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Mechanisms of cell death and survival in multiple myeloma (MM): Therapeutic implications

D. Chauhan and K. C. Anderson

The Jerome Lipper Multiple Myeloma Center, Department of Medical Oncology, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, USA

Multiple myeloma (MM), a hematologic malignancy, remains fatal despite all available therapies. Initial treatment with conventional drugs effectively induces MM cell death/apoptosis; however, prolonged drug exposures results in the development of *de novo* chemoresistance. Because MM is a bone marrow (BM) cancer, the progression of disease and drug efficacy is highly influenced by the BM microenvironment. Novel agents, such as proteasome inhibitors (PS-341), 2-methoxyestradiol (2ME2), thalidomide and its immunomodulatory derivatives (IMiDs), and histone deacetylase (HDAC) inhibitors target the MM cell in its BM microenvironment; thereby enhancing anti-MM activity as well as preventing development of drug-resistance. The transcriptional events and signaling pathways, which mediate these responses in MM cells are now being delineated, and may serve to identify novel therapeutic targets based upon interrupting MM cell growth or triggering MM cell death.

Keywords: apoptosis; mitochondria; multiple myeloma; protease inhibitor; protective signaling

Introduction

MM cells predominantly localize in the BM, where humoral factors facilitate MM cell growth and block the apoptotic effects of drugs.[1] For example, interleukin-6 (IL-6), and insulin growth factor (IGF) trigger growth and provide protection against Dexamethasone (Dex)-induced apoptosis in MM cells *via* activation of mitogen activated protein (MAP) kinase and phophatidylinositol-3 kinase/Akt kinase (PI3K/Akt) pathway (s).[2–4] Conversely, treatment with specific inhibitors of growth/survival signaling pathways abrogates MM cell growth and enhances anti-MM activity of drugs. Novel anti-MM agents target MM cell, and the BM microenvironment to overcome drug resistance.[3,4] The apoptotic mechanisms, which mediate the anti-MM effects of these novel drugs are now being defined.

Correspondence to: Kenneth C. Anderson, M557, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02215, USA. Tel.: (617)-632-2144; Fax: (617)-632-2140; e-mail: kenneth_anderson@dfci.harvard.edu

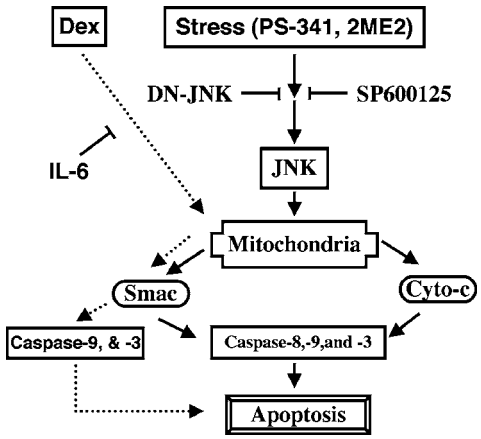
Programmed cell death (PCD) or apoptosis is associated with distinct morphological changes including membrane blebbing,[5] nuclear condensation,[6] chromatin aggregation, formation of apoptotic bodies,[7] and DNA fragmentation.[8] Induction of apoptosis occurs *via* sequential activation of initiator (procaspase-8, -9, -10) and effector caspases (caspase-3, -6, -7).[9,10] Caspases are present in cells as catalytically inactive zymogens.[10] Activation of effector caspases leads to cell death by proteolytic cleavage of various cellular targets: poly (ADP ribose) polymerase (PARP);[11] DNA-dependent protein kinase; protein kinase C-delta; and other substrates. Two potential upstream mechanisms trigger caspase cascades: first, by cross-linking of death receptors by their ligands; second, by release of mitochondrial apoptogenic proteins, cytochrome-c (cyto-c) and Smac (second mitochondriaderived activator of caspase).

In the context of MM, studies have shown that various anti-MM drugs trigger common apoptotic signals: reduction in the mitochondrial transmembrane potential (Deltapsim, $\Delta\Psi m$), caspase-3 and Poly (ADP ribose) polymerase (PARP) cleavage. However, these agents also induce differential and/or additional upstream signaling pathways that converge to common downstream pathways. Studies to date indicate occurrence of one or more of the following events during apoptosis: mitochondrial release of cyto-c and Smac; activation of stress activated protein kinase or c-Jun NH2-terminal kinase (SAPK/JNK) pathways; activation of death-receptor-initiated signals; and caspase-8, -9 and -3 activation. Apoptosis is also accompanied by downregulation of growth and anti-apoptotic signaling molecules: MAPK, PI3K, nuclear factor-kappa B (NF-κB), PKC, BCL2, and inhibitor of apoptosis proteins (IAPs). Here we summarize our current understanding of novel anti-MM agent-induced apoptotic signaling *vis-à-vis* conventional drug-triggered cell death signaling, as well as potential therapeutic implications in MM.

