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Preclinical Studies of Novel Targeted Therapies

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Summary

The bone marrow (BM) milieu confers drug resistance in multiple myeloma (MM) cells to conventional therapies. Therefore novel biologically-based therapies are needed. Preclinical studies have identified and validated molecular targeted therapeutics in MM. In particular, recognition of the biologic significance of the BM microenvironment both in MM pathogenesis and as a potential target for novel therapeutics has already derived several promising approaches. Thalidomide, lenalidomide (Revlimid®) and bortezomib (Velcade®) are directed not only at MM cells, but also BM milieu, and have rapidly from the bench to the bedside and FDA approval to treat MM.

Introduction

Despite advances in systemic and supportive therapies, MM remains incurable due to intrinsic or acquired chemotherapeutic resistance. High-dose chemotherapy with stem cell transplantation has significantly extended progression-free and overall survival, but cures few, if any, patients. Novel therapeutic approaches overcoming drug-resistance are therefore urgently needed in MM. The interaction of MM cells with extracellular matrix (ECM) proteins and BM stromal cells (SCs), as well as other components in the BM milieu (ie, osteoblast, osteoclast, vascular endothelial cells), plays a crucial role in MM cell pathogenesis and drug resistance. Importantly, novel biologically-based treatments which target not only the MM cell, but also the MM cell interaction with other accessory cells and cytokines/growth factors in the BM milieu, can overcome resistance to conventional therapies in both preclinical and clinical studies, and have great promise to improve patient outcome in MM.

The role of the BM microenvironment in MM

The BM microenvironment promotes MM cell growth, survival, migration and drug resistance. It is composed of different types of cellular component: including: hematopoietic stem cells; progenitor and precursor cells; immune cells; erythrocytes; BMSCs; BM endothelial cells (ECs); as well as osteoclasts and osteoblasts. These cells not only physically interact with MM cells, but also secrete growth and/or anti-apoptotic factors, such as interleukin (IL)-6, insulin-like growth factor (IGF)-1, vascular endothelial growth factor (VEGF), and tumor necrosis factor (TNF)-α, stromal cell-derived factor (SDF) 1α, and B-cell activating factor (BAFF). The interaction of these cellular components with growth/anti-apoptotic factors, several proliferative/anti-apoptotic signaling cascades in MM cells: phosphatidylinositol-3 kinase (PI3K)/Akt; Ras/Raf/mitogen-activated protein kinase (MAPK) kinase (MEK)/extracellular signal-related kinase (ERK); Janus kinase (JAK) 2/signal transducers and activators of

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transcription (STAT)-3; and nuclear factor (NF)-κB. These signaling cascades activate downstream target kinases and/or transcription factors which in turn regulate MM cell cycle progression, proliferation, and anti-apoptosis. Importantly, cytokines secreted from MM cells and BMSCs in turn further augment these signaling pathways [1–3]. Therefore, cytokines, their receptors, transcription factors and protein kinases represent potential targets for novel therapies (Figure 1).

Targeting growth factors and their receptors

1. IL-6

IL-6 mediates autocrine and paracrine growth of MM cells within the BM milieu (Figure 1). Specifically, some MM cells spontaneously secrete IL-6, and IL-6 secretion can be induced by CD 40 activation of tumor cells [4] or by cytokines (TNFα, VEGF, IL-1) within the BM microenvironment [5,6]. Most IL-6 in the BM milieu is secreted by BMSCs; importantly, transcription and secretion of IL-6 in BMSCs is upregulated both by binding of MM cells to BMSCs [7,8] and by secretion of cytokines (VEGF, TGF-β, TNFα) from MM cells [9–11]. IL-6-induced proliferation is associated with activation of Ras/Raf/mitogen-activated protein kinase kinase (MEK)/p42/44 MAPK signaling cascade [12,13], and can be abrogated by either MAPK antisense oligonucleotide or by the ERK or MEK inhibitor [14]. Survival of MM cells triggered by IL-6 is conferred via Janus kinase2 (JAK2)/signal transducers and activators of transcription (STAT) 3 signaling and downstream induction of Bcl-xL [15] and Mcl-1 expression [16,17]. IL-6 triggered drug (dexamethasone, Dex) resistance is mediated via phosphatidylinositol-3 kinase (PI3-K)/Akt signaling cascade, which can be neutralized by PI3K inhibitors (ie, wartmannin or LY294002). Specifically, Dex-mediated MM apoptosis is not associated with mitochondrial cytochrome c release [18], but is mediated by Second mitochondria activator of caspase (Smac) release, from mitochondria [19]; cytosolic Smac disrupts the inhibitor of apoptosis XIAP/caspase-9 complex, thereby allowing activation of caspase-9, caspase-3 cleavage, and apoptosis. IL-6 inhibits apoptosis triggered by Dex via PI3-K/Akt signaling [20]. We have used gene microarray profiling both to further delineate these cytokine-induced growth and anti-apoptotic pathways, and to derive targeted therapeutic strategies to overcome drug resistance based upon interrupting growth or triggering apoptotic signaling cascades [21]. For example, these studies have demonstrated that IL-6 induces the XBP-1 transcription factor [22], which is implicated in differentiation of normal B cells to plasma cells [23,24] and is markedly upregulated in freshly isolated MM patient samples.

Clinically, serum IL-6 and IL-6 receptors are prognostic factors which reflect the proliferative fraction of MM cells [25–27]. IL-6 or CRP, either alone or coupled with serum β2 microglobulin (β2m) as a measure of MM cell mass [28], provide one example of a biologically-based staging system in MM. Attempts to target IL-6 in treatment strategies to date have included antibodies to IL-6 and IL-6 receptor as well as IL-6 superantagonists (ie, Sant7) [29,30] which bind to IL-6R but do not trigger downstream signaling; although in vivo anti-MM activities have been observed, to date responses have only been transient.

