). Treatment of multiple myeloma cell lines with LC-1 resulted in decreased nuclear localization of the NF-kappaB subunit Rel A and the inhibition of NF-kappaB target genes. In addition, LC-1 showed synergy with melphalan, bortezomib, and doxorubicin (combination indices of 0.72, 0.61, and 0.78, respectively), and was more effective when cells were cultured on fibronectin. These data show that LC-1 has activity in multiple myeloma cell lines and primary multiple myeloma cells, and its ability to inhibit NF-kappaB seems important for its cytotoxic effects. Furthermore, LC-1-induced transcriptional suppression of survivin and MCL1 provides a potential explanation for its synergy with conventional agents

Markovina S, Callander NS, O'Connor SL et al. Bone marrow stromal cells from multiple myeloma patients uniquely induce bortezomib resistant NF-kappaB activity in myeloma cells. *Mol Cancer* 2010;9:176.

Abstract: BACKGROUND: Components of the microenvironment such as bone marrow stromal cells (BMSCs) are well known to support multiple myeloma (MM) disease progression and resistance to chemotherapy including the proteasome inhibitor bortezomib. However, functional distinctions between BMSCs in MM patients and those in disease-free marrow are not completely understood. We and other investigators have recently reported that NF-kappaB activity in primary MM cells is largely resistant to the proteasome inhibitor bortezomib, and that further enhancement of NF-kappaB by BMSCs is similarly resistant to bortezomib and may mediate resistance to this therapy. The mediating factor(s) of this bortezomib-resistant NF-kappaB activity is induced by BMSCs is not currently understood. RESULTS: Here we report that BMSCs specifically derived from MM patients are capable of further activating bortezomib-resistant NF-kappaB activity in MM cells. This induced activity is mediated by soluble proteinaceous factors secreted by MM BMSCs. Among the multiple factors evaluated, interleukin-8 was secreted by BMSCs from MM patients at significantly higher levels compared to those from non-MM sources, and we found that IL-8 contributes to BMSC-induced NF-kappaB activity. CONCLUSIONS: BMSCs from MM patients uniquely enhance constitutive NF-kappaB activity in MM cells via a proteinaceous secreted factor in part in conjunction with IL-8. Since NF-kappaB is known to potentiate MM cell survival and confer resistance to drugs including bortezomib, further identification of the NF-kappaB

activating factors produced specifically by MM-derived BMSCs may provide a novel biomarker and/or drug target for the treatment of this commonly fatal disease

Demchenko YN, Kuehl WM. A critical role for the NFkB pathway in multiple myeloma. *Oncotarget* 2010 May;1(1):59-68.

Abstract: NFkB transcription factors play a key role in the survival and proliferation of many kinds of B-cell tumors, including multiple myeloma (MM). It was shown that NFkB activation in MM tumors results mainly from extrinsic signaling by APRIL and BAFF ligands that stimulate receptors on normal plasma cells as well as on pre-malignant monoclonal gammopathy of undetermined significance (MGUS) and MM tumors. However, the mutations that occur during MM progression and that constitutively activate NFkB would be expected to decrease dependence of tumor cells on the bone marrow microenvironment. These mutations can activate the classical or alternative NFkB pathways selectively, but usually both pathways are activated in MM. Significantly, activation of either NFkB pathway leads to a similar response of MM cell lines. This frequent activation of the alternative pathway distinguishes MM from other B-cell tumors, which more frequently have mutations that are predicted to activate only the classical NFkB pathway. Given the strong dependence of MGUS and MM tumors on NFkB pathway activation, inhibition by a combination of targeting extrinsic signaling plus both NFkB pathways appears to be an attractive therapeutic approach in MM tumors

Gunn EJ, Williams JT, Huynh DT et al. The natural products parthenolide and andrographolide exhibit anti-cancer stem cell activity in multiple myeloma. *Leuk Lymphoma* 2011 June;52(6):1085-97.

Abstract: Multiple myeloma (MM) is an incurable plasma cell malignancy where nearly all patients succumb to a relapse. The current preclinical models of MM target the plasma cells, constituting the bulk of the tumor, leaving the cancer stem cells to trigger a relapse. Utilizing a three-dimensional tissue culture system where cells were grown in extracellular matrix designed to reconstruct human bone marrow, we tested the anti-multiple myeloma cancer stem cell (MM-CSC) potential of two natural product inhibitors of nuclear factor kappaB (NFkappaB). Here we show that parthenolide and andrographolide are potent anti-MM-CSC agents. Both natural products demonstrated preferential toxicity toward MM-CSCs over non-tumorigenic MM cells. Addition of the bone marrow stromal compartment abrogated andrographolide activity while having no effect on parthenolide cytotoxicity. This is the first report of a natural product with anti-CSC activity in myeloma, suggesting that it has the potential to improve the survival of patients with MM by eliminating the relapse-causing MM-CSCs

Kannaiyan R, Hay HS, Rajendran P et al. Celastrol inhibits proliferation and induces chemosensitization through down-regulation of NF-kappaB and STAT3 regulated gene products in multiple myeloma cells. *Br J Pharmacol* 2011 November;164(5):1506-21.

Abstract: BACKGROUND AND PURPOSE: Activation of pro-inflammatory transcription factors NF-kappaB and signal transducer and activator of transcription 3 (STAT3) is one of the major contributors to both pathogenesis and chemoresistance in multiple myeloma (MM), which results in high mortality rate. Thus, in the present study, we investigated whether celastrol could suppress the proliferation and induce chemosensitization of MM cells by interfering with NF-kappaB and STAT3 activation pathways. EXPERIMENTAL APPROACH: The effects of celastrol were investigated using both a virtual predictive

tumour cell system and different MM cell lines resistant to doxorubicin, melphalan and bortezomib. **KEY RESULTS:** Celastrol inhibited the proliferation of MM cell lines regardless of whether they were sensitive or resistant to bortezomib and other conventional chemotherapeutic drugs. It also synergistically enhanced the apoptotic effects of thalidomide and bortezomib. This correlated with the down-regulation of various proliferative and anti-apoptotic gene products including cyclin D1, Bcl-2, Bcl-xL, survivin, XIAP and Mcl-1. These effects of celastrol were mediated through suppression of constitutively active NF-kappaB induced by inhibition of IkappaBalpha kinase activation; and the phosphorylation of IkappaBalpha and of p65. Celastrol also inhibited both the constitutive and IL6-induced activation of STAT3, which induced apoptosis as indicated by an increase in the accumulation of cells in the sub-G1 phase, an increase in the expression of pro-apoptotic proteins and activation of caspase-3. **CONCLUSIONS AND IMPLICATIONS:** Thus, based on our experimental findings, we conclude that celastrol may have great potential as a treatment for MM and other haematological malignancies

Tagoug I, Sauty De CA, Dumontet C. Inhibition of IGF-1 signalling enhances the apoptotic effect of AS602868, an IKK2 inhibitor, in multiple myeloma cell lines. *PLoS ONE* 2011;6(7):e22641.

Abstract: Multiple myeloma (MM) is a B cell neoplasm characterized by bone marrow infiltration with malignant plasma cells. IGF-1 signalling has been explored as a therapeutic target in this disease. We analyzed the effect of the IKK2 inhibitor AS602868, in combination with a monoclonal antibody targeting IGF-1 receptor (anti-IGF-1R) in human MM cell lines. We found that anti-IGF-1R potentiated the apoptotic effect of AS602868 in LP1 and RPMI8226 MM cell lines which express high levels of IGF-1R. Anti-IGF-1R enhanced the inhibitory effect of AS602868 on NF-kappaB pathway signalling and potentiated the disruption of mitochondrial membrane potential caused by AS602868. These results support the role of IGF-1 signalling in MM and suggest that inhibition of this pathway could sensitize MM cells to NF-kappaB inhibitors

Kudo C, Yamakoshi H, Sato A et al. Novel curcumin analogs, GO-Y030 and GO-Y078, are multi-targeted agents with enhanced abilities for multiple myeloma. *Anticancer Res* 2011 November;31(11):3719-26.

Abstract: BACKGROUND: Multiple myeloma remains an incurable malignancy despite of the recent approval of new molecular-targeted agents. The complex molecular mechanism, composed of various signal networks, including nuclear factor-kappaB (NF-kappaB), phosphoinositide 3-kinase (PI3K)/AKT, Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3), and interferon regulatory factor 4 (IRF4) pathways, is a major reason for treatment failure. Curcumin can regulate these molecules, but its low bioavailability prevents its clinical application. **MATERIALS AND METHODS:** Growth-suppressive abilities of newly synthesized analogs, GO-Y030 and GO-Y078 were analyzed. Molecular-targeted abilities of the analogs for NF-kappaB, PI3K/AKT, JAK/STAT3, IRF4 pathways, as well as inhibition of interleukin-6 (IL-6) production, were also examined. **RESULTS:** GO-Y030 and GO-Y078 were 7 to 12-fold more potent growth suppressors for myeloma cells, and 6- to 15-fold stronger inhibitors of NF-kappaB, PI3K/AKT, JAK/STAT3, and IRF4 pathways than curcumin. GO-Y78 also 14-fold more potently inhibited IL-6 production. **CONCLUSION:** GO-Y030 and GO-Y078 are potential therapeutic candidates with enhanced abilities for multiple myeloma

Bai QX, Zhang XY. Curcumin Enhances Cytotoxic Effects of Bortezomib in Human Multiple Myeloma H929 Cells: Potential Roles of NF-kappaB/JNK. *Int J Mol Sci* 2012;13(4):4831-8.

Abstract: Combined curcumin and PS-341 treatment has been reported to enhance cytotoxicity and minimize adverse effects through ERK and p38MAPK mechanisms in human multiple myeloma cells. However, whether JNK plays similar role in this process remains unclear. In the present study, we found combined treatment altered NF-kappaB p65 expressions and distributions in multiple myeloma H929 cells. Western blot analysis showed combined treatment inactivated NF-kappaB while activated JNK signaling. Pre-treatment with JNK inhibitor SP600125 could attenuate NF-kappaB inactivation and restored H929 cells' survival. These results suggested that curcumin might enhance the cytotoxicity of PS-341 by interacting with NF-kappaB, at least in part, through JNK mechanism

Fabre C, Mimura N, Bobb K et al. Dual Inhibition of Canonical and Noncanonical NF-kappaB Pathways Demonstrates Significant Antitumor Activities in Multiple Myeloma. *Clin Cancer Res* 2012 September 1;18(17):4669-81.

Abstract: PURPOSE: NF-kappaB transcription factor plays a key role in the pathogenesis of multiple myeloma in the context of the bone marrow microenvironment. Both canonical and noncanonical pathways contribute to total NF-kappaB activity. Recent studies have shown a critical role for the noncanonical pathway: selective inhibitors of the canonical pathway present a limited activity, mutations of the noncanonical pathway are frequent, and bortezomib-induced cytotoxicity cannot be fully attributed to inhibition of canonical NF-kappaB activity. EXPERIMENTAL DESIGN: Multiple myeloma cell lines, primary patient cells, and the human multiple myeloma xenograft murine model were used to examine the biologic impact of dual inhibition of both canonical and noncanonical NF-kappaB pathways. RESULTS: We show that PBS-1086 induces potent cytotoxicity in multiple myeloma cells but not in peripheral blood mononuclear cells. PBS-1086 overcomes the proliferative and antiapoptotic effects of the bone marrow milieu, associated with inhibition of NF-kappaB activity. Moreover, PBS-1086 strongly enhances the cytotoxicity of bortezomib in bortezomib-resistant multiple myeloma cell lines and patient multiple myeloma cells. PBS-1086 also inhibits osteoclastogenesis through an inhibition of RANK ligand (RANKL)-induced NF-kappaB activation. Finally, in a xenograft model of human multiple myeloma in the bone marrow milieu, PBS-1086 shows significant in vivo anti-multiple myeloma activity and prolongs host survival, associated with apoptosis and inhibition of both NF-kappaB pathways in tumor cells. CONCLUSIONS: Our data show that PBS-1086 is a promising dual inhibitor of the canonical and noncanonical NF-kappaB pathways. Our preclinical study therefore provides the framework for clinical evaluation of PBS-1086 in combination with bortezomib for the treatment of multiple myeloma and related bone lesions. *Clin Cancer Res*; 18(17); 4669-81. (c)2012 AACR

Rushworth SA, Bowles KM, Barrera LN, Murray MY, Zaitseva L, Macewan DJ. BTK inhibitor ibrutinib is cytotoxic to myeloma and potently enhances bortezomib and lenalidomide activities through NF-kappaB. *Cell Signal* 2012 September 11.