Role of mitochondria

The findings that 1) anti-apoptotic protein BCL2 localizes to the outer membrane of mitochondria;[12] 2) a

Figure 1. Delineation of Dexamethasone (Dex) *versus* novel anti-MM agents 2-Methoyestradiol (2ME2) or Proteasome inhibitor (PS-341)-induced apoptotic signaling cascades in MM cells. 2ME2 or PS-341 induces activation of JNK, which translocates to mitochondria and triggers the release of cyto-c and Smac. Blockade of JNK by either DN-JNK or a specific inhibitor of JNK SP600125, disables JNK-mediated cyto-c and Smac release, as well as associated apoptosis (regular arrows: 2ME2-induced signal; dashed arrows: Dex-induced signal). By contrast, Dex-induced apoptotic signaling proceeds without concurrent JNK activation and cyto-c release; however, Dex triggers Smac release and downstream activation of caspase-9 and -3.



mitochondrial-rich fraction is required for the induction of apoptosis in a cell-free system;[13] and 3) $\Delta\Psi m$ is lost during early stages of apoptosis,[14] all suggest that mitochondria play an important role in apoptosis. Under stress, mitochondrial proteins cyto-c and Smac are released from mitochondria to cytosol and activate caspase-9 by two distinct upstream pathways: first, cytosolic cyto-c binds to Apaf-1, resulting in Apaf-1 oligomerization and subsequent Capase-9 activation; second, cytosolic Smac binds to XIAP (an inhibitor of apoptosis protein) and thereby eliminates its inhibitory effects on caspase-9.[15,16] Our study showed that anti-MM agents use either one or both of these pathways to activate caspase-9. For example, 2ME2 and PS-341 activate caspase-9 *via* triggering the release of both cyto-c and Smac, whereas Dex activates caspase-9 only through Smac release (Figure 1). IL-6, a growth factor for MM, block Dex, but not 2ME2 or PS-341-induced apoptosis *via* preventing the release of Smac. Irradiation and IMiDs also trigger the release of cyto-c in MM cells, indicating that cyto-c is required for most drug-triggered cell death.[17] The findings that Dex does not induce cyto-c release, and that IL-6 protects MM cells against Dex-induced apoptosis, suggest that combining Dex with novel agents which trigger cyto-c release will enhance anti-MM activity of Dex.

Role of c-Jun NH2-terminal kinase (JNK)

The Stress activated protein kinase (SAPK) or JNK has been linked to apoptosis.[18,19] Previous studies have shown that stress stimuli (Irradiation, tumor necrosis factor, sphingomyelinase and UV light) activate JNK.[18–20] The mechanism whereby JNK triggers downstream apoptotic signaling is less well characterized. A recent study demonstrated that JNK mediates the release of cyto-c during apoptosis.[21] Studies in MM cells showed that 2ME2 or PS-341-induced apoptosis is associated with activation of JNK, translocation of JNK from cytosol to mitochondria, and release of both cyto-c and Smac from mitochondria to cytosol. Blocking JNK, either by dominant-negative mutant (DN-JNK) or cotreatment with a specific JNK inhibitor SP600125, abrogates both stress-induced release of Smac and induction of apoptosis (Figure 1).* The mechanism whereby JNK regulates the release of cyto-c is unclear. Studies in other cell types have shown that anti-apoptotic protein Bc12/Bclx, which are present constitutively in the outer membrane of mitochondria represent potential sites of regulation by JNK.[22,23] BH3 members of the BCL2 family can engage BAX and Bak to release cyto-c.[24] Importantly, the findings that Bax protein subfamily is essential for JNK-dependent apoptosis,[25] and that JNK is required for cyto-c release,[21] suggest an essential role of Bax in mediating JNK-dependent release of cyto-c. Whether similar mechanisms regulate the 2ME2 or PS-341-induced cyto-c or Smac release in MM cells remains to be determined. Nevertheless our findings demonstrate that activation of JNK is an obligatory event for release of cyto-c and Smac during 2ME2 or PS-341-induced apoptosis in MM cells.