2. IGF1

Insulin-like growth factor-1 is a multifunctional peptide that regulates cell proliferation, differentiation, and apoptosis [31,32]. In the circulation, IGF-1 binds mainly to the main IGF binding protein (IGFBP-3). Several studies suggest that high concentrations of circulating IGF-1 are associated with an increased risk of prostate, breast, lung, and colorectal cancer, whereas high IGFBP-3 concentrations are associated with a decreased risk [32]. However, the direct relationship of serum IGF-1 level and prognosis in MM has not yet been clarified. Standal et al reported that the mean IGF-1 level did not differ between MM patients and controls. However, IGF-1 was a strong indicator of prognosis: median survival of patients with low

levels (<13 nmol/l) of serum IGF-1 had not been reached at 80 months [33]. Previous studies have delineated the biological sequelae of IGF-1 in MM cells. Specifically, IGF-1 augments the proliferative and anti-apoptotic effects of IL-6 [34]. In contrast to IL-6, IGF-1 activates only Ras/Raf/MAPK kinase/ERK and PI3K/Akt signalling, but not JAK2/STAT3 pathways, via type1 IGF receptor (IGF1R) [35].

IGF-1 stimulates sustained activation of PI3K/Akt and NFjB; induces phosphorylation of FKHR (forkhead) transcription factor; upregulates a series of intracellular anti-apoptotic proteins including FLIP, survivin, cIAP-2, A1/Bfl-1, and XIAP; as well as decreases drug sensitivity of MM cells [36]. IGF-1 primes MM cell responsiveness to IL-6 and stimulates production of angiogenic cytokines [37]. Importantly, it is more potent than IL-6 in mediating these effects, setting the stage for novel MM treatments targeting IGF-1. IGF-1 also mediates MM cell migration via activation of PI3K/Akt signalling cascade [38]. The anti-apoptotic effect of IGF-1 has also been studied using an in vitro model system of MM cells in the BM milieu. Specifically, IGF-1 inhibits Dex-induced apoptosis in MM cell lines, without altering Bcl-2 or Bcl-XL proteins, associated with activation of ERK and PI3K/Akt signalling pathways [36]. IGF-1 mediates MM cell growth and survival in MM cells both in vitro [34] and in vivo [31]. Recently, we showed that caveolin-1, which is usually absent in blood cells, is expressed in MM cells and plays a crucial role in both IL-6 and IGF-1-mediated signalling cascades [39]. Preclinical studies of IGF1R targeted strategies have shown efficacy comparable with that of other antineoplastic strategies, i.e. proteasome inhibitors and IMiDs, which have proven to be clinically useful [32]. Small-molecule IGF1R kinase inhibitor NVP-ADW742 [31], anti-IGF1R antibodies, or anti-IGF-1 ligand antibodies, will be evaluated in clinical trials in several cancers, including MM [40].

3. VEGF

VEGF is a known angiogenic factor in both solid tumors and haematological malignancies [41]. In MM, VEGF is produced both by MM cells and BMSCs and may account, at least in part, for the increased angiogenesis in MM patient BM. Our recent studies show that VEGF triggers ERK activation, proliferation, and migration of MM cells [42,43], which can be neutralised by VEGF receptor tyrosine kinase inhibitors PTK787 [44] and GW654652 [45]. VEGF also triggers Src-dependent phosphorylation of caveolin-1, which is required for p130Cas phosphorylation and MM cell migration [46]. Recently, we have shown that VEGF upregulates Mcl-1 expression in MM cell lines and MM patient cells; conversely, pan-VEGF inhibitor GW654652 inhibits VEGF-induced upregulation of Mcl-1, associated with decreased proliferation and induction of apoptosis [47].

We have also shown that a VEGF receptor inhibitor pazopanib (GW786034B) inhibits VEGF-triggered signaling pathways in both tumor and endothelial cells [48]. Humanized monoclonal antibody against VEGF Bevacizumab (Avastin) was recently approved by the FDA for the therapy of metastatic colorectal cancer, and ongoing studies in MM are evaluating the efficacy of bevacizumab, with or without thalidomide, in patients with relapsed or refractory MM [41].

4. FGF

MM cells express and secrete bFGF, which contributes to the increased angiogenic potential of BM plasma cells in progressive MM [49]. BMSCs from MM patients and control subjects express high-affinity FGF receptors R1–R4. Importantly, stimulation of BMSCs with bFGF induces a time- and dose-dependent increase in IL-6 secretion; conversely, stimulation with IL-6 enhances bFGF expression and secretion by MM cell lines, as well as MM patient cells [50]. In MM, dysregulation of fibroblast growth factor receptor 3 (FGFR3) by the t(4;14) translocation is a primary event in 10–20% MM patients and confers poor prognosis [51–54]. As a surface receptor, FGFR3 can be targeted by monoclonal antibodies [55,56] or be inhibited

by selective tyrosine kinase inhibitors (SU5402, SU10991, PD173074, or PKC412) [57,58]. Preclinical studies have validate FGFR3 as a therapeutic target in t(4;14) MM, and FGFR3 inhibitors are currently under clinical evaluation to improve prognosis of this patient subgroup.