Abstract: Ibrutinib (previously known as PCI-32765) has recently shown encouraging clinical activity in chronic lymphocytic leukaemia (CLL) effecting cell death through inhibition of Bruton's tyrosine kinase (BTK). In this study we report for the first time that ibrutinib is cytotoxic to malignant plasma cells from patients with multiple myeloma (MM) and furthermore that treatment with ibrutinib significantly augments the cytotoxic activity of

bortezomib and lenalidomide chemotherapies. We describe that the cytotoxicity of ibrutinib in MM is mediated via an inhibitory effect on the nuclear factor-kappaB (NF-kappaB) pathway. Specifically, ibrutinib blocks the phosphorylation of serine-536 of the p65 subunit of NF-kappaB, preventing its nuclear translocation, resulting in down-regulation of anti-apoptotic proteins Bcl-xL, FLIP(L) and survivin and culminating in caspase-mediated apoptosis within the malignant plasma cells. Taken together these data provide a platform for clinical trials of ibrutinib in myeloma and a rationale for its use in combination therapy, particularly with bortezomib

Hedgehog Pathway Inhibitors

The hedgehog pathway, driven by hedgehog ligands secreted by stromal cells in the bone marrow, lymph nodes, and spleen, often promotes chemoresistance and stem cell proliferation in multiple myeloma. Although anti-cancer drugs specifically designed to address the hedgehog pathway have not yet been approved, the anti-fungal agent itraconazole has recently been reported to inhibit hedgehog signaling, in vitro and in vivo, in concentrations that appear clinically feasible.

Peacock CD, Wang Q, Gesell GS et al. Hedgehog signaling maintains a tumor stem cell compartment in multiple myeloma. *Proc Natl Acad Sci U S A* 2007 March 6;104(10):4048-53.

Abstract: The cancer stem cell hypothesis suggests that malignant growth depends on a subset of tumor cells with stem cell-like properties of self-renewal. Because hedgehog (Hh) signaling regulates progenitor cell fate in normal development and homeostasis, aberrant pathway activation might be involved in the maintenance of such a population in cancer. Indeed, mutational activation of the Hh pathway is associated with medulloblastoma and basal cell carcinoma; pathway activity is also critical for growth of other tumors lacking such mutations, although the mechanism of pathway activation is poorly understood. Here we study the role and mechanism of Hh pathway activation in multiple myeloma (MM), a malignancy with a well defined stem cell compartment. In this model, rare malignant progenitors capable of clonal expansion resemble B cells, whereas the much larger tumor cell population manifests a differentiated plasma cell phenotype that pathologically defines the disease. We show that the subset of MM cells that manifests Hh pathway activity is markedly concentrated within the tumor stem cell compartment. The Hh ligand promotes expansion of MM stem cells without differentiation, whereas the Hh pathway blockade, while having little or no effect on malignant plasma cell growth, markedly inhibits clonal expansion accompanied by terminal differentiation of purified MM stem cells. These data reveal that Hh pathway activation is heterogeneous across the spectrum of MM tumor stem cells and their more differentiated progeny. The potential existence of similar relationships in other adult cancers may have important biologic and clinical implications for the study of aberrant Hh signaling

Dierks C, Grbic J, Ziriklik K et al. Essential role of stromally induced hedgehog signaling in B-cell malignancies. *Nat Med* 2007 August;13(8):944-51.

Abstract: Interaction of cancer cells with their microenvironment generated by stromal cells is essential for tumor cell survival and influences the localization of tumor growth. Here we

demonstrate that hedgehog ligands secreted by bone-marrow, nodal and splenic stromal cells function as survival factors for malignant lymphoma and plasmacytoma cells derived from transgenic Emu-Myc mice or isolated from humans with these malignancies. Hedgehog pathway inhibition in lymphomas induced apoptosis through downregulation of Bcl2, but was independent of p53 or Bmi1 expression. Blockage of hedgehog signaling in vivo inhibited expansion of mouse lymphoma cells in a syngeneic mouse model and reduced tumor mass in mice with fully developed disease. Our data indicate that stromally induced hedgehog signaling may provide an important survival signal for B- and plasma-cell malignancies in vitro and in vivo. Disruption of this interaction by hedgehog pathway inhibition could provide a new strategy in lymphoma and multiple myeloma therapy

Agarwal JR, Matsui W. Multiple myeloma: a paradigm for translation of the cancer stem cell hypothesis. *Anticancer Agents Med Chem* 2010 February;10(2):116-20.

Abstract: Despite recent advances in drug development, multiple myeloma (MM) remains incurable for the majority of patients due to relapse and disease progression. The cancer stem cell (CSC) hypothesis may provide an explanation for these clinical findings. It suggests that the long-term proliferative potential responsible for disease initiation, maintenance, and relapse is contained within specific subpopulations of biologically distinct tumor cells. Data in MM suggest that CSCs represent a rare cell population phenotypically resembling normal memory B cells. Compared to MM plasma cells, MM CSCs also appear to be relatively resistant to a wide variety of standard anti-cancer agents suggesting they may persist following treatment and mediate tumor re-growth and relapse. A unique property CSCs share with their normal counterparts is the potential for self-renewal that likely maintains the malignant clone over time. The development of therapeutic strategies targeting the signaling elements contributing to cancer cell self-renewal has been limited primarily because the cellular processes involved are poorly understood. However, it is common that the signaling pathway components regulating normal stem cell self-renewal are aberrantly activated in human cancers and may serve as potential therapeutic targets. One class of shared regulatory pathways are those active during normal embryonic patterning and organ formation such as Hedgehog (Hh), Notch and Wingless (Wnt), and emerging data suggest that these may play a role in CSCs. Here we review the identification and characterization of MM CSCs, the role of Hh in MM, and issues to be considered during the early clinical testing of CSC targeting agents

Blotta S, Jakubikova J, Calimeri T et al. Canonical and non canonical Hedgehog pathway in the pathogenesis of multiple myeloma. *Blood* 2012 July 20.

Abstract: The Hedgehog (Hh)-pathway is required for cell-fate determination during the embryonic life, as well as cell growth and differentiation in the adult organism, where the inappropriate activation has been implicated in several cancers. Here, we demonstrate that Hh-signaling plays a significant role in growth and survival of multiple myeloma (MM) cells. We observed that CD138(+) MM cells express Hh-genes and confirmed Smoothed (Smo)-dependent Hh-signaling in MM using a novel synthetic Smo-inhibitor, NVP-LDE225 (Novartis), which decreased MM cell viability by inducing specific down-regulation of Gli1 and Ptch1, hallmarks of Hh-activity. Additionally, we detected a nuclear localization of Gli1 in MM cells, which is completely abrogated by Forskolin, a Gli1 modulating compound, confirming Smo-independent mechanisms leading to Hh-activation in MM. Finally, we identified that bone-marrow stromal cells (BMSCs) are a source of Shh-ligand, although they are resistant to Hh-inhibitor due to defective Smo expression and Ptch1 up-regulation. Further in vitro as well as in vivo studies showed anti-tumor efficacy of NVP-LDE225 in combination with Bortezomib. All together, our data demonstrate activation of both canonical and non

canonical Hh-pathway in MM, thus providing the rationale for testing Hh-inhibitors in clinical trials, in order to improve MM patient outcome

Kim J, Tang JY, Gong R et al. Itraconazole, a commonly used antifungal that inhibits Hedgehog pathway activity and cancer growth. *Cancer Cell* 2010 April 13;17(4):388-99.

Abstract: In a screen of drugs previously tested in humans we identified itraconazole, a systemic antifungal, as a potent antagonist of the Hedgehog (Hh) signaling pathway that acts by a mechanism distinct from its inhibitory effect on fungal sterol biosynthesis. Systemically administered itraconazole, like other Hh pathway antagonists, can suppress Hh pathway activity and the growth of medulloblastoma in a mouse allograft model and does so at serum levels comparable to those in patients undergoing antifungal therapy. Mechanistically, itraconazole appears to act on the essential Hh pathway component Smoothed (SMO) by a mechanism distinct from that of cyclopamine and other known SMO antagonists, and prevents the ciliary accumulation of SMO normally caused by Hh stimulation

Shi W, Nacev BA, Aftab BT, Head S, Rudin CM, Liu JO. Itraconazole side chain analogues: structure-activity relationship studies for inhibition of endothelial cell proliferation, vascular endothelial growth factor receptor 2 (VEGFR2) glycosylation, and hedgehog signaling. *J Med Chem* 2011 October 27;54(20):7363-74.

Abstract: Itraconazole is an antifungal drug that was recently found to possess potent antiangiogenic activity and anti-hedgehog (Hh) pathway activity. To search for analogues of itraconazole with greater potency and to understand the structure-activity relationship in both antiangiogenic and Hh targeting activity, 25 itraconazole side chain analogues were synthesized and assayed for inhibition of endothelial cell proliferation and Gli1 transcription in a medulloblastoma (MB) culture. Through this analysis, we have identified analogues with increased potency for inhibiting endothelial cell proliferation and the Hh pathway, as well as VEGFR2 glycosylation that was recently found to be inhibited by itraconazole. An SAR analysis of these activities revealed that potent activity of the analogues against VEGFR2 glycosylation was generally driven by side chains of at least four carbons in composition with branching at the alpha or beta position. SAR trends for targeting the Hh pathway were divergent from those related to HUVEC proliferation or VEGFR2 glycosylation. These results also suggest that modification of the sec-butyl side chain can lead to enhancement of the biological activity of itraconazole

Nelfinavir

The HIV protease inhibitor drug nelfinavir has shown cancer-retardant activity in a range of cancers, including MM. Like bortezomib and carfilzomib, it can inhibit the chymotrypsin-like protease activity of proteasomes, in clinically relevant concentrations. But for some reason it markedly boosts the cytotoxic activity of bortezomib in cell cultures, apparently by potentiating ER stress. This combination is notably effective in a MM xenograft model in nude mice, suggesting that this effect may be clinically relevant.

Kawabata S, Gills JJ, Mercado-Matos JR et al. Synergistic effects of nelfinavir and bortezomib on proteotoxic death of NSCLC and multiple myeloma cells. *Cell Death Dis* 2012;3:e353.

Abstract: Exploiting protein homeostasis is a new therapeutic approach in cancer. Nelfinavir (NFV) is an HIV protease inhibitor that induces endoplasmic reticulum (ER) stress in cancer cells. Under conditions of ER stress, misfolded proteins are transported from the ER back to the cytosol for subsequent degradation by the ubiquitin-proteasome system. Bortezomib (BZ) is a proteasome inhibitor and interferes with degradation of misfolded proteins. Here, we show that NFV and BZ enhance proteotoxicity in non-small cell lung cancer (NSCLC) and multiple myeloma (MM) cells. The combination synergistically inhibited cell proliferation and induced cell death. Activating transcription factor (ATF)3 and CCAAT-enhancer binding protein homologous protein (CHOP), markers of ER stress, were rapidly increased, and their siRNA-mediated knockdown inhibited cell death. Knockdown of double-stranded RNA activated protein kinase-like ER kinase, a signal transducer in ER stress, significantly decreased apoptosis. Pretreatment with the protein synthesis inhibitor, cycloheximide, decreased levels of ubiquitinated proteins, ATF3, CHOP, and the overall total cell death, suggesting that inhibition of protein synthesis increases cell survival by relieving proteotoxic stress. The NFV/BZ combination inhibited the growth of NSCLC xenografts, which correlated with the induction of markers of ER stress and apoptosis. Collectively, these data show that NFV and BZ enhance proteotoxicity in NSCLC and MM cells, and suggest that this combination could tip the precarious balance of protein homeostasis in cancer cells for therapeutic gain

Bono C, Karlin L, Harel S et al. The human immunodeficiency virus-1 protease inhibitor nelfinavir impairs proteasome activity and inhibits the proliferation of multiple myeloma cells in vitro and in vivo. *Haematologica* 2012 July;97(7):1101-9.

Abstract: BACKGROUND: Multiple myeloma is characterized by the accumulation of tumor plasma cells in the bone marrow. Despite therapeutic improvements brought by proteasome inhibitors such as bortezomib, myeloma remains an incurable disease. In a variety of human cancers, human immunodeficiency virus protease inhibitors (e.g. nelfinavir) effectively inhibit tumor progression, but their impact on myeloma is unknown. We assessed the in vitro and in vivo effects of nelfinavir on multiple myeloma. DESIGN AND METHODS: The effects of nelfinavir (1-10 μ M) on proteasome activity, proliferation and viability of myeloma cell lines and plasma cells from patients were assessed by measuring PERK, AKT, STAT3 and ERK1/2 phosphorylation and CHOP expression with immunoblotting or flow cytometry. The in vivo effect was assessed in NOD/SCID mice injected with luciferase expressing human myeloma cell lines and treated with nelfinavir at a dose of 75 mg/kg/day. Tumor progression was evaluated using a bioluminescent system. RESULTS: Nelfinavir inhibited 26S chymotrypsin-like proteasome activity, impaired proliferation and triggered apoptosis of the myeloma cell lines and fresh plasma cells. It activated the pro-apoptotic unfolded protein response pathway by inducing PERK phosphorylation and CHOP expression. Cell death triggered by nelfinavir treatment correlated with decreased phosphorylation of AKT, STAT3 and ERK1/2. Nelfinavir enhanced the anti-proliferative activity of bortezomib, dexamethasone and histone deacetylase inhibitors and delayed tumor growth in a myeloma mouse model. CONCLUSIONS: These results suggest that nelfinavir, used at a pharmacological dosage, alone or in combination, may be useful in the treatment of myeloma. Our data provide a preclinical basis for clinical trials using nelfinavir in patients with myeloma

Ikezoe T, Saito T, Bandobashi K, Yang Y, Koeffler HP, Taguchi H. HIV-1 protease inhibitor induces growth arrest and apoptosis of human multiple myeloma cells via inactivation of signal transducer and activator of transcription 3 and extracellular signal-regulated kinase 1/2. *Mol Cancer Ther* 2004 April;3(4):473-9.