JNK phosphorylates mitochondrial proteins during apoptosis; however, not all apoptotic agents trigger an activation of JNK and related mitochondrial pathways. For instance, our prior studies showed that Dex-induced apoptosis in MM cells occurs independent of JNK activation, but is associated with Smac release.[17,26,27] The observation that 2ME2 induces JNK activation suggests that JNK signaling is not defective in these cells. These findings are consistent with other studies demonstrating that anti-Fas-induced apoptosis is associated with the release of cyto-c in JNK-deficient MEF cells whereas UV-induced apoptosis in these same cells requies JNK activation for release of cyto-c.[21] Thus JNK activation and JNK-dependent release of mitochondrial proteins is stimulus specific (Figure 1).

*Chauhan D, Li G, Hideshima T, Podar K, Mitsiades C, Mitsiades N, Munshi N, Kharbanda S, Anderson KC. JNK-dependent release of mitochondrial protein, Smac, during apoptosis in multiple myeloma (MM) cells. *J Biol Chem* 2003 May 16; **278**(20): 17593–17596.

Signaling pathways mediating *de novo* drug resistance

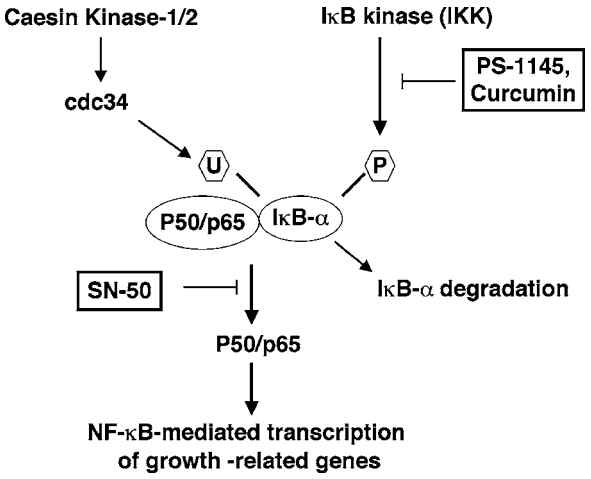
Conventional chemotherapy achieves responses in many MM patients, but eventually MM cells acquire drug resistance. For instance, Dex therapy is associated with acquired Dex-resistance in MM cells.[28] The factors regulating the development of drug-resistance include both binding to BM stromal cells (BMSCs) and the related cytokine network, which not only triggers MM cell growth but also blocks the apoptotic effects of various agents. For example, our earlier study showed the mechanism whereby IL-6 (a BMSCs-derived MM cell growth factor) prevents Dex-induced apoptosis in MM cells: Dex-induced apoptosis is mediated by activation of related adhesion focal tyrosine kinase (RAFTK); adhesion of MM cells to BMSCs induces transcription and secretion of IL-6; IL-6 induces activation of SHP2, a tyrosine phosphatase which dephosphorylates RAFTK, and thereby blocking Dex-induced apoptosis and promoting MM cell survival.[2] Therefore, growth factors present in the BM microenvironment interrupt chemotherapy-induced apoptotic signaling in MM cells. In this context, a recent study also showed that human MM cells upon binding to BMSCs become independent of IL-6/GP130/STAT3,[29–31] suggesting that BM-derived cytokines besides IL-6 also trigger growth/survival of MM cells and block anti-MM activity of drugs. Therefore, the key factor in determining the efficacy of a drug is its ability to inhibit growth/survival signaling and overcome the protective effects of the BM microenvironment (Figure 3).

Adhesion-mediated survival signaling *via* Nuclear factor-kappa B (NF-κB)

Adhesion of MM cells to BMSCs induces the paracrine secretion of cytokines that support MM cell growth and survival.[32,33] Adhesion of MM cells to fibronectin also confers a survival advantage for MM cells by inhibiting drug-induced apoptosis.[34] Our earlier study showed that adhesion of MM cells to BMSCs stimulates IL-6 transcription and secretion *via* activation of NF-κB,[35,36] which in turn triggers the proliferation of MM cells and protects them against Dex-induced apoptosis. Other studies on MM cells also confirmed the role of NF-κB in their growth and survival.