5. BAFF

B-lymphocyte stimulating factor (Blys) is a TNF family member, which plays a critical role for maintenance of normal B-cell development and homeostasis. B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), another TNF family members sharing significant homology, are both expressed on MM cells [59,60]. Three receptors for BAFF have been identified: B-cell maturation antigen (BCMA), transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI), and BAFF-receptor. TACI and BCMA can also bind to APRIL, whereas BAFF-R is specific for BAFF. It has been shown that the serum levels of BAFF and APRIL are increased in patients with MM [61]. BAFF and APRIL promote MM cell growth and activate NF-κB, PI3K/Akt, and Ras/Raf/MAPK pathways with upregulation of Mcl-1 and Bcl- 2 anti-apoptotic proteins, leading to protection of MM cells against Dex-induced apoptosis [59]. Therefore blockade of BAFF/BAFR axis represents a potential therapeutic target.

6. Wnt

Wnt signalling regulates various developmental processes and can lead to malignant transformation. Wnts are a family of secreted cysteine-rich glycoproteins that act as short-range ligands locally and bind to frizzled transmembrane receptors. Intracellularly, a canonical Wnt/β-catenin signaling cascade inhibits GSK-3β activity, thereby blocking β-catenin phosphorylation and degradation by proteasomes. In MM, Wnt/β-catenin pathway is activated following treatment with Wnt-3a. MM cells highly express β-catenin, which is consistent with active β-catenin/T-cell factor (TCF)-mediated transcription [62]. Further accumulation and nuclear localisation of β-catenin, and/or increased cell proliferation, is achieved by stimulation of Wnt signaling with either the Wnt-3a or the constitutively active mutant of β-catenin [62]. Recent studies have shown that inhibition of β-catenin and TCF-4 interaction by PKF115-584 induces cytotoxicity in both patient MM cells and MM cell lines and mouse xenograft models of human MM [63].

In the BM microenvironment, Wnt signaling is involved in osteblastogenesis. MM cells in patient BM-biopsy specimens express dickkopf 1 (DKK1), a negative regulator of the Wnt/β-catenin signaling cascade [64]. Moreover, elevated DKK1 levels in BM plasma and peripheral blood from patients with MM correlate with DKK1 gene expression patterns and were associated with focal bone lytic lesions [65]. Importantly, recent studies have shown that anti-DKK1 neutralizing Ab increases numbers of osteocalcin-expressing osteoblasts and bone mineral density of implanted bone in SCID mice [66].

7. CD40

CD40 is a TNFα super family member. CD40 ligand (L) triggers p53-dependent MM cell proliferation, as well as PI3K/Akt/NFκB-dependent migration in MM cells [67,68]. In BMSCs, CD40 triggers secretion of IL-6 and VEGF, which further promotes MM cell growth in the BM milieu. Therefore, inhibition of CD40-CD40L interaction is a possible therapeutic strategy in MM. Indeed, anti-CD40 antibodies (SGN-40, CHIR-12.12) modestly inhibit MM cell proliferation [69]. Importantly, these antibodies can induce antibody-dependent cell-mediated cytotoxicity (ADCC) against CD40-positive MM cells, which can be further enhanced by lenalidomide [70,71].

8. Others

Serotherapy directed against CD20 targets only a minority of MM patient tumor cells, since CD20 expression is not common in MM (20% CD20+). The anti-CD20 monoclonal antibody (Rituximab) achieved response in 32% previously treated MM patients, all of whom had CD20 + tumor cells [72]. CS1 (CD2 subset 1) is a member of the CD2 family of cell surface glycoproteins and highly expresses on myeloma cells. Recent studies have shown that a novel humanized anti-CS1 mAb, HuLuc63, induces significant ADCC against MM cells including drug-resistant cells, and inhibited their interaction with BMSCs [73]

Targeting intracellular molecules

1. Proteasome

Ubiquitin-proteasome pathway is a protein degradation system which maintains intracellular protein homeostasis. It plays a central role in the targeted degradation of cellular proteins, including cell cycle regulatory proteins and apoptosis associated proteins. Ubiquitin is a small protein (76 amino acids). The C-terminus of ubiquitin forms an isopeptide bond with the amino group of a lysine side chain in a target protein. After attaching multiple copies of ubiquitin to target proteins, the protein will be degraded by 26S proteasome, which consists of a proteolytic core, the 20S proteasome, sandwiched between two 19S regulatory complexes. The 20S proteasome has multiple active sites, including caspase-like, trypsin-like, and chymotrypsin-like sites. Since Ubiquitin-proteasome pathway is crucial for survival of cancer cells, its inhibition represents a novel therapeutic strategy in cancer. The proteasome inhibitors are classified as reversible and irreversible according to their inhibition of chymotrypsin-like, trypsin-like, and/or caspase-like activities. Bortezomib is a reversible inhibitor of chymotrypsin-like activity, and has demonstrated significant anti-tumor activity in preclinical and clinical studies in MM.

a. Bortezomib (Velcade®)—Bortezomib (N-pyrazinecarbonyl-L-phenylalanine-L-leucine boronicacid) is a boronic acid dipeptide which inhibits β1, β1i, and β5 subunits of the 20S proteasome core in the 26S proteasome complex [74]. The initial rationale to use bortezomib in MM is its inhibitory effect of NF-κB, which plays a crucial role in the pathogenesis in cancer cells including MM. The NF-κB complex is a dimer of different combinations of Rel family proteins, including p65 (RelA), RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2). Recent studies have revealed that NF-κB activity is mediated via two distinct pathways. In the canonical pathway, NF-κB is typically a heterodimer composed of p50 and p65 subunits [75], and its activity is regulated by association with IκB family proteins [76]. Following stimulation by various factor, including cytokines (ie, TNFα, IL-1β, IGF-1), IκB protein is phosphorylated by IκB kinase (IKK), typically IKKβ. Phosphorylated IκB is subsequently poly-ubiquitinated and degraded by the 26S proteasome [77,78], which allows p50/p65 NF-κB nuclear translocation. Bortezomib inhibits degradation of IκB and blocks NF-κB activity.