Abstract: We previously showed that HIV-1 protease inhibitors slowed the proliferation of

human myeloid leukemia cells and enhanced their differentiation in the presence of all-trans retinoic acid (ATRA). In this study, we found that protease inhibitors, including ritonavir, saquinavir, and nelfinavir, but not indinavir, induced growth arrest and apoptosis of U266, RPMI8226, and ARH77 human multiple myeloma (MM) cells in association with down-regulation of antiapoptotic protein Mcl-1. Also, protease inhibitors inhibited the survival of freshly isolated MM cells from patients. In contrast, these protease inhibitors did not affect survival of normal B cells and colony formation of myeloid committed stem cells (CFU-GM) from healthy volunteers. In addition, we found that all of the protease inhibitors, except for indinavir, blocked interleukin-6 (IL-6)-stimulated phosphorylation of both signal transducer and activator of transcription 3 (STAT 3) and extracellular signal-regulated kinase 1/2 in U266 and RPMI8226 MM cells. Moreover, the protease inhibitors inhibited both the basal and IL-6-stimulated STAT 3/DNA binding activity in U266 cells as measured by an ELISA-based assay. Furthermore, ritonavir inhibited production of vascular endothelial growth factor one of the targets of STAT 3, in U266 and RPMI8226 cells as measured by ELISA. Taken together, protease inhibitors might be useful for treatment of individuals with MM

Histone Deacetylase Inhibitors

A broad range of histone deacetylase (HDAC) inhibitors show growth retardant and pro-apoptotic effects on multiple myeloma cell lines. Moreover, these agents typically interact additively or synergistically with cytotoxic agents and bortezomib against MM. The clinically available agent vorinostat is now being studied in formal clinical trials in MM. As a single agent, it promotes stable disease. Its effects are more impressive when used in conjunction with bortezomib; a phase I study reports a 42% response rate, including partial responses in a third of patients who had become refractory to bortezomib. This finding has been confirmed in an open clinical report. Other currently available agents that can function clinically as HDACs include valproic acid and phenylbutyrate; each of these likewise has been found to aid control of MM in pre-clinical studies.

Khan SB, Maududi T, Barton K, Ayers J, Alkan S. Analysis of histone deacetylase inhibitor, depsipeptide (FR901228), effect on multiple myeloma. *Br J Haematol* 2004 April;125(2):156-61.

Abstract: Multiple myeloma (MM) is a neoplastic proliferation of plasma cells and remains an incurable disease because of the development of drug resistance. Histone deacetylase (HDAC) inhibitors are a new class of chemotherapeutic reagents that cause growth arrest and apoptosis of neoplastic cells. Depsipeptide, a new member of the HDAC inhibitors, was found to be safe in humans and has been shown to induce apoptosis in various cancers. In order to evaluate the effects of depsipeptide, a MM cell line, U266 [interleukin (IL)-6 dependent], was analysed for viability and apoptosis. The combined effect of depsipeptide with melphalan and changes in BCL-2 family proteins (BCL-2, BCL-XL, BAX and MCL-1) were also investigated. In addition, the RPMI 8226 cell line (IL-6 independent), and primary patient myeloma cells were also analysed for apoptosis after depsipeptide treatment. Depsipeptide induced apoptosis in both U266 and RPMI 8226 cell lines in a time- and dose-dependent fashion, and in primary patient myeloma cells. We also demonstrated that depsipeptide had an additive effect with melphalan (10 micromol/l). BCL-2, BCL-XL and MCL-1 showed decreased expression in depsipeptide-treated samples. Based on recent

clinical trials demonstrating minimal clinical toxicity, our study supports the future clinical utilization of depsipeptide in the management of MM

Catley L, Weisberg E, Tai YT et al. NVP-LAQ824 is a potent novel histone deacetylase inhibitor with significant activity against multiple myeloma. *Blood* 2003 October 1;102(7):2615-22.

Abstract: Histone deacetylase (HDAC) inhibitors are emerging as a promising new treatment strategy in hematologic malignancies. Here we show that NVP-LAQ824, a novel hydroxamic acid derivative, induces apoptosis at physiologically achievable concentrations (median inhibitory concentration [IC50] of 100 nM at 24 hours) in multiple myeloma (MM) cell lines resistant to conventional therapies. MM.1S myeloma cell proliferation was also inhibited when cocultured with bone marrow stromal cells, demonstrating ability to overcome the stimulatory effects of the bone marrow microenvironment. Importantly, NVP-LAQ824 also inhibited patient MM cell growth in a dose- and time-dependent manner. NVP-LAQ824-induced apoptotic signaling includes up-regulation of p21, caspase cascade activation, and poly (adenosine diphosphate [ADP]) ribose (PARP) cleavage. Apoptosis was confirmed with cell cycle analysis and annexin-propidium iodide staining. Interestingly, treatment of MM cells with NVPLAQ824 also led to proteasome inhibition, as determined by reduced proteasome chymotrypsin-like activity and increased levels of cellular polyubiquitin conjugates. Finally, a study using NVP-LAQ824 in a preclinical murine myeloma model provides in vivo relevance to our in vitro studies. Taken together, these findings provide the framework for NVP-LAQ824 as a novel therapeutic in MM

Kaiser M, Zavrski I, Sterz J et al. The effects of the histone deacetylase inhibitor valproic acid on cell cycle, growth suppression and apoptosis in multiple myeloma. *Haematologica* 2006 February;91(2):248-51.

Abstract: The aim of this study was to evaluate the effects of valproic acid (VPA), as a histone deacetylase inhibitor, on myeloma cell lines and on sorted human bone marrow multiple myeloma cells. VPA induced accumulation of acetylated histones, potently inhibited proliferation in a dose-dependent manner and induced apoptosis in all myeloma cell lines tested as well as in sorted primary multiple myeloma cells. Cell cycle analysis indicated an arrest in G0/1 phase in response to VPA. Accumulation of p21 and reduced levels of cyclin D1 were detected. The production of vascular endothelial growth factor was significantly inhibited by VPA. These results provide the framework for clinical trials

Maiso P, Carvajal-Vergara X, Ocio EM et al. The histone deacetylase inhibitor LBH589 is a potent antimyeloma agent that overcomes drug resistance. *Cancer Res* 2006 June 1;66(11):5781-9.

Abstract: Multiple myeloma represents an incurable disease, for which development of new therapies is required. Here, we report the effect on myeloma cells of LBH589, a new hydroxamic acid-derived histone deacetylase inhibitor. LBH589 was a potent antimyeloma agent (IC(50) < 40 nmol/L) on both cell lines and fresh cells from multiple myeloma patients, including cells resistant to conventional chemotherapeutic agents. In addition, LBH589 potentiated the action of drugs, such as bortezomib, dexamethasone, or melphalan. Using gene array, quantitative PCR, and Western analyses, we observed that LBH589 affected a large number of genes involved in cell cycle and cell death pathways. LBH589 blocked cell cycle progression, and this was accompanied by p21, p53, and p57 up-regulation. LBH589 induced cell death through an increase in the mitochondrial outer membrane permeability.

LBH589 favored apoptosome formation by inducing cytochrome c release, Apaf-1 up-regulation, and caspase-9 cleavage. In addition, LBH589 stimulated a caspase-independent pathway through the release of AIF from the mitochondria. LBH589 down-regulated Bcl-2 and particularly Bcl-X. Moreover, overexpression of Bcl-X in multiple myeloma cells prevented LBH589-induced cell death. All these data indicate that LBH589 could be a useful drug for the treatment of multiple myeloma patients

Feng R, Oton A, Mapara MY, Anderson G, Belani C, Lentzsch S. The histone deacetylase inhibitor, PXD101, potentiates bortezomib-induced anti-multiple myeloma effect by induction of oxidative stress and DNA damage. *Br J Haematol* 2007 November;139(3):385-97.

Abstract: Clinical trials have shown the high anti-myeloma activity of the proteasome inhibitor bortezomib. The present study examined the activity of bortezomib combined with PXD101, a histone deacetylase inhibitor, against multiple myeloma (MM) and osteoclastogenesis. Treatment of myeloma cell lines with combinations of bortezomib and PXD101 led to synergistic inhibition of proliferation and induction of cell death. The combination significantly decreased the viability of primary human CD138(+) myeloma cells but not of bone marrow mononuclear cells. Further studies showed a dose-dependent activation of caspases-3, -8 and -9 and nuclear fragmentation in myeloma cells. Bortezomib/PXD101 treatment markedly triggered reactive oxygen species (ROS) generation that was accompanied by p53, H2A.X and p38-mitogen-activated protein kinase phosphorylation. ROS generation could be blocked by the free radical scavenger N-acetyl-L-cysteine. The combination of bortezomib and PXD101 also resulted in synergistic inhibition of osteoclast formation. In conclusion, bortezomib and PXD101 have different molecular targets. The combination induces cell death in myeloma cells via ROS-mediated DNA damage and also inhibits osteoclastogenesis. Therefore, this study provides the rationale for the clinical evaluation of bortezomib combined with PXD101 in patients with MM

Dong XF, Song Q, Li LZ, Zhao CL, Wang LQ. Histone deacetylase inhibitor valproic acid inhibits proliferation and induces apoptosis in KM3 cells via downregulating VEGF receptor. *Neuro Endocrinol Lett* 2007 December;28(6):775-80.

Abstract: The expression of vascular endothelial growth factor receptor 1(VEGFR-1) in human multiple myeloma KM3 cells in vitro, effects of valproic acid (VPA), as a histone deacetylase inhibitor, on cell proliferation and apoptosis and the underlying molecular mechanism were investigated. The effects of VPA on the growth of KM3 cells were studied by MTT assay. The apoptosis rate was determined with flow cytometry. The mRNA level of VEGFR was determined by RT-PCR; and immunocytochemistry was used to detect the protein level of ac-H4 and VEGFR. VPA inhibited proliferation of KM3 cells in a time- and dose-dependent manner. Treatment with VPA (4, 2, 1 and 0.5 mmol/L) for 48h, the apoptosis rates of KM3 cells were (13.27+/-3.54)%, (22.13+/-1.20)%, (24.41+/-2.23)% and(40.62+/-4.28)% respectively. The expression of VEGFR-1 in KM3 cells were decreased in VPA-treated group by the immunochemistry and RT-PCR, whereas the acetylated histone H4(ac-H4) accumulated. It suggested VPA could decrease the expression of VEGFR-1 in KM3 cells, and it might play an important role in regulating the proliferation and apoptosis of multiple myeloma cell line KM3 cells. These results provide the framework for clinical trials

Prince HM, Bishton M, Harrison S. The potential of histone deacetylase inhibitors for the treatment of multiple myeloma. *Leuk Lymphoma* 2008 March;49(3):385-7.

Abstract: Preclinical evidence supports the investigation of histone deacetylase inhibitors for the treatment of myeloma. Results from early studies demonstrate clinical activity and further studies investigating combination strategies should be explored

Richardson P, Mitsiades C, Colson K et al. Phase I trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) in patients with advanced multiple myeloma. *Leuk Lymphoma* 2008 March;49(3):502-7.

Abstract: A Phase I trial (NCT00109109) of oral vorinostat 200, 250 or 300 mg twice daily for 5 days/week/4-week cycle or 200, 300, or 400 mg twice daily for 14 days/3-week cycle until progressive disease or intolerable toxicity was conducted. Patients with measurable, relapsed/refractory multiple myeloma were eligible. The objectives were to determine maximum tolerated doses (MTDs) and assess activity and safety. Thirteen patients (median age, 63 years; median prior therapies, 3) were enrolled. MTDs were not determined due to early study termination by sponsor decision. One patient (250 mg twice daily 5 days/week) developed dose-limiting toxicity (DLT; grade 3 fatigue). There were no other DLTs and the maximum administered doses were 250 mg twice daily for 5 days/week/4-week cycle and 200 mg twice daily for 14 days/3-week cycle. Drug-related adverse experiences included fatigue, anorexia, dehydration, diarrhea, and nausea and were mostly grade ≤ 2 . Of 10 evaluable patients, 1 had a minimal response and 9 had stable disease, demonstrating modest single-agent activity in relapsed/refractory multiple myeloma

Neri P, Tagliaferri P, Di Martino MT et al. In vivo anti-myeloma activity and modulation of gene expression profile induced by valproic acid, a histone deacetylase inhibitor. *Br J Haematol* 2008 November;143(4):520-31.