Activation of NF-κB involves the following sequential events: activation of IκB-α kinase (IKK) > IκB phosphorylation > Ubiquitination and degradation of IκB > disassociation of p50/65 NF-κB from IκB > nuclear translocation of p50/65 NF-κB[37] (Figure 2). Several anti-MM agents including Dex, thalidomide, and PS-341, inhibit NF-κB activity. Curcumin (diferuloylmethane) decreases constitutive activation of IKK in MM cells, leading to suppression of proliferation and induction of apoptosis.[38]

Figure 2. Blockade of NF-κB pathway in MM cells. Activation of NF-κB occurs as follows: induction of IκB kinase (IKK) activity → IκB phosphorylation → Ubiquitination and degradation of IκB → disassociation of p50/65 NF-κB from IκB → nuclear translocation of p50/65 NF-κB → NF-κB activation. PS-1145 or Curcumin, block IKK activation; prevents IκB phosphorylation-dependent ubiquitination and degradation of IκB, thereby not allowing disassociation of IκB from p50/65 NF-κB and NF-κB activation. SN-50 binds to p50 subunit of NF-κB and prevents its nuclear translocation and subsequent NF-κB activation. Of note, SN-50 does not inhibit IKK activation.



However, the effect of specific inhibition of NF-κB in MM has only been recently defined. Our studies have utilized two specific inhibitors of NF-κB to provide direct evidence for the role of NF-κB in MM (Figure 2). In particular, we observed high human telomerase reverse transcriptase (hTERT) catalytic subunit activity in MM cells compared to normals, consistent with its known function in cancer cells. Our results also showed that hTERT protein interacts directly with NF-κB p65. Importantly, treatment of MM cells with a cell-permeable specific inhibitor of NF-κB activity SN-50 abrogated interaction of activated NF-κB with hTERT, as well as nuclear translocation of this complex, thereby inhibiting telomerase function.[39] Another study demonstrated that SN-50 triggered apoptosis in MM cell lines and patient cells is associated with: downregulation of Bcl-2, A1, XIAP, cIAP-1 and -2, and survivin; upregulation of Bax; cytochrome c release; and caspase-9, -3, but not caspase-8, cleavage.[40] Furthermore, SN50 sensitized MM cells to both TNF-α and TNF-related apoptosisinducing ligand (TRAIL)/Apo2L-induced apoptosis. Finally, SN50 inhibited TNF-α-induced expression of another NF-κB target gene, intercellular adhesion molecule-1 (ICAM-1), which mediates homotypic adhesion of MM cells.[41]

In another study, we used an IκB kinase (IKK) inhibitor (PS-1145) as an inhibitor of NF-κB. PS-1145 and PS-341 blocked TNF-α-induced NF-κB activation by inhibiting phosphorylation and degradation of IκB-α.[37] Dex increased IκB-α protein and enhanced blockade

of NF-κB activation by PS-1145. PS-1145 also blocks the protective effect of IL-6 against Dex-induced apoptosis. Moreover, PS-1145 inhibited both IL-6 secretion from BMSCs triggered by MM cell adhesion, and proliferation of MM cells adherent to BMSCs.[37] However, in contrast to PS-341, PS-1145 only partially (20–50%) inhibited MM cell proliferation, suggesting that NF-κB blockade cannot account for all of the anti-MM activity of PS-341. Together, these studies demonstrate that specific targeting of NF-κB can overcome the growth and survival advantage conferred both by MM 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-κB.