Although NF-κB is a major target of bortezomib, it also has other target molecules. First, it directly induces apoptosis of human MM cell lines and freshly isolated patient MM cells despite induction of p53-independent $p21^{Cip1}$ and $p27^{Kip1}$. Second, it triggers apoptosis even in drug resistant cells, and adds to the anti-MM activity of Dex. Importantly, IL-6 and other growth factors do not overcome bortezomib -induced apoptosis, which is triggered by activation of caspase-3 via caspase-8/9 [79,80]. Third, bortezomib cleaves DNA repair enzymes (DNA-PKcs, ATM) [81,82], and enhances sensitivity of MM cells to conventional chemotherapeutic agents, especially to DNA damaging agents (ie, doxorubicin, melphalan) [81]. Forth, previous studies have also shown that normal plasma cells, as well as MM cells, produce and secrete abundant immunoglobulins, which require a highly developed endoplasmic reticulum (ER) and chaperone proteins (ie, heat shock proteins (Hsps)) that effect proper translation and folding.

The unfolded protein response ensures that the plasma cells can catabolize immunoglobulins, therefore proteasome inhibition is an ideal novel therapeutic strategy for MM [83]. Fifth, bortezomib induces a stress response in MM cells. For example, bortezomib upregulates HSPs and c-Jun NH_2-terminal kinase (JNK) which mediate apoptosis triggered by unfolded proteins. While bortezomib directly induces caspase-dependent apoptosis, it also targets the BM microenvironment. Specifically, in MM cells, it triggers downregulation of gp130 [84], which is phosphorylated after IL-6 binding to its receptor, thereby inhibiting phosphorylation of ERK, STAT3, and Akt induced by either IL-6 or by binding of MM cells to BMSCs. Sixth, bortezomib also inhibits VEGF-triggered caveolin-1 phosphorylation and markedly decreases caveolin-1 expression, thereby inhibiting VEGF-induced MM cell migration [46]. Seventh, expression of adhesion molecules (ie, ICAM-1, VCAM-1) on both MM cells and BMSCs is also regulated by NF-κB, inhibition of NF-κB by bortezomib decreases adhesion and thereby enhances susceptibility of MM cells to therapeutic agents [85,86] (Figure 2). Importantly, bortezomib also inhibits the paracrine growth of human MM cells in the BM milieu by decreasing their adherence to BMSCs and related NF-κB dependent induction of IL-6 secretion in BMSCs (Figure 1).

Most recently, the effects of bortezomib in bone remodeling, specifically on osteoblasts and osteoclasts, have been reported [87,88]. Bortezomib significantly induced a stimulatory effect on osteoblast markers in human mesenchymal cells without affecting the number of osteoblast progenitors in bone marrow cultures or the viability of mature osteoblasts, associated with upregulated Runx2/Cbfa1 activity in human osteoblast progenitors and osteoblasts. Importantly, numbers of osteoblastic cells was significantly increased by bortezomib. Specifically, Runx2/Cbfa1-positive osteoblastic cells was observed in MM patients responded to bortezomib treatment [88]. Moreover, bortezomib inhibited osteoclast differentiation and bone resorption activity. The mechanisms of action targeting early osteoclast differentiation was related to the inhibition of p38 MAPK pathways, whereas targeting the later phase of differentiation and activation was due to inhibition of p38 MAPK, AP-1 and NF-κB activation [89].

Other proteasome inhibitors

NPI-0052 is a novel proteasome inhibitor from Salinospora tropica, a marine actinomycete. Although bortezomib only blocks chymotryptic activity, NPI-0052 inhibits chymotryptic, trypsin-like and caspase-like activities. NPI-0052-induced cytotoxicity is predominantly triggered by caspase-dependent apoptosis. It induces cytotoxicity in MM cells resistant to conventional agents. Importantly, it is also able to overcome bortezomib resistance in vitro [90]. NPI-0052 triggers reactive oxygen species/caspase-8-dependent apoptosis, which can be enhancd by histone deacetylase inhibitor in ALL cells [91].

PR-171 is another novel epoxyketone-based irreversible proteasome inhibitor, which primarily inhibits chymotriptic activity of 20S proteasome. It triggers JNK/caspase-dependent apoptosis. In comparison to bortezomib, PR-171 exhibits equal potency but greater selectivity for the chymotrypsin-like activity of the proteasome. In cell culture, PR-171 is more cytotoxic than bortezomib following brief treatments that mimic the in vivo pharmacokinetics of both molecules.[92]. Multicenter phase I studies to evaluate the safety, tolerability, and clinical response to intensive dosing with PR-171 in patients with relapsed or refractory hematological malignancies has already been reported. In this study, 51 patients are enrolled, and 17 out of 21 myeloma patients were previously treated with bortezomib; importantly, 4 myeloma patients responded to PR-171 treatment (PR, 19%) [93].