Abstract: Valproic acid (VPA) is a well-tolerated anticonvulsant that exerts anti-tumour activity as a histone deacetylase inhibitor. This study investigated the in vitro and in vivo activity of VPA against multiple myeloma (MM) cells. In vitro exposure of interleukin-6-dependent or -independent MM cells to VPA inhibited cell proliferation in a time- and dose-dependent manner and induced apoptosis. In a cohort of severe combined immunodeficiency mice bearing human MM xenografts, VPA induced tumour growth inhibition and survival advantage in treated animals versus controls. Flow cytometric analysis performed on MM cells from excised tumours showed increase of G(0)-G(1) and a decreased G(2)/M- and S-phase following VPA treatment, indicating in vivo effects of VPA on cell cycle regulation. Gene expression profiling of MM cells exposed to VPA showed downregulation of genes involved in cell cycle progression, DNA replication and transcription, as well as upregulation of genes implicated in apoptosis and chemokine pathways. Pathfinder analysis of gene array data identified cell growth, cell cycle, cell death, as well as DNA replication and repair as the most important signalling networks modulated by VPA. Taken together, our data provide the preclinical rationale for VPA clinical evaluation as a single agent or in combination, to improve patient outcome in MM

Kitazoe K, Abe M, Hiasa M et al. Valproic acid exerts anti-tumor as well as anti-angiogenic effects on myeloma. *Int J Hematol* 2009 January;89(1):45-57.

Abstract: Multiple myeloma is still an incurable disease, most commonly occurring in the elderly. The myeloma-induced bone marrow microenvironment protects myeloma cells from drug-induced apoptosis. Therefore, the development of novel and tolerable therapeutic alternatives to overcome the drug resistance is an important clinical issue. Valproic acid

(VPA), a safe and widely used anti-epileptic agent, is revisited as a class I- and IIa-specific histone deacetylase inhibitor. In the present study, we evaluated the effect as well as a mechanism of actions of VPA on myeloma cell growth and survival, with special reference to the myeloma-induced bone marrow microenvironment. VPA at therapeutic concentrations for epilepsy induced cell death in primary CD138-positive myeloma cells as well as myeloma cell lines, but not in CD138-negative bone marrow cells. VPA suppressed osteoclastogenesis as well as osteoclast-mediated myeloma cell growth. VPA also inhibited vascular tubule formation enhanced by co-cultures of myeloma cells and osteoclasts in concert with thalidomide. In addition, VPA induced both caspase-dependent and -independent cell death in myeloma cells, and potentiated the anti-myeloma effects of melphalan and dexamethasone. Collectively, VPA is suggested to exert multi-factorial anti-myeloma actions, and may serve as a safe adjuvant to be included in conventional chemotherapies against myeloma

Galli M, Salmoiraghi S, Golay J et al. A phase II multiple dose clinical trial of histone deacetylase inhibitor ITF2357 in patients with relapsed or progressive multiple myeloma. *Ann Hematol* 2010 February;89(2):185-90.

Abstract: ITF2357, an orally effective member of the family of histone deacetylase inhibitors, is a potent inducer of apoptosis and death of multiple myeloma (MM) cells. We performed a phase-II, multiple-dose clinical trial in 19 patients with relapsed or progressive MM to determine the maximum tolerated dose (MTD) of ITF2357 administered twice daily for four consecutive days every week for 4 weeks (i.e., first cycle). The first six patients received 150 mg ITF2357 twice daily. Since two of them experienced a dose-limiting toxicity (DLT) during the first cycle, the subsequent patients received 100 mg ITF2357 twice daily. This was the MTD, as only one DLT occurred. Up to 12 weeks (i.e., three cycles) of treatment were scheduled. Oral dexamethasone was allowed to a maximum weekly amount of 20 mg. Median duration of treatment was 6 weeks, ranging from two (two patients) to 12 weeks (five patients). Four patients suffered from serious adverse events. Three patients experienced grade 3-4 gastro-intestinal toxicity and three had transient electrocardiographic abnormalities. Thrombocytopenia occurred in all but one patient (grade 3-4 in ten patients). At last follow-up, five patients were in stable disease, five had disease progression, and nine had died all of progressive MM. In conclusion, when given at a dose of 100 mg twice daily alone or combined with dexamethasone, ITF2357 proved tolerable but showed a modest clinical benefit in advanced MM

Badros A, Burger AM, Philip S et al. Phase I study of vorinostat in combination with bortezomib for relapsed and refractory multiple myeloma. *Clin Cancer Res* 2009 August 15;15(16):5250-7.

Abstract: PURPOSE: Vorinostat, a histone deacetylase inhibitor, enhances cell death by the proteasome inhibitor bortezomib in vitro. We sought to test the combination clinically. EXPERIMENTAL DESIGN: A phase I trial evaluated sequential dose escalation of bortezomib at 1 to 1.3 mg/m² i.v. on days 1, 4, 8, and 11 and vorinostat at 100 to 500 mg orally daily for 8 days of each 21-day cycle in relapsed/refractory multiple myeloma patients. Vorinostat pharmacokinetics and dynamics were assessed. RESULTS: Twenty-three patients were treated. Patients had received a median of 7 prior regimens (range, 3-13), including autologous transplantation in 20, thalidomide in all 23, lenalidomide in 17, and bortezomib in 19, 9 of whom were bortezomib-refractory. Two patients receiving 500 mg vorinostat had prolonged QT interval and fatigue as dose-limiting toxicities. The most common grade >3 toxicities were myelo-suppression (n = 13), fatigue (n = 11), and diarrhea (n = 5). There were no drug-related deaths. Overall response rate was 42%, including three partial responses

among nine bortezomib refractory patients. Vorinostat pharmacokinetics were nonlinear. Serum Cmax reached a plateau above 400 mg. Pharmacodynamic changes in CD-138+ bone marrow cells before and on day 11 showed no correlation between protein levels of NF-kappaB, IkappaB, acetylated tubulin, and p21CIP1 and clinical response.

CONCLUSIONS: The maximum tolerated dose of vorinostat in our study was 400 mg daily for 8 days every 21 days, with bortezomib administered at a dose of 1.3 mg/m² on days 1, 4, 8, and 11. The promising antimyeloma activity of the regimen in refractory patients merits further evaluation

Aouali N, Palissot V, El-Khoury V et al. Peroxisome proliferator-activated receptor gamma agonists potentiate the cytotoxic effect of valproic acid in multiple myeloma cells. *Br J Haematol* 2009 December;147(5):662-71.

Abstract: The main challenge in using chemotherapy to treat multiple myeloma (MM) is drug resistance. In order to evaluate the anti-neoplastic properties of a new drug combination in MM, two clinically available drugs, valproic acid (VPA) a histone deacetylase (HDAC) inhibitor and pioglitazone, a peroxisome proliferator-activated receptor gamma (PPARgamma) agonist, were tested in vitro on MM cell lines and MM patient cells. The sensitivity towards VPA alone was observed on several MM cell lines tested and also on primary myeloma cells and peripheral blood mononuclear cells from healthy donors. Importantly, the addition of a PPARgamma agonist to the VPA treatment increased the cytotoxic effect of VPA in a synergistic/additive manner on the different MM cell lines and MM patient cells. This effect was observed at the physiological range of VPA used to treat epileptic patients. The mechanisms underlying this increase induced a cell cycle arrest and caspase-dependent apoptosis. The potentiation of the effect of VPA by pioglitazone was mediated by higher acetylation levels of histones H3 and H4 compared to levels induced by HDAC inhibitors alone. This association reveals a new promising chemotherapeutic combination to be tested in MM

Campbell RA, Sanchez E, Steinberg J et al. Vorinostat enhances the antimyeloma effects of melphalan and bortezomib. *Eur J Haematol* 2010 March;84(3):201-11.

Abstract: **OBJECTIVES:** Examine the antitumor activity of the histone deacetylase inhibitor vorinostat's antitumor activity against multiple myeloma (MM) using cell lines and a murine xenograft model. **METHODS:** RPMI8226, U266, and MM1S cells were cultured for 48 h in the presence of media, vorinostat, melphalan, or bortezomib alone, or combinations of vorinostat with melphalan or bortezomib. Cell proliferation was measured using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfphophenyl)-2H-te trazolium, inner salt] assay. Severe combined immunodeficient mice bearing LAGkappa-1B tumors were treated with vorinostat [30, 60, or 100 mg/kg daily for five consecutive days per week (qdx5d), 100 or 300 mg/kg daily for 2 d/wk (qdx2d)], melphalan (1, 3, or 10 mg/kg qdx1d), bortezomib (0.25 or 0.5 mg/kg qdx2d), or combinations thereof for 35 d. Tumor growth was determined via measurement of human immunoglobulin G (hIgG) levels and tumor volume. **RESULTS AND CONCLUSIONS:** Vorinostat enhanced the anti-MM effects of melphalan and bortezomib in vitro. Synergism was observed with vorinostat and melphalan in RPMI8226 and U266 cell lines. Vorinostat 100 mg/kg in combination with melphalan 3 mg/kg resulted in significant inhibition of tumor growth in vivo, compared with control (tumor volume P = 0.0001; hIgG P = 0.0001), single-agent vorinostat (tumor volume P = 0.0025; hIgG P = 0.0137), and single-agent melphalan (tumor volume P = 0.0043; hIgG P = 0.0426). Vorinostat also enhanced the antimyeloma effects of bortezomib in vivo. Vorinostat enhances the anti-MM activity of melphalan and bortezomib in vitro and in vivo.

This study provides rationale for further evaluation of vorinostat in combination with chemotherapeutic agents and bortezomib for the treatment of MM

Mandl-Weber S, Meinel FG, Jankowsky R, Oduncu F, Schmidmaier R, Baumann P. The novel inhibitor of histone deacetylase resminostat (RAS2410) inhibits proliferation and induces apoptosis in multiple myeloma (MM) cells. *Br J Haematol* 2010 May;149(4):518-28.

Abstract: Inhibition of histone deacetylase (HDAC) is a promising mechanism for novel, anti-myeloma agents. We investigated the effects of the novel HDAC inhibitor resminostat on multiple myeloma (MM) cells in vitro. Resminostat is a potent inhibitor of HDACs 1, 3 and 6 [50% inhibitory concentration (IC₅₀)=43-72 nmol/l] representing HDAC classes I and II and induces hyperacetylation of histone H4 in MM cells. Low micromolar concentrations of resminostat abrogated cell growth and strongly induced apoptosis (IC₅₀=2.5-3 micromol/l in 3 out of 4 MM cell lines) in MM cell lines as well as primary MM cells. At 1 micromol/l, resminostat inhibited proliferation and induced G₀/G₁ cell cycle arrest in 3 out of 4 MM cell lines accompanied with decreased levels of cyclin D1, cdc25a, Cdk4 and pRb as well as upregulation of p21. Resminostat decreased phosphorylation of 4E-BP1 and p70S6k indicating an interference with Akt pathway signalling. Treatment with resminostat resulted in increased protein levels of Bim and Bax and decreased levels of Bcl-xL. Caspases 3, 8 and 9 were activated by resminostat. Furthermore, synergistic effects were observed for combinations of resminostat with melphalan and the proteasome inhibitors bortezomib and S-2209. In conclusion, we have identified potent anti-myeloma activity for this novel HDAC inhibitor

Mazumder A, Vesole DH, Jagannath S. Vorinostat plus bortezomib for the treatment of relapsed/refractory multiple myeloma: a case series illustrating utility in clinical practice. *Clin Lymphoma Myeloma Leuk* 2010 April;10(2):149-51.

Abstract: INTRODUCTION: Increasing numbers of patients are presenting with relapsed/refractory multiple myeloma (MM) following treatment with bortezomib. Therefore, there is a need for effective and well-tolerated treatment strategies after failure of bortezomib-based regimens. Vorinostat, a histone deacetylase inhibitor, has demonstrated antiproliferative and proapoptotic activity alone and in combination with bortezomib in preclinical models of MM. Preliminary results from ongoing phase I trials have demonstrated the clinical activity of vorinostat in combination with bortezomib in patients with MM. This case series reports our experience of combined vorinostat and bortezomib in 6 patients with relapsed/refractory MM after previous bortezomib. MATERIALS AND METHODS: Patients received oral vorinostat 300 mg or 400 mg once daily (days 1-14) and bortezomib 1.3 mg/m² on days 1, 4, 8, and 11 in a 21-day cycle. RESULTS: All patients derived clinical benefit from combined vorinostat and bortezomib, with objective response observed in 5 of the 6 patients (> or = minimal response), including 1 very good partial response; stable disease was observed in the remaining patient. Patients remained on therapy until disease progression. Combined vorinostat and bortezomib therapy was well tolerated: grade 2 nausea and diarrhea were the only adverse events reported. No patients discontinued therapy because of toxicity, and no dose adjustments were required for either agent. CONCLUSION: These results suggest that combined vorinostat and bortezomib therapy is effective in patients with relapsed/refractory MM after failure of previous bortezomib-based regimens and support further evaluation of this combination in randomized trials

Kikuchi J, Wada T, Shimizu R et al. Histone deacetylases are critical targets of bortezomib-induced cytotoxicity in multiple myeloma. *Blood* 2010 July 22;116(3):406-17.