MAPK, PI3K/Akt signaling during apoptosis

Apoptosis is associated with concurrent dysfunction of growth and survival pathways: growth factor/adhesion-induced MAPK, JAK/STAT, PI3K/Akt, or Src kinase signaling cascades; cell-cycle-related pathways; NF-κB signaling; as well as BCL2-mediated protective pathways.[3,4,42] Our earlier study demonstrated that Dexinduced apoptosis in MM cells is associated with downregulation of conventional growth pathway: Ras > Raf > MAPK together with downregulation of $p70^{rsk}$. Multiple studies have made similar observations using various conventional anti-MM drugs. We and others have shown that IL-6 and IGF induce growth of MM cells *via* Ras > Raf > MAPK and/or PI3-K/Akt signaling pathways in MM cells[43,44] (Figure 3). IL-6 induces activation of Akt/PKB activation and its downstream targets, including Bad, GSK-3β, and forkhead transcriptional factor (FKHR). Inhibition of Akt activation by the PI3K inhibitor LY294002 blocks both IL-6 triggered MEK/MAPK activation and proliferation; as well as Dex-induced apoptosis and associated caspase activation. We also showed that IL-6 triggered PI3-K/Akt signaling in MM.1S cells and downstream phosphorylation of FKHR, with related G1/S phase transition; conversely, LY294002 blocked this signaling, resulting in upregulation of p27 (KIP1) and G1 growth arrest. IGF-1 similarly stimulated sustained activation of NF-κB and Akt; induced phosphorylation of FKHRL-1; upregulated a series of intracellular anti-apoptotic proteins including FLIP, survivin, cIAP-2, A1/Bf1-1, and XIAP; and decreased Apo2L/TRAIL-sensitivity of MM cells.[45] Importantly, these studies provide the basis for future clinical trials combining conventional or novel agents with strategies designed to neutralize IGF-1 (Figure 3). In this context, a recent study demonstrated that cotreatment of MM cells with IL-6 superantagonist Sant-7 and Dex induced synergistic growth arrest and apoptosis.[46] Humanized anti-interleukin-6 receptor monoclonal antibody has been shown to induce apoptosis of both fresh and cloned human myeloma cells *in vitro*.[47]

Chemotherapy-induced protective signaling

Our recent study using oligonucleotide arrays showed that Dex not only triggered a cell death signal, but also simultaneously induced a protective/defensive signal.[48] For example, treatment of MM cells with Dex induced

Figure 3. Strategies to block growth and survival signaling pathways in MM cell. IL-6 and IGF-1 induce growth and survival by activating MAPK, PI3K/Akt, and JAK/STAT pathways. These pathways can be interrupted at various potential sites by using specific biochemical inhibitors, neutralizing antibodies, small peptides, or anti-sense oligonucleotides (ODNs) against the molecules mediating growth and survival.

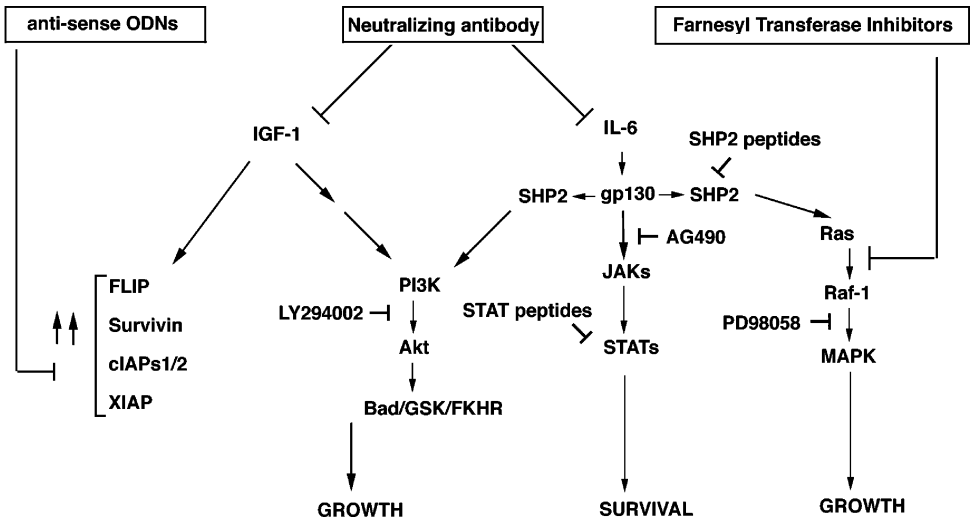
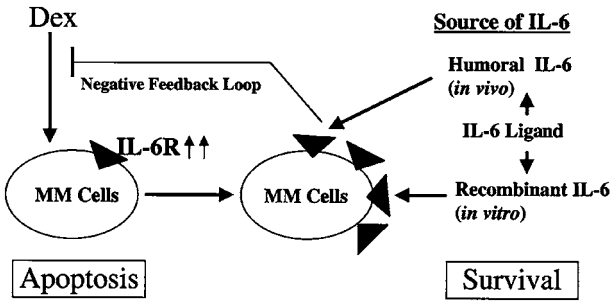


Figure 4. Schema showing a simultaneous induction of both cell death and protective signaling in response to Dex in MM cells. Treatment of MM cells with Dexamethasone (Dex) not only induce apoptotic signaling, but also concurrently triggers a protective/defense signaling, whereby increased IL-6R on the MM cell surface allow increased IL-6 binding and associated protection against Dex-induced apoptosis.