2. Lenalidomide (Revlimid®)

Although lenalidomide, an immunomodulatory derivative of thalidomide has multiple mechanisms of anti-MM activities: including directly inducing G1 growth arrest or apoptosis; inhibits MM cell adherence to BMSCs; decreasing production of cytokines; inhibiting BM angiogenesis which is increased in MM patients; and enhancing anti-MM immunity with stimulation of T cell and natural killer cell responses. We and others have recently studied the mechanism of anti-MM activity of thalidomide derivatives known as immunomodulatory drugs (IMiDs), which have significantly higher potency at inducing apoptosis or growth arrest in MM cells resistant to melphalan, doxorubicin and dexamethasone [94]. The IMiDs reduce the secretion of IL-6 and VEGF triggered by the binding of MM cells to BMSCs, and inhibit angiogenesis [11]. We and others demonstrated that the IMiDs stimulated T-cell proliferation via T cell co-stimulatory mechanism. Specifically, IMiDs trigger tyrosine phosphorylation of CD28 on T cells, followed by activation of nuclear factor of activated T cell 2 (NFAT2) and production of IL-2 [70,95]. Moreover, IMiDs induce NK cell cytotoxicity, since both NK cell proliferation and antibody-dependent cell-mediated cytotoxicity (ADCC) activity were enhanced by IL-2 production from T cells triggered by IMiDs [70,71,96] (Figure 2). These data provide the cellular and molecular basis for use of IMiDs as an adjuvant in immunotherapeutic treatment strategies for MM.

3. Histone deacetylase (HDAC)

HDAC inhibitors are members of novel class of anti-tumor agents for malignancies, and a large number of structurally diverse HDAC inhibitors have been purified from natural sources or synthetically developed. HDAC inhibitors can be divided into six classes based on their chemical structure. These classes are short-chain fatty acid, hydroxamate, benzamide, cyclic tetrapeptide, electrophilic ketone and the others [97]. Accumulated histone acetylation by HDAC inhibitors attenuates their electrostatic interaction with the negatively charged DNA backbone, promoting the unfolding of histone–DNA complex, thereby modulating access of transcription factors to their binding sites of action and transcription of their target genes [98–100] (Figure 1). Previous studies have shown that deletions or inactivating mutations of HATs which decrease histone acetylation are involved in development of human neoplasms [101,102]. In contrast, inhibition of HDAC activity triggers growth arrest and/or apoptosis of tumor cells. Possible mechanisms of anti-tumor activities of HDAC inhibitors have recently been comprehensively described [97]; however, their mechanisms of growth inhibitory effects in MM cells have not yet been fully characterized.

a. Suberoylanilide hydroxamic acid (SAHA)—SAHA is prototype class I, II HDAC inhibitor which directly interacts with the catalytic site of HDAC like protein and inhibits its enzymatic activity. Inhibition of HDAC activity by SAHA therefore results in alteration of gene expression in various cell types including MM [103]. Like other HDAC inhibitors, SAHA upregulates $p21^{WAF1}$ expression [104,105], thereby inhibiting tumor cell growth. In MM, SAHA: modulates gene expression and inhibits of tumor cell growth [103,106]; induces upregulation of $p21^{WAF1}$; upregulates p53 protein expression; and dephosphorylates Rb, followed by apoptosis. Importantly, upregulation of $p21^{WAF1}$ occurs prior to p53 induction, suggesting that $p21^{WAF1}$ upregulation is independent of p53 activity [103]. SAHA-induced apoptosis in MM cells is associated with Bcl-2 interacting protein Bid; conversely, overexpression of Bcl-2 blocks SAHA-induced apoptosis, suggesting that Bcl-2 plays a crucial role regulating SAHA-induced apoptosis in MM cells. Interestingly, SAHA does not trigger caspase activation, and the caspase inhibitor does not protect against SAHA-induced cytotoxicity. However, poly (ADP) ribose polymerase (PARP) is significantly cleaved by SAHA, suggesting that SAHA triggers atypical PARP cleavage in MM cells [103]. Importantly, SAHA suppresses expression and activity of the proteasome and its subunits, providing the rationale for its use in combination with bortezomib to enhance its cytotoxicity [106]. It has also

shown that SAHA enhances tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cytotoxicity, associated with upregulation of the proapoptotic proteins (Bim, Bak, Bax, Noxa, and PUMA) and downregulation of anti-apoptotic proteins (Bcl-2 and Bcl-xL) [107].

b. MVP-LAQ824 (LAQ824)—LAQ824 is a member of hydroxamate HDAC inhibitor which blocks class I and II HDAC activity. LAQ824 inhibits proliferation of cancer cell lines with IC50s of 10–150nM ranges in vitro, indicating that anti-proliferative potency of LAQ824 is up to 200-fold higher than that of SAHA. [108,109]. Anti-tumor activity of LAQ824 has been extensively studied in leukemia cells [110–114]. In MM, LAQ824 induces apoptosis at IC50 of 100 nM at 24 hour in most MM cell lines and patient tumor cells. Importantly, LAQ824 is effective in cells which are resistant to conventional therapies (dexamethasone, doxorubicin, melphalan). Moreover, LAQ824 inhibits cell growth in vivo in a preclinical murine myeloma model. Unlike SAHA, LAQ824-induced apoptosis is associated with caspase activation [115].

c. LBH589—LBH589 is a hydroxamic acid analog which blocks class I and II HDAC activity. LBH589 has been studied in many malignancies as a single agent, as well as combined with other anticancer agents [116–119]. LBH589 has also been shown to inhibit angiogenesis in vitro [120]. In MM, LBH589 blocks cell cycle progression, associated with upregulation of $p21^{WAF1}$, p53, and p57, and induces cytotoxicity through an increase in mitochondrial outer membrane permeability [121]. The IC50 of LBH589 is 40–80 nM in most MM cell lines [121, 122]. LBH589-induced cytotoxicity is associated with caspase/PARP cleavage; however, interestingly, LBH589 also triggers a caspase-independent apoptotic pathway through the release of apoptosis-inducing factor (AIF) from mitochondria [121]. Synergistic cytotoxicity against MM cells is observed with LBH589 in combination with bortezomib [122]. Phase II clinical trials of LBH589 are ongoing in MM, and a clinical trial of bortezomib with LBH589 to block proteasomal and aggresomal breakdown of protein, respectively, is soon to begin.