Abstract: Bortezomib is now widely used for the treatment of multiple myeloma (MM); however, its action mechanisms are not fully understood. Despite the initial results, recent investigations have indicated that bortezomib does not inactivate nuclear factor-kappaB activity in MM cells, suggesting the presence of other critical pathways leading to cytotoxicity. In this study, we show that histone deacetylases (HDACs) are critical targets of bortezomib, which specifically down-regulated the expression of class I HDACs (HDAC1, HDAC2, and HDAC3) in MM cell lines and primary MM cells at the transcriptional level, accompanied by reciprocal histone hyperacetylation. Transcriptional repression of HDACs was mediated by caspase-8-dependent degradation of Sp1 protein, the most potent transactivator of class I HDAC genes. Short-interfering RNA-mediated knockdown of HDAC1 enhanced bortezomib-induced apoptosis and histone hyperacetylation, whereas HDAC1 overexpression inhibited them. HDAC1 overexpression conferred resistance to bortezomib in MM cells, and administration of the HDAC inhibitor romidepsin restored sensitivity to bortezomib in HDAC1-overexpressing cells both in vitro and in vivo. These results suggest that bortezomib targets HDACs via distinct mechanisms from conventional HDAC inhibitors. Our findings provide a novel molecular basis and rationale for the use of bortezomib in MM treatment

Schmitt S, Ho AD, Goldschmidt H. The oral histone deacetylase inhibitor LBH589 is a potential and promising therapeutic agent in multiple myeloma after at least two lines of chemotherapy including bortezomib or lenalidomide. *Onkologie* 2010;33(4):183-6.

Abstract: BACKGROUND: Multiple myeloma as the second most common hematological malignancy is characterized by proliferation of monoclonal plasma cells. This entity still remains a non-curable disorder leading, amongst others, to complications as myeloma bone disease, bleeding events, kidney failure and neurological impairment. LBH589 is a histone deacetylase inhibitor with an epigenetic mechanism of action and the potential for treatment in myeloma. CASE REPORT: We report here about the successful treatment of a 44-year-old woman suffering from progressive myeloma with LBH589 after five different chemotherapies. During the 9 years after first diagnosis of myeloma in April 2000, our patient twice underwent an autologous stem cell transplantation and was also treated with the new substances bortezomib, thalidomide and lenalidomide. RESULTS: A rapid decline of myeloma activity parameters could be reached, with an approximately exponential decrease of kappa light chains in the 24-h urine. As a consequence, a near-complete remission was determined after about 6 months of LBH589 treatment. Additionally, the adverse event profile was acceptable, and the patient's quality of life showed a considerable advancement of well-being. CONCLUSIONS: LBH589 may be very effective in multiple myeloma after a multitude of preceding treatments that could not induce a long-term anti-myeloma effect. Future trials with LBH589 should search for the specific characteristics of responding patients

Lee CK, Wang S, Huang X, Ryder J, Liu B. HDAC inhibition synergistically enhances alkylator-induced DNA damage responses and apoptosis in multiple myeloma cells. *Cancer Lett* 2010 October 28;296(2):233-40.

Abstract: Histone deacetylase (HDAC) inhibitors induce chromatin destabilization. We sought to determine whether HDAC inhibition may amplify alkylator-induced mitotic cell death in multiple myeloma (MM) cells. The combination of SNDX-275, a class I HDAC inhibitor, with melphalan, showed a powerful synergism on growth inhibition with the combination index ranged from 0.27 to 0.75 in MM1.S and RPMI8226 cells. Their

combinations as compared with either agent alone promoted much more caspase-dependent apoptosis. Flow cytometry analysis showed that SNDX-275 had minimal effects on cell cycle progression of MM1.S cells, but clearly increased the percentage of S phase in RPMI8226 cells associated with an upregulation in p21(waf1) and a reduction in cyclin D1 and E2F1. Melphalan alone significantly arrested both MM1.S and RPMI8226 cells at S phase and enhanced expression of p53 and p21(waf1). Furthermore, studies on DNA damage response revealed that phospho-histone H2A.X (gammaH2A.X), a hall marker of DNA double strand break, along with phosphorylated CHK1 (P-CHK1) and CHK2 (P-CHK2) was dramatically induced by SNDX-275 or melphalan. The increase in gammaH2A.X and P-CHK1 was considerably higher on combination than either agent alone. These molecular changes correlated well with the significant increase in mitotic catastrophe. Our data indicate that SNDX-275 synergistically enhances melphalan-induced apoptosis in MM cells via intensification of DNA damage, suggesting that SNDX-275 in combination with melphalan may be a novel therapeutic strategy for MM

Jagannath S, Dimopoulos MA, Lonial S. Combined proteasome and histone deacetylase inhibition: A promising synergy for patients with relapsed/refractory multiple myeloma. *Leuk Res* 2010 September;34(9):1111-8.

Abstract: Multiple myeloma (MM) is an incurable disease characterized by the accumulation of malignant plasma cells in the bone marrow. Recently, an improved understanding of the biology of the disease has led to the development of targeted agents such as the proteasome inhibitor bortezomib and the immunomodulatory agents thalidomide and lenalidomide; however, MM remains incurable. The combination of bortezomib and an HDAC inhibitor synergistically induces MM cell apoptosis and may be of value in the treatment of patients with relapsed/refractory MM. This review examines the potential of combined proteasome and HDAC inhibition in the treatment of relapsed/refractory MM

Sanchez E, Shen J, Steinberg J et al. The histone deacetylase inhibitor LBH589 enhances the anti-myeloma effects of chemotherapy in vitro and in vivo. *Leuk Res* 2011 March;35(3):373-9.

Abstract: Panobinostat (LBH589) is a potent histone deacetylase inhibitor (HDACi) that has shown anti-tumor activity in preclinical studies in both solid and hematological malignancies. We evaluated the anti-multiple myeloma (MM) effects of LBH589 alone and with melphalan or doxorubicin using MM cell lines and our human MM xenograft model LAGlambda-1. LBH589 treatment resulted in increased acetylation of histones, induction of caspase cleavage, inhibition of cell proliferation and synergistic anti-MM effects with melphalan or doxorubicin in vitro. LBH589 with melphalan or doxorubicin also showed significantly enhanced anti-myeloma activity in vivo. These findings provide the basis for clinical development of these combination therapies

Zhang S, Suvannasankha A, Crean CD, White VL, Chen CS, Farag SS. The novel histone deacetylase inhibitor, AR-42, inhibits gp130/Stat3 pathway and induces apoptosis and cell cycle arrest in multiple myeloma cells. *Int J Cancer* 2011 July 1;129(1):204-13.

Abstract: Multiple myeloma (MM) remains incurable with current therapy, indicating the need for continued development of novel therapeutic agents. We evaluated the activity of a novel phenylbutyrate-derived histone deacetylase inhibitor, AR-42, in primary human myeloma cells and cell lines. AR-42 was cytotoxic to MM cells at a mean LC(50) of 0.18 +/- 0.06 mumol/l at 48 hr and induced apoptosis with cleavage of caspases 8, 9 and 3, with cell

death largely prevented by caspase inhibition. AR-42 downregulated the expression of gp130 and inhibited activation of STAT3, with minimal effects on the PI3K/Akt and MAPK pathways, indicating a predominant effect on the gp130/STAT-3 pathway. AR-42 also inhibited interleukin (IL)-6-induced STAT3 activation, which could not be overcome by exogenous IL-6. AR-42 also downregulated the expression of STAT3-regulated targets, including Bcl-xL and cyclin D1. Overexpression of Bcl-xL by a lentivirus construct partly protected against cell death induced by AR-42. The cyclin dependent kinase inhibitors, p16 and p21, were also significantly induced by AR-42, which together with a decrease in cyclin D1, resulted in G(1) and G(2) cell cycle arrest. In conclusion, AR-42 has potent cytotoxicity against MM cells mainly through gp130/STAT-3 pathway. The results provide rationale for clinical investigation of AR-42 in MM

Niesvizky R, Ely S, Mark T et al. Phase 2 trial of the histone deacetylase inhibitor romidepsin for the treatment of refractory multiple myeloma. *Cancer* 2011 January 15;117(2):336-42.

Abstract: BACKGROUND: Epigenetic dysregulation is a hallmark of cancer, including multiple myeloma. Inhibitors of histone deacetylases (HDACs) induce DNA hyperacetylation by inhibiting removal of acetyl groups from amino tails on histone proteins, thereby uncoiling condensed chromatin favoring transcription of silenced genes, including tumor suppressor genes. Romidepsin is an HDAC inhibitor that exhibits antiproliferative and apoptotic effects against multiple myeloma cell lines. METHODS: A phase 2 trial was performed of romidepsin in patients with multiple myeloma who were refractory to standard therapy. Treatment was comprised of romidepsin (13 mg/m²) given as a 4-hour intravenous infusion on Days 1, 8, and 15 every 28 days. Thirteen patients received a median of 2 cycles of therapy (range, 1-7 cycles). RESULTS: Although no patients had an objective response, 4 of 12 patients with secretory myeloma exhibited evidence of M-protein stabilization, and several other patients experienced improvement in bone pain and resolution of hypercalcemia. CONCLUSIONS: The results of the current study demonstrate that romidepsin, as a single agent, is unlikely to be associated with a response rate of $\geq 30\%$ in patients with refractory myeloma, although there was some clinical evidence suggesting a biological effect associated with therapy

Bai LY, Omar HA, Chiu CF, Chi ZP, Hu JL, Weng JR. Antitumor effects of (S)-HDAC42, a phenylbutyrate-derived histone deacetylase inhibitor, in multiple myeloma cells. *Cancer Chemother Pharmacol* 2011 August;68(2):489-96.

Abstract: PURPOSE: Epigenetic agents are among the newly targeted therapeutic strategies being studied with intense interest for patients with multiple myeloma. Here, we demonstrate the antitumor activity of a phenylbutyrate-based histone deacetylase (HDAC) inhibitor, (S)-HDAC42, and identify its possible targets in myeloma cells. METHODS: The antiproliferative effect of (S)-HDAC42 was compared with suberoylanilide hydroxamic acid (SAHA) in three myeloma cell lines, IM-9, RPMI-8226, and U266. Flow cytometry and terminal transferase dUTP nick-end labeling (TUNEL) assay were used to demonstrate the induction of apoptosis by (S)-HDAC42. Moreover, the proposed mechanisms of action, such as modulation of Akt, NF-kappaB pathway, and cell cycle-related proteins, were investigated by western blotting. RESULTS: (S)-HDAC42 exhibited four- to sevenfold higher potency relative to SAHA in suppressing myeloma cell viabilities. The apoptotic effect induced by (S)-HDAC42 was through both intrinsic and extrinsic pathways, as evidenced by increased cleavage of caspase-3, caspase-8, and caspase-9 and release of cytochrome c from mitochondria. In addition to HDAC inhibition, (S)-HDAC42 also disturbed signaling pathways governing cell survival, including downregulating Akt phosphorylation and

NF-kappaB signaling. The modulation of cell cycle-related proteins by (S)-HDAC42 suggested its inhibitory effect on cell cycle propagation. CONCLUSION: These data suggest the translational value of (S)-HDAC42 in developing new therapeutic strategies for myeloma, which warrants further investigations

Terpos E. The synergistic effect of panobinostat (LBH589) with melphalan or doxorubicin on multiple myeloma cells; rationale for the use of combination regimens in myeloma patients. *Leuk Res* 2011 March;35(3):295-6.

Cheriyath V, Kuhns MA, Kalaycio ME, Borden EC. Potentiation of apoptosis by histone deacetylase inhibitors and doxorubicin combination: cytoplasmic cathepsin B as a mediator of apoptosis in multiple myeloma. *Br J Cancer* 2011 March 15;104(6):957-67.

Abstract: BACKGROUND: Although inhibitors of histone deacetylase inhibitors (HDACis) in combination with genotoxins potentiate apoptosis, the role of proteases other than caspases in this process remained elusive. Therefore, we examined the potentiation of apoptosis and related mechanisms of HDACis and doxorubicin combination in a panel of myeloma cell lines and in 25 primary myelomas. RESULTS: At IC(50) concentrations, sodium butyrate (an HDACi) or doxorubicin alone caused little apoptosis. However, their combination potentiated apoptosis and synergistically reduced the viability of myeloma cells independent of p53 and caspase 3-7 activation. Potentiated apoptosis correlated with nuclear translocation of apoptosis-inducing factor, suggesting the induction of caspase 3- and 7-independent pathways. Consistent with this, butyrate and doxorubicin combination significantly increased the activity of cytoplasmic cathepsin B. Inhibition of cathepsin B either with a small-molecule inhibitor or downregulation with a siRNA reversed butyrate- and doxorubicin-potentiated apoptosis. Finally, ex vivo, clinically relevant concentrations of butyrate or SAHA (suberoylanilide hydroxamic acid, vorinostat, an HDACi in clinical testing) in combination with doxorubicin significantly ($P < 0.0001$) reduced the survival of primary myeloma cells. CONCLUSIONS: Cathepsin B has a prominent function in mediating apoptosis potentiated by HDACi and doxorubicin combinations in myeloma. Our results support a molecular model of lysosomal-mitochondrial crosstalk in HDACi- and doxorubicin-potentiated apoptosis through the activation of cathepsin B

Dai Y, Chen S, Wang L et al. Disruption of IkappaB kinase (IKK)-mediated RelA serine 536 phosphorylation sensitizes human multiple myeloma cells to histone deacetylase (HDAC) inhibitors. *J Biol Chem* 2011 September 30;286(39):34036-50.