an early transient increase in IL-6 receptor (IL-6R) transcripts and protein levels. An increase in the availability of IL-6R molecules on the MM cell surface allowed enhanced binding of humoral IL-6 to its receptors, thereby triggering growth/anti-apoptotic signaling in MM cells (Figure 4). Furthermore, an increase in the TGF-βRII occurred early after Dex treatment of MM cells, consistent with a protective mechanism whereby increased TGF-βRII expression on the MM cell surface facilitated enhanced TGF-β binding, thereby triggering transcription and secretion of IL-6 and related protection against Dex-induced apoptosis. These data suggest that combination of Dex with agents that block the initiation of protective signaling may enhance the sensitivity as well as development of Dex-resistance. For example, addition of neutralizing antibodies against IL-6R, or IGF-receptor may disrupt the Dex-induced protective signaling, thereby increasing the anti-MM activity of Dex. Alternatively, using Dex with specific biochemical inhibitor of IL-6 or IGF-induced growth signaling pathways may provide synergistic anti-MM effects.

Role of BCL2 proteins

Our study has shown that treatment of MM cells with peptides targeting the BH3 domain of BCL2 enhanced PS-341-induced apoptosis.[49] Overexpression of BCL2 inhibited Bid cleavage and associated apoptosis.[50] Another recent study demonstrated that an anti-sense oligodeoxynucleotide (ODNs) complementary to the first six codons of the Bc1-2 mRNA, G3139 (oblimersen sodium; Genasense) downregulated the expression of BCL2 as well as enhanced sensitivity to Dex and Doxorubicin-induced apoptosis in MM cells. These data support further clinical evaluation of G3139 therapy in MM.[51] On the contrary, anti-sense ODNs to MCL1, but not BCL2 or Bclx, induced apoptosis, associated with a decrease in $\Delta\Psi$m.[52] Furthermore, inhibition of MCL1 also sensitized MM cells to Dex-induced apoptosis. Together, these findings suggest that disabling the function of BCL2 members using an anti-sense strategy adds to the apoptotic effect of anti-MM agents.

Newly identified anti-MM agents

A recent study showed that Farnesy1 transferase inhibitors induced apoptosis in MM cells in the presence of BMSCs, with complete inhibition of Ras/MAPK pathway. Blockade of cell cycle and growth signaling pathways by UCN-01 and MEK/2 inhibitors induced apoptosis and overcame drug resistance in MM cells.[53] Biochemical inhibitors of JAK-STAT pathways, piceatannol (JAK1/STAT3 inhibitor) and tyrphostin AG490 (JAK2/STAT3 inhibitor) sensitized MM cells to apoptosis induced by various therapeutic drugs including cisplatin, fludarabine, Adriamycin, and vinblastine.[54] Moreover, inhibition of histone deacetylase (HDAC) with suberoylanilide hydroxamic acid (SAHA) triggered apoptosis in MM and overcame cell-adhesion-mediated drug-resistance.[50] Another study demonstrated that targeting the BM microenvironment by inhibiting the interaction between receptor activator of nuclear factor-kappaB (RANK) ligand and RANK with recombinant osteoprotegerin (rOPG) not only inhibited the development of myeloma bone disease but also decreased tumor growth and increased survival.[55]

Conclusion

Delineation of cellular growth and apoptotic signaling cascades facilitates identification of molecular targets to interrupt of MM cell growth. For example, anti-MM drugs induce apoptosis *via* release of mitochondrial apoptogenic proteins cyto-c or Smac, suggesting that active peptides against these molecules may sensitize MM cells to various anti-MM agents. The observation that conventional drugs such as Dex induce apoptotic signaling, without cyto-c release or JNK activation, provides the preclinical rationale for combining Dex with novel agents that trigger both cyto-c release and JNK activation, in order to enhance anti-MM activity. Similarly, the combination of biochemical inhibitors of growth/survival signaling pathways with conventional or novel drugs will augment MM cell death. Furthermore, disabling antiapoptotic signaling (Mc11, Bc12, SHP2, or IAPs) using an antisense strategy may improve patient outcome. Finally, since most anti-MM drugs decrease mitochondrial function ($\Delta\Psi$m), novel mitochondria-targeted agents may become valuable tools to overcome resistance to conventional therapies. Further *in vitro* and *in vivo* studies in patient MM cells using gene expression profiling and proteomic analysis

will both define the role of specific regulators of apoptosis and provide the framework for novel targeted treatment strategies to enhance MM cell death.

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