e. Other HDAC inhibitors—Tubacin is a hydroxamic acid HDAC inhibitor and inhibits only HDAC6 activity [123]. Previous studies have characterized the aggresome as an alternative system to the proteasome for degradation of polyubiquitinated proteins. The aggresome pathway therefore likely provides a novel system for delivery of aggregated proteins from the cytoplasm to lysosomes for degradation [124]. In this aggresomal protein degradation pathway, HDAC6 has an essential role, since it can bind both polyubiquitinated proteins and dynein motors, thereby acting to recruit protein cargo to dynein motors for transport to aggresomes [125]. We have demonstrated that blockade of both proteasomal and aggresomal protein degradation by bortezomib and tubacin, respectively, synergistically enhances cytotoxicity in MM cells in vitro [126]. Depsipeptide (FR901228, FK228) is a class of cyclic tetrapeptide and inhibits only class I HDAC activity [127]. Depsipeptide induces apoptosis in MM cell lines and in primary patient tumor cells, associated with downregulation of Bcl-2, BCL-xL and Mcl-1 expression [128]. PXD101 is a hydroxamate class HDAC inhibitor [129] which has antiproliferative activity in MM cell lines, and shows additive and/or synergistic effects with conventional agents used in MM. MS-275 belongs to the benzamide class and inhibits class I and II HDACs. KD5170 is non-hydroxamate, orally bioavailable HDAC inhibitor which significantly inhibits osteoclast formation at lower μM range and triggers apoptosis in MM cells [130].

4. Heat shock protein (Hsp) 90

Hsp90 is a molecular chaperone which facilitates intracellular protein trafficking, conformational maturation, and 3-dimensional folding required for protein function. Intracellular overexpression of Hsp90 proteins are observed in most MM tumor cells, but not in monoclonal gammopathy of undetermined significance (MGUS) or in normal plasma cells

[131]. The ansamycin antibiotic geldanamycin (GA) and its analogs bind to the critical ATP-binding site of Hsp90, thereby abrogating its chaperoning activity in the MM BM milieu; decreasing IGF-1R and IL-6R expression on MM cells; depleting growth kinases (e.g., Akt, IKK, Raf) and anti-apoptotic proteins (FLIP, XIAP, cIAP, telomerase); as well as inhibiting both constitutive and cytokine-induced activation of NF-κB and telomerase (hTERT) in the BM milieu [132]. GA and other Hsp90 inhibitors induce apoptosis of MM cell lines and patient cells which are resistant to Dex, anthracyclines, Thal or IMiDs, TRAIL/Apo2L, and bortezomib. Moreover, a geldanamycin analog 17-AAG suppresses in MM cells the expression and/or function of multiple levels of insulin-like growth factor receptor (IGF-1R) and interleukin-6 receptor (IL-6R) signaling (eg, IKK/NF-κB, PI-3K/Akt, and Raf/MAPK) and downstream effectors (eg, proteasome, telomerase, and HIF-1α activities)in MM cells [132]. Most recently, Hsp90 inhibitors have been reported to induce myeloma cell death, at least in part, via ER stress and the unfolded protein response death pathway [133].

IPI-504 is a hydroquinone hydrochloride derivative of 17-AAG. In MM, IPI-504 inhibits MM cell growth in vitro and in mouse models. Like other Hsp90 inhibitors, IPI-504 synergistically enhances cytotoxicity of bortezomib [134]. 17-dimethylaminoethylamino-17-demethoxygeldanamycin hydrochloride (17-DMAG) is also a water soluble novel Hsp90 inhibitor 17-DMAG, which attenuates the levels of STAT3 and phospho-ERK, as well as decreases the viability of MM cells [131].

5. Akt (protein kinase B)

Akt signaling mediates MM cell resistance to conventional therapeutics [20,36,135], therefore, biologically-based treatments targeting Akt are a promising therapeutic strategy in MM. Perifosine is a synthetic novel alkylphospholipid which inhibits Akt activation. In MM cells, we have shown that Perifosine inhibits both baseline and cytokine (IL-6, IGF-1)-triggered Akt activation. Importantly, Perifosine triggers significant cytotoxicity even of MM cells adherent to BM stromal cells (SCs) and therefore overcomes CAM-DR. Furthermore, Perifosine augments both conventional agent- and bortezomib-induced MM cell cytotoxicity. Importantly, we have also demonstrated in vivo anti-MM activity of Perifosine in a human plasmacytoma mouse model, associated with downregulation of Akt phosphorylation in tumor cells [136]. Perifosine has been shown to induce selective apoptosis in MM cells by recruitment of death receptors, such as TNF-related apoptosis-inducing ligand (TRAIL)-R1/DR4 and TRAIL-R2/DR5 [137]. Most recently, we have shown that Perifosine-indiced cytotoxicity is strongly associated with downregulation of survivin [138].