Abstract: Post-translational modifications of RelA play an important role in regulation of NF-kappaB activation. We previously demonstrated that in malignant hematopoietic cells, histone deacetylase inhibitors (HDACIs) induced RelA hyperacetylation and NF-kappaB activation, attenuating lethality. We now present evidence that IkappaB kinase (IKK) beta-mediated RelA Ser-536 phosphorylation plays a significant functional role in promoting RelA acetylation, inducing NF-kappaB activation, and limiting HDACi lethality in human multiple myeloma (MM) cells. Immunoblot profiling revealed that although basal RelA phosphorylation varied in MM cells, Ser-536 phosphorylation correlated with IKK activity. Exposure to the pan-HDACIs vorinostat or LBH-589 induced phosphorylation of IKKalpha/beta (Ser-180/Ser-181) and RelA (Ser-536) in MM cells, including cells expressing an IkappaBalpha "super-repressor," accompanied by increased RelA nuclear translocation, acetylation, DNA binding, and transactivation activity. These events were substantially blocked by either pan-IKK or IKKbeta-selective inhibitors, resulting in marked apoptosis.

Consistent with these events, inhibitory peptides targeting either the NF-kappaB essential modulator (NEMO) binding domain for IKK complex formation or RelA phosphorylation sites also significantly increased HDACI lethality. Moreover, IKKbeta knockdown by shRNA prevented Ser-536 phosphorylation and significantly enhanced HDACI susceptibility. Finally, introduction of a nonphosphorylatable RelA mutant S536A, which failed to undergo acetylation in response to HDACIs, impaired NF-kappaB activation and increased cell death. These findings indicate that HDACIs induce Ser-536 phosphorylation of the NF-kappaB subunit RelA through an IKKbeta-dependent mechanism, an action that is functionally involved in activation of the cytoprotective NF-kappaB signaling cascade primarily through facilitation of RelA acetylation rather than nuclear translocation

Hideshima T, Richardson PG, Anderson KC. Mechanism of action of proteasome inhibitors and deacetylase inhibitors and the biological basis of synergy in multiple myeloma. *Mol Cancer Ther* 2011 November;10(11):2034-42.

Abstract: Novel agents, including the proteasome inhibitor bortezomib, have significantly improved the response and survival of patients with multiple myeloma over the last decade. Despite these advances, many patients relapse or do not benefit from the currently available therapies; thus, multiple myeloma remains an incurable disease. Deacetylase inhibitors (DACi), including panobinostat and vorinostat, have recently emerged as novel agents being evaluated in the treatment of multiple myeloma. Deacetylases are a group of enzymes with effects on various intracellular proteins, including histones, transcription factors, and molecular chaperones. Although DACi inhibit cell growth and induce apoptosis in multiple myeloma cells as a single agent, synergistic activity has been observed when they were used in combination with bortezomib. The mechanistic basis of synergy is multifactorial and includes disruption of protein degradation and inhibition of the interaction of multiple myeloma cells with the tumor microenvironment. This review summarizes recent advancements in the understanding of the mechanism of action of proteasome inhibitors and DACi in multiple myeloma and examines the biological basis of their synergistic effects. Data from the studies summarized here have been used as the rationale for the implementation of phase II and III clinical trials of DACi, alone and combined with bortezomib, in relapsed and refractory multiple myeloma

Baumann P, Junghanns C, Mandl-Weber S, Strobl S, Oduncu F, Schmidmaier R. The pan-histone deacetylase inhibitor CR2408 disrupts cell cycle progression, diminishes proliferation and causes apoptosis in multiple myeloma cells. *Br J Haematol* 2012 March;156(5):633-42.

Abstract: In view of the fact that histone deacetylases (HDACs) are promising targets for myeloma therapy, we investigated the effects of the HDAC inhibitor CR2408 on multiple myeloma (MM) cells in vitro. CR2408 is a direct pan-HDAC inhibitor and inhibits all known 11 HDACs with a 50% inhibitory concentration (IC(50)) of 12 nmol/l (HDAC 6) to 520 nmol/l (HDAC 8). Correspondingly, CR2408 induces hyperacetylation of histone H4, inhibits cell growth and strongly induces apoptosis (IC(50) = 0.1-0.5 μmol/l) in MM cell lines and primary MM cells. CR2408 leads to fragmentation of cells and induces an accumulation in the subG1 phase accompanied with moderately decreased levels of cyclin D1 and cdk4 and strongly decreased levels of cdc25a, pRb and p53. Interruption of the cell cycle is reflected by inhibition of cell proliferation and is accompanied by decreased phosphorylation of 4E-BP1 and p70S6k. Treatment with CR2408 results in increased protein levels of Bim and pJNK and downregulation of Bad and Bcl-xL and activation of Caspases 3, 8 and 9. Furthermore, as HDAC inhibitors have shown synergism with other drugs, these effects were investigated and

synergism was observed for combinations of CR2408 with doxorubicin and bortezomib. In conclusion, we have identified potent anti-myeloma activity for this novel HDAC inhibitor that gives further insights into the biological sequelae of HDAC inhibition in MM

Neri P, Bahlis NJ, Lonial S. Panobinostat for the treatment of multiple myeloma. *Expert Opin Investig Drugs* 2012 May;21(5):733-47.

Abstract: INTRODUCTION: Multiple myeloma (MM) is a B-cell malignancy characterized by proliferation of monoclonal plasma cells in the bone marrow. Although new therapeutic options have been introduced and response rates have improved in recent years, MM still remains incurable and new treatment options are urgently needed. The histone deacetylase inhibitors (HDACi) are a new class of anticancer agents in early clinical development in many malignancies including MM. HDACi target the enzyme histone deacetylase (HDAC) involved in the deacetylation of histone and non-histone cellular proteins that play important roles in epigenetic regulation of gene expression inducing death, apoptosis and cell cycle arrest in cancer cells. Panobinostat (LBH589) is a highly potent HDACi with demonstrated antitumor activities at low nanomolar concentration in several preclinical studies and its clinical efficacy is currently under investigation in several clinical trials. AREA COVERED: In this review the authors discuss the role of HDACs in the regulation of gene expression and the biological mechanisms mediating the anticancer effects of HDACi with particular focus on the recent development of panobinostat as anti-MM agent in preclinical and clinical studies. EXPERT OPINION: As a 'multi-target' drug, panobinostat appears attractive as potential anti-MM therapeutic for its ability to modulate a variety of biological pathways essential in MM biology. This 'multi-target' property of panobinostat may also be one its major shortcomings, and a better understanding of its mechanisms of action and targets will permit to identify the best combination therapies that will ultimately overcome and improve outcomes in MM patients

Cea M, Cagnetta A, Gobbi M et al. New Insights into the Treatment of Multiple Myeloma with Histone Deacetylase Inhibitors. *Curr Pharm Des* 2012 September 26.

Abstract: Multiple Myeloma (MM) is a common hematologic malignancy of plasma cells representing an excellent model of epigenomics dysregulation in human disease. Importantly, these findings, in addition to provide a better understanding of the underlying molecular changes leading to this malignance, furnish the basis for an innovative therapeutic approach. Histone deacetylase inhibitors (HDACIs), including Vorinostat and Panobinostat, represent a novel class of drugs targeting enzymes involved in epigenetic regulation of gene expression, which have been evaluated also for the treatment of multiple myeloma. Although the clinical role in this setting is evolving and their precise utility remains to be determined, to date that single-agent anti-MM activity is modest. More importantly, HDACIs appear to be synergistic both in vitro and in vivo when combined with other anti-MM agents, mainly proteasome inhibitors including bortezomib. The molecular basis underlying this synergism seems to be multifactorial and involves interference with protein degradation as well as the interaction of myeloma cells with microenvironment. Here we review molecular events underling antitumor effects of HDACIs and the most recent results of clinical trials in relapsed and refractory MM

Witzig TE, Timm M, Stenson M, Svingen PA, Kaufmann SH. Induction of apoptosis in malignant B cells by phenylbutyrate or phenylacetate in combination with chemotherapeutic agents. *Clin Cancer Res* 2000 February;6(2):681-92.

Abstract: Phenylacetate (PA) and phenylbutyrate (PB) are aromatic fatty acids that are

presently undergoing evaluation as potential antineoplastic agents. In vitro, PA and PB cause differentiation or growth inhibition of malignant cells. Clinical trials of these drugs as single agents indicate that they are not myelosuppressive; therefore, combinations with other chemotherapy agents may be possible. The goals of this study were to determine whether PA and PB (a) are cytotoxic to malignant B cells from patients with non-Hodgkin's lymphoma and B-cell chronic lymphocytic leukemia and (b) exhibit additive or synergistic induction of apoptosis when administered to myeloma cell lines in combination with conventional drugs. In the clinical specimens, cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and percent apoptosis was measured using 7-aminoactinomycin D and flow cytometry. Viability was decreased by > 50% in 7% (1/15) of non-Hodgkin's lymphoma samples treated with 5 mM PA, 27% treated with 1 mM PB, and 60% treated with 2 mM PB. Likewise, viability was decreased by > 50% in 44% (4/9) of chronic lymphocytic leukemia samples treated with 5 mM PA, 67% treated with 1 mM PB, and 100% treated with 2 mM PB. Studies in the myeloma cell lines demonstrated that PB treatment induced activation of caspases 3, 7, and 9 accompanied by cleavage of their substrates and internucleosomal DNA degradation. Combinations of PA or PB with conventional drugs (cytarabine, topotecan, doxorubicin, etoposide, chlorambucil, melphalan, fludarabine, carboplatin, and cisplatin) were examined for synergism (combination index < 1 in median effect analysis) in inducing apoptosis of both the MY5 and 8226 human myeloma cell lines. At concentrations that killed > 50% of cells, most combinations were additive; however, PB was synergistic with cytarabine, etoposide, and topotecan, with the combination index < 1 at each of the 50, 75, and 95% apoptosis levels. These observations indicate that PA and PB can induce apoptosis in malignant B cells and enhance the cytotoxicity of agents used in the treatment of these malignancies

Autophagy Inhibitors

Cytotoxic chemotherapy typically triggers autophagy in multiple myeloma and other cancers, and more often than not this autophagic response suppresses apoptosis and aids cancer survival. Hence, autophagy inhibitors, such as the anti-malarial drug hydroxychloroquine, have potential as adjuvants to chemotherapy of MM. However, there is one report that that autophagy inhibitors 3AM and chloroquine, although they are directly toxic to MM cells, actually suppress the apoptotic response to bortezomib. Hence, it may not be prudent to use hydroxychloroquine in conjunction with proteasome inhibitors until this issue is further clarified.

Pan Y, Gao Y, Chen L et al. Targeting autophagy augments in vitro and in vivo antimyeloma activity of DNA-damaging chemotherapy. *Clin Cancer Res* 2011 May 15;17(10):3248-58.

Abstract: PURPOSE: Although autophagy occurs in most tumor cells following DNA damage, it is still a mystery how this DNA-damaging event turns on the autophagy machinery in multiple myeloma (MM) and how the functional status of autophagy impacts on its susceptibility to death in response to DNA-damaging chemotherapy. EXPERIMENTAL DESIGN: We investigate the effects of DNA damage on autophagy in MM cells and elucidate its underlying molecular mechanism. Then, we examined the impacts of pharmacologic or genetic inhibition of autophagy on DNA damage-induced apoptosis. Furthermore, the antimyeloma activity of autophagy inhibitor in combination with DNA-damaging agents was evaluated in MM xenograft models. RESULTS: We showed that DNA-damaging drugs, doxorubicin and melphalan, induce caspase-dependent apoptosis and concurrently trigger

Beclin 1-regulated autophagy in human MM cell lines H929 and RPMI 8226. Mechanistically, association of autophagy execution proteins Beclin 1 with class III phosphoinositide 3-kinase, which is inhibited by Bcl-2 recruitment, contributes directly to the autophagic process. Importantly, targeting suppression of autophagy by minimally toxic concentrations of pharmacologic inhibitors (hydroxychloroquine and 3-methyladenine) or short hairpin RNAs against autophagy genes, Beclin 1 and Atg5, dramatically augments proapoptotic activity of DNA-damaging chemotherapy both in vitro using MM cell lines or purified patient MM cells and in vivo in a human plasmacytoma xenograft mouse model. CONCLUSION: These data can help unravel the underlying molecular mechanism of autophagy in DNA-damaged MM cells and also provide a rationale for clinical evaluation of autophagy inhibitors in combination with DNA-damaging chemotherapy in MM

Zang Y, Thomas SM, Chan ET et al. The next generation proteasome inhibitors carfilzomib and oprozomib activate prosurvival autophagy via induction of the unfolded protein response and ATF4. *Autophagy* 2012 September 20;8(12).