6. Mammalian target of rapamycin (mTOR)

mTOR is a serine/threonine protein kinase that regulates transcription, cell proliferation, and survival. Inhibition of mTOR by its inhibitors therefore induces potent cytotoxicity in MM cells [139,140]. Specifically, rapamycin induced G0/G1 arrest, associated with an increase of the cyclin-dependent kinase inhibitor p27 and a decrease of cyclins D2 and D3 in MM cells [141].. Interestingly, PTEN-negative myeloma cells are more sensitive to mTOR inhibition that PTEN-positive cells [142]. Rapamycin shows synergistic cytotoxicity in combination with dexamethasone Stromberg, 2004 #5217} and lenalidomide [143]. CCI-779 is a rapamycin analog which demonstrates inhibition of proliferation and induction of apoptosis, associated with cyclin D1 and c-myc downregulation and up-regulation of $p27^{Kip1}$ in OPM-2 cells [144]. Moreover, CCI-779 downregulates VEGF translation, and ultimately blocks angiogenesis [145].

7. MAPK kinase (MEK)

MEK/ERK pathway is one of the major signaling cascades which can be activated by many cytokines (ie, IL-6, IGF-1, SDF1α, BAFF) in MM cells. We have shown that inhibition of ERK

by antisense oligonucleotide blocks MM cell proliferation [12,13]. Therefore inhibition of MEK/ERK signaling is a promising therapeutic strategy.

Recent studies have shown that clinical grade novel MEK1/2 inhibitor AZD6244 (ARRY-142886) induces apoptosis in MM cell lines and patient MM cells, associated with caspase-3 activation. Importantly, AZD6244 down-regulates the expression/secretion of osteoclast (OC)-activating factors from MM cells and inhibits in vitro differentiation of MM patient PBMCs to OCs [14].

8. Bcl2 and Bcl-xL

Bcl2 family members have a crucial role in protecting cells from apoptotic stimuli. In MM, Bcl-2 antisense oligonucleotide (G3139) [146,147], and Bcl2/Bcl-XL inhibitor (ABT-737) [148,149] induce strong anti-MM activities as single agents and in combination with Dex [150].

Future directions

Although each of novel agent demonstrates significant preclinical anti-MM activity in vitro and using an in vivo mouse model of human MM, treatment with single agents may not achieve sufficient clinical efficacy. Therefore, treatments combining novel agents with conventional and/or novel agents to overcome clinical drug resistance are required. Among these combination therapies, thalidomide with dexamethasone, bortezomib with dexamethasone, and bortezomib with doxorubicin have shown promising results in clinical studies based upon our preclinical studies. Our recent preclinical studies indicate that other novel agents enhance cytotoxicity induced by conventional agents. For example, bortezomib induces stress response-related proteins such as heat shock proteins hsp27, hsp70, and hsp90. Blockade of Hsp90 or Hsp27 by their inhibitors restores sensitivity to bortezomib. Recent studies have demonstrated that unfolded and ubiquitinated proteins are degraded not only by proteasomes, but also by aggresomes dependent on HDAC6 activity. Inhibition of both proteasome and aggresome mechanisms using bortezomib and HDAC6 specific inhibitor tubacin induces accumulation of ubiquitinated proteins, followed by significant cell stress and cytotoxicity in MM cells. Most recently, we demonstrated that the potent Akt inhibitor perifosine augments bortezomib-induced cytotoxicity in MM. These preclinical studies of combination therapies of bortezomib with novel agents provide the rational framework for clinical evaluation of these treatment options.

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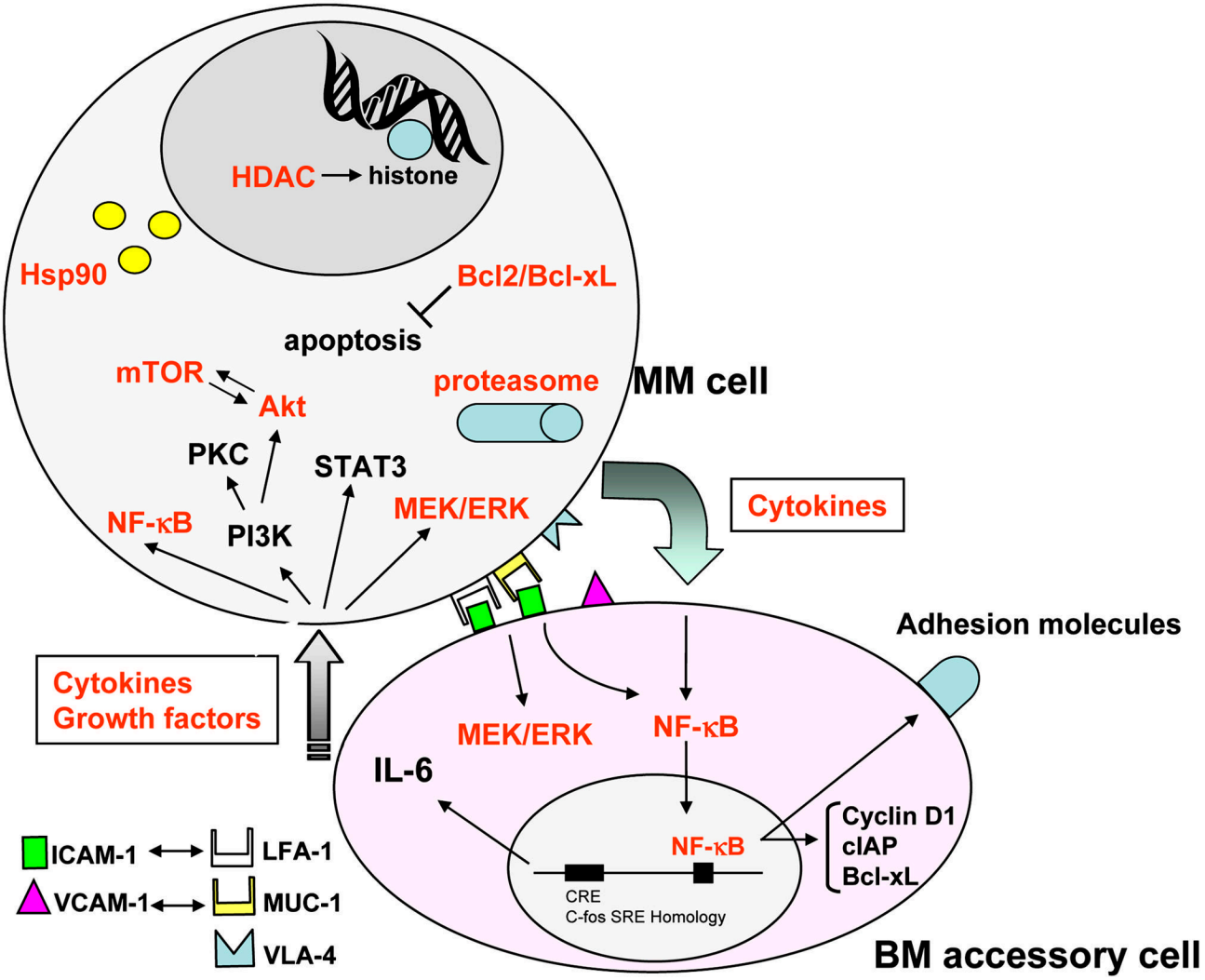