Abstract: The proteasome inhibitor bortezomib has shown remarkable clinical success in the treatment of multiple myeloma. However, the efficacy and mechanism of action of bortezomib in solid tumor malignancies is less well understood. In addition, the use of this first-in-class proteasome inhibitor is limited by several factors, including off-target effects that lead to adverse toxicities. We recently reported the impact and mechanisms of carfilzomib and oprozomib, second-in-class proteasome inhibitors with higher specificities and reduced toxicities, against head and neck squamous cell carcinoma (HNSCC). Carfilzomib and oprozomib potently inhibit HNSCC cell survival and the growth of HNSCC tumors. Both compounds promote upregulation of proapoptotic BIK and antiapoptotic MCL1, which serves to mediate and attenuate, respectively, the killing activities of these proteasome inhibitors. Both compounds also induce complete autophagic flux that is partially dependent on activation of the unfolded protein response (UPR) and upregulation of ATF4. Carfilzomib- and oprozomib-induced autophagy acts to promote HNSCC cell survival. Our study indicates that the therapeutic benefit of these promising proteasome inhibitors may be improved by inhibiting MCL1 expression or autophagy

Kawaguchi T, Miyazawa K, Moriya S et al. Combined treatment with bortezomib plus bafilomycin A1 enhances the cytotoxic effect and induces endoplasmic reticulum stress in U266 myeloma cells: crosstalk among proteasome, autophagy-lysosome and ER stress. *Int J Oncol* 2011 March;38(3):643-54.

Abstract: Bortezomib (BZ), a first line 26S proteasome inhibitor, induces a potent cytotoxic effect with caspase-3 activation in multiple myeloma (MM) cell lines. Since I κ B is a substrate of the proteasome, the initial rationale for using BZ in MM has been to inhibit NF- κ B. However, BZ rather activated NF- κ B activity in U266 cells. BZ induces autophagy as well as endoplasmic reticulum (ER) stress in various cell lines tested. Inhibition of initial autophagosome formation by treatment with either 3-methyladenine or siRNA for LC3B in U266 cells and knockdown of the atg5 gene in a murine embryonic fibroblastic cell line all resulted in attenuation of BZ-induced cell death. In contrast, combined treatment with BZ and bafilomycin A1 (BAF), which is a specific inhibitor of vacuolar-ATPase and is used as an autophagy inhibitor at the late stage, resulted in synergistic cytotoxicity, compared with that by either BZ or BAF alone. BAF treatment also induced ER stress, but the kinetics of inductions of ER stress-related genes [e.g. CHOP (GADD153) and GRP78] completely differed between BZ- and BAF-treatments: BZ induced these ER stress markers within 8 h, whereas treatment with BAF required more than 48 h in U266 cells. In order to synchronize ER stress,

we pre-treated U266 cells with BAF for 48 h, followed with BZ for 48 h. The sequential treatment with BAF and BZ induced a further enhanced cytotoxicity, compared with the simultaneous combination of BAF and BZ. These data suggest crosstalk among the ubiquitin-proteasome system, the autophagy-lysosome system, and ER stress. Controlling these interactions and kinetics appears to have important implications for optimizing clinical cancer treatment including MM-therapy

Hoang B, Benavides A, Shi Y, Frost P, Lichtenstein A. Effect of autophagy on multiple myeloma cell viability. *Mol Cancer Ther* 2009 July;8(7):1974-84.

Abstract: Because accumulation of potentially toxic malformed protein may be extensive in immunoglobulin-producing multiple myeloma (MM) cells, we investigated the phenomenon of autophagy in myeloma, a physiologic process that can protect against malformed protein under some circumstances. Autophagy in MM cell lines that express and secrete immunoglobulin and primary specimens was significantly increased by treatment with the endoplasmic reticulum stress-inducing agent thapsigargin, the mammalian target of rapamycin inhibitor rapamycin, and the proteasome inhibitor bortezomib. Inhibition of basal autophagy in these cell lines and primary cells by use of the inhibitors 3-methyladenine and chloroquine resulted in a cytotoxic effect that was associated with enhanced apoptosis. Use of small interfering RNA to knock down expression of beclin-1, a key protein required for autophagy, also inhibited viable recovery of MM cells. Because the data suggested that autophagy protected MM cell viability, we predicted that autophagy inhibitors would synergize with bortezomib for enhanced antimyeloma effects. However, the combination of these drugs resulted in an antagonistic response. In contrast, the autophagy inhibitor 3-methyladenine did synergize with thapsigargin for an enhanced cytotoxic response. These data suggest that autophagy inhibitors have therapeutic potential in myeloma but caution against combining such drugs with bortezomib

AMPK Activators

Metformin, which shows intriguing promise for the prevention and treatment of many cancers, has not yet been studied, pre-clinically or clinically, in multiple myeloma. However, there is a report that activation of AMPK, its clinical target, can slow cell cycle progression of MM cell lines, via S-phase arrest. In addition, activation of Akt, Erk, and mTOR is decreased, effects which could make MM more vulnerable to chemotherapy. Intriguingly, there are many reports that resveratrol, many of whose intriguing “pro-longevity” effects in rodents are now known to be mediated by AMPK activation, can suppress growth of MM cell lines, and also has an anti-osteoclast effect potentially useful in clinical MM. While the pharmacokinetics of resveratrol render it of little value for clinical use, metformin might be able to replicate its favorable effects on MM control.

Baumann P, Mandl-Weber S, Emmerich B, Straka C, Schmidmaier R. Activation of adenosine monophosphate activated protein kinase inhibits growth of multiple myeloma cells. *Exp Cell Res* 2007 October 1;313(16):3592-603.

Abstract: The role of adenosine monophosphate activated protein kinase (AMPK) in regulating multiple myeloma (MM) cell growth is not yet clear. In this study, we show that the AMPK activators 5-aminoimidazole-4-carboxamide riboside (AICAr) and D942 inhibit cell growth in MM cell lines. AICAr also induced an S-phase cell cycle arrest in all four tested cell lines and

led to phosphorylation and thus activation of AMPK. Furthermore, the inhibition of a nucleoside transporter by nitrobenzyl-thio-9-beta-d-ribofuranosylpurine (NBTI), inhibition of the adenosine kinase by iodotubericidine and inhibition of AMPK by AMPKI Compound C reversed AICAr effects, indicating that the cellular effects of AICAr were mediated by AMPK. Activation of AMPK inhibited basal extracellular signal-regulated kinase (ERK), mammalian target of rapamycin (mTOR) and P70S6 kinase (P70S6K) as well as AKT phosphorylation, and blocked IL-6, IGF-1, and HS-5 stromal cell conditioned medium-induced increase of cell growth. Troglitazone, which has previously been shown to activate AMPK, similarly inhibited MM cell growth, activated AMPK, and decreased ERK and P70S6K phosphorylation. Our results suggest that activation of AMPK inhibits MM cell growth despite stimulation with IL-6, IGF-1, or HS-5 stromal cell conditioned medium and represents a potential new target in the therapy of MM

Wang FM, Galson DL, Roodman GD, Ouyang H. Resveratrol triggers the pro-apoptotic endoplasmic reticulum stress response and represses pro-survival XBP1 signaling in human multiple myeloma cells. *Exp Hematol* 2011 October;39(10):999-1006.

Abstract: OBJECTIVE: Resveratrol, trans-3, 4', 5,-trihydroxystilbene, suppresses multiple myeloma (MM). The endoplasmic reticulum (ER) stress response component inositol-requiring enzyme 1alpha (IRE1alpha)/X-box binding protein 1 (XBP1) axis is essential for MM pathogenesis. We investigated the molecular action of resveratrol on IRE1alpha/XBP1 axis in human MM cells. MATERIALS AND METHODS: Human MM cell lines ANBL-6, OPM2, and MM.1S were utilized to determine the molecular signaling events following treatment with resveratrol. The stimulation of IRE1alpha/XBP1 axis was analyzed by Western blot and reverse transcription polymerase chain reaction. The effect of resveratrol on the transcriptional activity of spliced XBP1 was assessed by luciferase assays. Chromatin immunoprecipitation was performed to determine the effects of resveratrol on the DNA binding activity of XBP1 in MM cells. RESULTS: Resveratrol activated IRE1alpha as evidenced by XBP1 messenger RNA splicing and phosphorylation of both IRE1alpha and its downstream kinase c-Jun N-terminal kinase in MM cells. These responses were associated with resveratrol-induced cytotoxicity of MM cells. Resveratrol selectively suppressed the transcriptional activity of XBP1s while it stimulated gene expression of the molecules that are regulated by the non-IRE1/XBP1 axis of the ER stress response. Luciferase assays indicated that resveratrol suppressed the transcriptional activity of XBP1s through sirtuin 1, a downstream molecular target of resveratrol. Chromatin immunoprecipitation studies revealed that resveratrol decreased the DNA binding capacity of XBP1 and increased the enrichment of sirtuin 1 at the XBP1 binding region in the XBP1 promoter. CONCLUSIONS: Resveratrol exerts its chemotherapeutic effect on human MM cells through mechanisms involving the impairment of the pro-survival XBP1 signaling and the activation of pro-apoptotic ER stress response

Kupisiewicz K. Biological aspects of altered bone remodeling in multiple myeloma and possibilities of pharmacological intervention. *Dan Med Bull* 2011 May;58(5):B4277.

Abstract: Multiple myeloma is a fatal B cell neoplasm often resulting in focal and in some cases more diffuse destruction of bone. The bone destruction is a result of increased activity of bone resorbing cells--multinucleated osteoclasts emerging through of multiple fusions. In multiple myeloma, clonally expanding cancer cells provide a stimulatory signal for osteoclast recruitment, differentiation and excessive bone resorption. The stimulatory actions of myeloma cells are believed to be mediated via the production of cytokines and local factors or by modulating bone microenvironment in order to stimulate osteoclastic bone resorption. However, our recent study revealed potentially a novel and more intimate contribution of

myeloma cells to the bone destruction. Our analysis of the bone biopsies from myeloma patients showed fully integrated malignant nuclei inside osteoclasts, which were transcriptionally active. As a result, about 30% of the osteoclasts in the bone marrow biopsies from myeloma patients were in fact osteoclast-myeloma cell hybrids. As the functional relevance of this novel cell type remained uncertain, the aim of my PhD study became to 1) strengthen the evidence of the existence of hybrid cells, 2) elucidate the functional differences between hybrid cells and non-hybrid OCs and 3) relate these findings to the pathogenesis of osteolytic disease in multiple myeloma. To this end, I developed a culture model of osteoclast-myeloma cell fusion between (pre)osteoclasts already committed to fuse and myeloma cells selected for adherence. The model was applied for testing of the bone resorptive properties of hybrid cells identified by labelling with green fluorescence. When comparing the highly fluorescent and non-fluorescent OCs on bone slices, it seemed that the frequency of highly fluorescent osteoclasts actively resorbing bone was increased as compared with non-fluorescent osteoclasts. This was assessed in two independent ways. Furthermore, these fluorescent osteoclasts appear to resorb deeper compared to non-fluorescent osteoclasts. The preliminary data that need to be confirmed suggest that formation of hybrid cells by fusion of myeloma cells with osteoclasts may result in reprogramming of the osteoclasts and contribute to the more aggressive bone resorption by osteoclasts as it is typically seen in myeloma patients. Another aspect of multiple myeloma and associated bone disease is the unmet need for novel and more efficient therapeutic regimens. Resveratrol (trans-3, 4', 5-trihydroxystilbene; RSV) is a natural compound shown to target the key players of myeloma bone disease: bone resorbing osteoclasts, bone forming osteoblasts and myeloma cells. Our in vitro study on RSV showed that it possessed this ideal triad of properties appearing and thus might be of interest as a potential drug for the treatment of multiple myeloma. RSV suppresses the growth and survival of myeloma cells, inhibits osteoclasts and stimulates the formation of osteoblasts. However, the need for high concentrations combined with low biological availability after oral administration and risk of important side effects stimulated a search for RSV derivatives with the same spectrum of actions but safer and with better bioavailability. As the other task of my PhD, I screened structurally modified RSV analogues in cultures of myeloma cells, osteoblasts and osteoclasts. Compared to resveratrol, some analogues showed an up to 5,000-times increased potency to inhibit osteoclast differentiation and could still promote osteoblast maturation but they did not antagonize myeloma cells. The potency of the best-performing candidate in vitro was tested in vivo in an ovariectomy-induced model of osteoporosis, but effect on bone loss could not be detected. During my PhD, I also participated in the studies of the effect of the proteasome inhibitor - bortezomib on osteoclasts conducted at the department. Based on its potent activity in multiple myeloma, bortezomib was accepted as a front-line treatment of myeloma patients by EMEA for the European Union. In our study we assessed the effect of bortezomib on osteoclasts in cultures under the conditions that mimic the pulse-treatment regime used for myeloma patients. The pulse administration of bortezomib significantly inhibited OC activity and, moreover, significantly but transiently reduced levels of two bone resorption markers measured in serum of treated myeloma patients. In MM the clonal expansion of malignant plasma cells results in the unbalanced bone remodelling, therefore it is essential to understand the molecular mechanisms governing the actions of osteoclasts and osteoblasts. During my PhD, I was involved in the investigations of mesenchymal stem cells over-expressing delta like protein - 1(Dlk-1) previously shown to inhibit the differentiation of mesenchymal stem cells (MSC) into osteoblasts. In results, the over-expression of Dlk-1 evoked pro-inflammatory phenotype in MSC suggesting the involvement of Dlk-1 in the immune response

Hu Y, Sun CY, Huang J, Hong L, Zhang L, Chu ZB. Antimyeloma effects of resveratrol through inhibition of angiogenesis. *Chin Med J (Engl)* 2007 October 5;120(19):1672-7.