Figure 1.

Novel biologically-based therapies targeting MM cells and the BM microenvironment. Novel agents A. directly inhibit MM cell growth; B. inhibit angiogenesis; C. inhibit MM cell adhesion to BM accessory cells; D. decrease cytokine production and sequelae in the BM microenvironment; and E. enhance host anti-MM immunity.

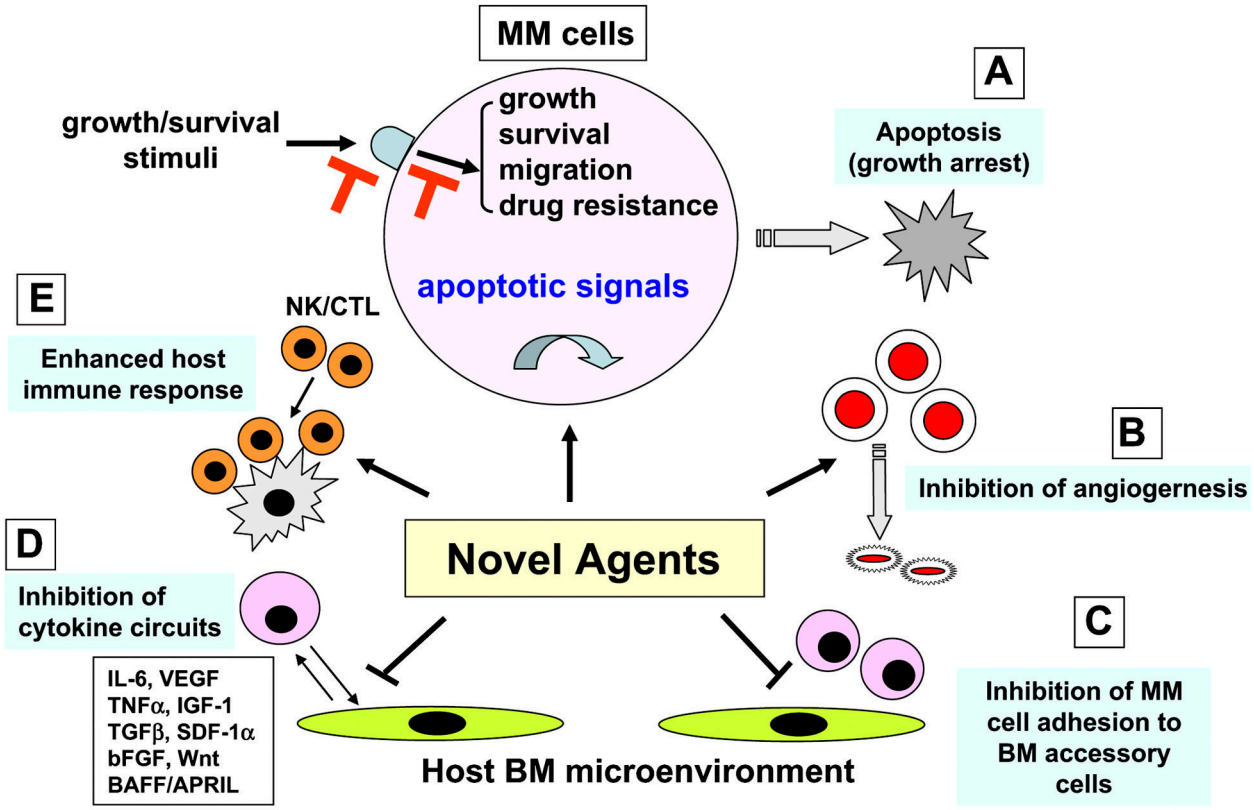

Figure 2.

Cell surface and intracellular targets of novel therapeutic agents. Novel agents block signaling cascade triggered by MM cell-BM accessory cell interaction and induce growth inhibition in the BM microenvironment. Novel agents; inhibit interaction of cytokines/growth factors and their receptors expressed on MM cell; inhibit receptor tyrosine kinase activity; intracellular molecules (kinases, anti-apoptotic proteins, molecular chaperons, transcription factors).