Abstract: BACKGROUND: In multiple myeloma (MM), bone marrow angiogenesis parallels tumour progression and correlates with disease activity. Recent studies have proved resveratrol possesses antiangiogenic activity in vitro and in vivo. In this study, we examined the effects of resveratrol on myeloma cell dependent angiogenesis and the effects of resveratrol on some important angiogenic factors of RPMI 8226 cells. METHODS: RPMI 8226 cells were cocultured with human umbilical vein endothelial cells (HUVECs) to evaluate the effects of myeloma cells on angiogenesis. The RPMI 8226 cells were treated with various concentrations of resveratrol (6.25 - 50.00 micromol/L) for different times (12 - 72 hours). Reverse transcriptase polymerase chain reaction (RT-PCR) was used to assay vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), metalloproteinases (MMP)-2 and MMP-9 mRNA. Gelatin zymography was used to analyze MMP-2 and MMP-9 activity. VEGF and bFGF proteins secreted by the cells in the medium were quantified by enzyme linked immunosorbent assay (ELISA). RESULTS: Cell proliferation, migration and differentiation of HUVECs markedly increased by coculture with RPMI 8226 cells. Resveratrol inhibited proliferation, migration and tube formation of HUVECs cocultured with myeloma cells in a dose dependent manner. Treatment of RPMI 8226 cells with resveratrol caused a decrease in MMP-2 and MMP-9 activity. Resveratrol inhibited VEGF and bFGF protein expression in a dose and time dependent manner. Furthermore, decreased levels of VEGF, bFGF, MMP-2 and MMP-9 mRNA from cells treated with various concentrations of resveratrol confirmed its antiangiogenic action at the level of gene expression. CONCLUSIONS: Resveratrol inhibits multiple myeloma angiogenesis by regulating expression and secretion of VEGF, bFGF, MMP-2 and MMP-9. Resveratrol may be a potential candidate for the treatment of multiple myeloma

Bhardwaj A, Sethi G, Vadhan-Raj S et al. Resveratrol inhibits proliferation, induces apoptosis, and overcomes chemoresistance through down-regulation of STAT3 and nuclear factor-kappaB-regulated antiapoptotic and cell survival gene products in human multiple myeloma cells. *Blood* 2007 March 15;109(6):2293-302.

Abstract: Whether resveratrol, a component of red grapes, berries, and peanuts, could suppress the proliferation of multiple myeloma (MM) cells by interfering with NF-kappaB and STAT3 pathways, was investigated. Resveratrol inhibited the proliferation of human multiple myeloma cell lines regardless of whether they were sensitive or resistant to the conventional chemotherapy agents. This stilbene also potentiated the apoptotic effects of bortezomib and thalidomide. Resveratrol induced apoptosis as indicated by accumulation of sub-G(1) population, increase in Bax release, and activation of caspase-3. This correlated with down-regulation of various proliferative and antiapoptotic gene products, including cyclin D1, cIAP-2, XIAP, survivin, Bcl-2, Bcl-xL, Bfl-1/A1, and TRAF2. In addition, resveratrol down-regulated the constitutive activation of AKT. These effects of resveratrol are mediated through suppression of constitutively active NF-kappaB through inhibition of IkappaBalpha kinase and the phosphorylation of IkappaBalpha and of p65. Resveratrol inhibited both the constitutive and the interleukin 6-induced activation of STAT3. When we examined CD138(+) plasma cells from patients with MM, resveratrol inhibited constitutive activation of both NF-kappaB and STAT3, leading to down-regulation of cell proliferation and potentiation of apoptosis induced by bortezomib and thalidomide. These mechanistic findings suggest that resveratrol may have a potential in the treatment of multiple myeloma

Sun CY, Hu Y, Guo T et al. Resveratrol as a novel agent for treatment of multiple myeloma with matrix metalloproteinase inhibitory activity. *Acta Pharmacol Sin* 2006 November;27(11):1447-52.

Abstract: AIM: To examine the in vitro antitumor activity of resveratrol against multiple myeloma (MM) cell lines (RPMI 8226, U266, and KM3), and the mechanisms involved. **METHODS:** The growth inhibition of resveratrol was determined by 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The effect of resveratrol on the apoptosis was investigated by combined annexin V-propidium iodide staining. The effect of resveratrol on the invasion through Matrigel matrix was detected by transwell invasion analyses. The activity of matrix metalloproteinase (MMP)-2 and -9 proteins were determined by gelatin zymography analysis. The expression of MMP-2, MMP-9, Bcl-2, Bcl-x(L), XIAP and Bax protein were detected using Western blotting analysis. **RESULTS:** Resveratrol inhibited proliferation of MM cells in a dose- and time-dependent manner. Incubation of MM cells with resveratrol resulted in apoptotic cell death. Resveratrol down-regulated the expression of the antiapoptotic proteins Bcl-2, Bcl-x(L) and XIAP and up-regulated the expression of the proapoptotic protein Bax. Furthermore, resveratrol inhibited invasion of RPMI 8226, U266, and KM3 cells with IC50 values of 64+/-8 micromol/L, 93+/-11 micromol/L, and 153+/-11 micromol/L, respectively. Resveratrol inhibited the constitutive expression of MMP-2 and -9 proteins of MM cells and suppressed its gelatinolytic activity. **CONCLUSION:** Resveratrol inhibits the proliferation of MM cells by inducing apoptotic cell death. Resveratrol also inhibits MM cell invasion. The inhibition of invasion may be associated with the attenuation of the enzymatic activities of MMP-2 and -9

7Sun C, Hu Y, Liu X et al. Resveratrol downregulates the constitutional activation of nuclear factor-kappaB in multiple myeloma cells, leading to suppression of proliferation and invasion, arrest of cell cycle, and induction of apoptosis. *Cancer Genet Cytogenet* 2006 February;165(1):9-19.

Abstract: Resveratrol has been proposed to act as a chemopreventive agent in numerous epidemiologic studies and has been shown to inhibit proliferation of various tumor cells in vitro. In the present study, we investigated the antitumor effects of resveratrol on multiple myeloma (MM) cells and the mechanisms involved. Our findings indicated that resveratrol inhibited proliferation of tumor cells in a dose- [corrected] dependent manner by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and [3H]thymidine incorporation assay. Resveratrol also enhanced the inhibitory effect of dexamethasone on the growth of MM cells by MTT assay. Flow cytometric analysis demonstrated that resveratrol arrested the cells at the G1 and S phases of the cell cycle. Because nuclear factor-kappaB (NF-kappaB) plays a key role in cell survival and proliferation of human MM cells, we tested the effect of resveratrol on NF-kappaB expression by Western blot analysis and immunofluorescence. NF-kappaB was constitutively active in all human MM cell lines examined, and resveratrol down-regulated NF-kappaB expression in all cell lines. Resveratrol also down-regulated the expression of NF-kappaB-regulated gene products by Western blot analysis, gelatin zymography, and enzyme-linked immunosorbent assay, including interleukin-6, Bcl-2, Bcl-xL, XIAP, c-IAP, vascular endothelial growth factor (VEGF), and matrix metalloproteinase-9 (MMP-9), which modulates an array of signals controlling cellular survival and proliferation and tumor promotion. Indeed, annexin V-fluoroisothiocyanate and Transwell invasion analyses revealed that incubation of MM cells with resveratrol resulted in apoptotic cell death and inhibition of invasion. In conclusion, these data suggest that resveratrol is an effective in vitro inhibitor of NF-kappaB in human MM cells. Resveratrol plays a role in suppressing the proliferation of MM cells and induces apoptosis, thus providing the molecular basis for the treatment of MM patients with this compound

Boissy P, Andersen TL, Abdallah BM, Kassem M, Plesner T, Delaisse JM. Resveratrol inhibits myeloma cell growth, prevents osteoclast formation, and promotes osteoblast differentiation. *Cancer Res* 2005 November 1;65(21):9943-52.

Abstract: Multiple myeloma is characterized by the accumulation of clonal malignant plasma cells in the bone marrow, which stimulates bone destruction by osteoclasts and reduces bone formation by osteoblasts. In turn, the changed bone microenvironment sustains survival of myeloma cells. Therefore, a challenge for treating multiple myeloma is discovering drugs targeting not only myeloma cells but also osteoclasts and osteoblasts. Because resveratrol (trans-3,4',5-trihydroxystilbene) is reported to display antitumor activities on a variety of human cancer cells, we investigated the effects of this natural compound on myeloma and bone cells. We found that resveratrol reduces dose-dependently the growth of myeloma cell lines (RPMI 8226 and OPM-2) by a mechanism involving cell apoptosis. In cultures of human primary monocytes, resveratrol inhibits dose-dependently receptor activator of nuclear factor-kappaB (NF-kappaB) ligand-induced formation of tartrate-resistant acid phosphatase (TRACP)-positive multinucleated cells, TRACP activity in the medium, up-regulation of cathepsin K gene expression, and bone resorption. These inhibitions are associated with a down-regulation of RANK expression at both mRNA and cell surface protein levels and a decrease of NFATc1 stimulation and NF-kappaB nuclear translocation, whereas the gene expression of *c-fms*, CD14, and CD11a is up-regulated. Finally, resveratrol promotes dose-dependently the expression of osteoblast markers like osteocalcin and osteopontin in human bone marrow mesenchymal stem cells (hMSC-TERT) and stimulates their response to 1,25(OH)₂ vitamin D₃ [1,25(OH)₂D₃]. Moreover, resveratrol up-regulates dose-dependently the expression of 1,25(OH)₂D₃ nuclear receptor. Taken together, these results suggest that resveratrol or its derivatives deserve attention as potential drugs for treating multiple myeloma

Jazirehi AR, Bonavida B. Resveratrol modifies the expression of apoptotic regulatory proteins and sensitizes non-Hodgkin's lymphoma and multiple myeloma cell lines to paclitaxel-induced apoptosis. *Mol Cancer Ther* 2004 January;3(1):71-84.

Abstract: Resveratrol (trans-3,4,5-trihydroxystilbene) has received attention for its potential chemopreventive and antitumor effects in experimental systems. Recent evidence suggests that paclitaxel, alone or in combination with other drugs, can be effectively used in the treatment of non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM). This study investigated whether resveratrol can sensitize NHL and MM cell lines to paclitaxel-mediated apoptosis and to delineate the underlying molecular mechanism of sensitization. Both resveratrol and paclitaxel negatively modulated tumor cell growth by arresting the cells at the G(2)-M phase of the cell cycle. Low concentrations of resveratrol exerted a sensitizing effect on drug-refractory NHL and MM cells to apoptosis induced by paclitaxel. Resveratrol selectively down-regulated the expression of antiapoptotic proteins Bcl-x(L) and myeloid cell differentiation factor-1 (Mcl-1) and up-regulated the expression of proapoptotic proteins Bax and apoptosis protease activating factor-1 (Apaf-1). Paclitaxel down-regulated the expression of Bcl-x(L), Mcl-1, and cellular inhibitor of apoptosis protein-1 antiapoptotic proteins and up-regulated Bid and Apaf-1. Combination treatment resulted in apoptosis through the formation of tBid, mitochondrial membrane depolarization, cytosolic release of cytochrome c and Smac/DIABLO, activation of the caspase cascade, and cleavage of poly(adenosine diphosphate-ribose) polymerase. Combination of resveratrol with paclitaxel had minimal cytotoxicity against quiescent and mitogenically stimulated human peripheral blood mononuclear cells. Inhibition of Bcl-x(L) expression by resveratrol was critical for chemosensitization and its functional impairment mimics resveratrol-mediated sensitization to paclitaxel-induced apoptosis. Inhibition of Bcl-x(L) expression by resveratrol was due to the inhibition of the extracellular signal-regulated

kinase 1/2 (ERK1/2) pathway and diminished activator protein-1-dependent Bcl-x(L) expression. The findings by resveratrol were corroborated with inhibitors of the ERK1/2 pathway. This study demonstrates that in resistant NHL and MM cell lines resveratrol and paclitaxel selectively modify the expression of regulatory proteins in the apoptotic signaling pathway and the combination, via functional complementation, results in synergistic apoptotic activity