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Nagaraju Anreddy
West Virginia University

Lori A. Hazlehurst
West Virginia University

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Targeting intrinsic and extrinsic vulnerabilities for the treatment of multiple myeloma

Nagaraju Anreddy and **Lori A Hazlehurst***

Department of Pharmaceutical Sciences, School of Pharmacy, West Virginia University, Morgantown, 26506

Abstract

Multiple myeloma (MM) is a malignant plasma cell disorder, clinically characterized by osteolytic lesions, immunodeficiency, and renal disease. Over the past decade, MM therapy is significantly improved by the introduction of novel therapeutics such as immunomodulatory agents (thalidomide, lenalidomide, and pomalidomide), proteasome inhibitors (bortezomib, carfilzomib, and ixazomib), monoclonal antibodies (daratumumab and elotuzumab), histone deacetylase (HDAC) inhibitors (Panobinostat). The clinical success of these agents has clearly identified vulnerabilities intrinsic to the MM cell- as well as targets that emanate from the tumor microenvironment. Despite these significant improvements, MM remains incurable due to the development of drug resistance. This perspective will discuss more recent strategies which take advantage of multiple targets within the proteome recycling pathway, chromatin remodeling, and disruption of nuclear export. In addition, we will review the development of strategies designed to block opportunistic survival signaling that occurs between the MM cell and the tumor microenvironment including strategies for inhibiting myeloma-induced immune suppression. It has become clear that MM tumors continue to evolve on therapy leading to drug resistance. It will be important to understand the mechanism and additional vulnerabilities that occur due to the development of clinical resistance.

Keywords

Multiple Myeloma; Bone marrow microenvironment; Drug resistance; Proteasome; Integrins; CD44

Introduction

Multiple myeloma (MM) is a plasma cell malignancy clinically characterized by greater than 10% bone marrow (BM) resident plasma cells and increased monoclonal protein in the blood and/or urine. MM leads to end-organ damage diseases such as anemia, hypocalcemia, renal insufficiency, or osteolytic bone lesions. Monoclonal proliferations of plasma cells residing in the bone marrow can be detected at a pre-malignant stage referred to as at as monoclonal gammopathy of undetermined significance (MGUS). Paradoxically, MGUS

*Corresponding Author: Lori A Hazlehurst, Department of Pharmaceutical Sciences, School of Pharmacy, West Virginia University, Morgantown, 26506. lahazlehurst@hsc.wvu.edu, Phone: 304-293-3398, Fax: 304-293-2576.

lacks the clinical characteristics of MM, yet can harbor the same genetic alterations of symptomatic myeloma [Brousseau et al., 2007]. The rate of development of myeloma from MGUS is very low, and it has been estimated to be 1% per year. The introduction of several novel agents such as immunomodulatory (lenalidomide, and pomalidomide) agents which target the bone marrow microenvironment (BME), proteasome inhibitors (bortezomib, carfilzomib, and ixazomib), monoclonal antibodies that target cell surface receptors (daratumumab and elotuzumab), and histone deacetylase (HDAC) inhibitors (Panobinostat) significantly improved outcomes for patients with MM. The promise of these agents led to a rapid explosion of inhibitors, which target these pathways. The goal of this perspective is to discuss novel therapeutic strategies which target pathways intrinsic to MM cell (ie., i: protein homeostasis; ii: epigenetic regulation; iii: disruption of nuclear export homeostasis) and the supportive BME (i:adhesion receptors; ii: chemokines/cytokines and iii: immune suppression). Many of these strategies are in early clinical development and efforts to define optimal combination strategies are ongoing to provide MM patients with better outcomes.

1.0: Agents that target protein homeostasis

Myeloma cells are terminally differentiated plasma cells, and as such have an efficient and well-developed secretory machinery to support the physiological function of antibody production of the plasma cell in the humoral immune response. However, the function of secreting high levels of immunoglobulin occurs at a cost to the cell and contributes to the relatively short life span of a normal plasma cell [Smith et al., 1996]. Myeloma cells can tolerate the consistent production of secretory antibodies; however, coordinated and coupled degradation/recycling of proteins are essential to support the survival of MM cells. Understanding and delineation of the degradation/recycling proteome machinery has provided key targets for intervention that provide specificity toward the MM cell. Chaperones such as heat shock proteins assist with the folding of newly synthesized proteins and refolding of proteins destructed by stress and cellular injury. Misfolded proteins are targeted for degradation by the ubiquitin-proteasome system (UPS). UPS involve a two-step process, ubiquitination and proteasome degradation. Ubiquitination involves a three-step enzymatic cascade as shown in Figure1. First, E1 utilizes ATP to adenylate Ub to create a thioester bond between the Ub C-terminal group and the thiol group of a cysteine residue. The activated Ub is then shifted to the cysteine residue of an E2 by a thioester linkage. Ultimately, an E3 ligase recruits a charged E2 and facilitates the transfer of Ub to the target protein to form mono-or poly-Ub chains, which can lead to protein degradation mediated by the proteasome. Deubiquitinating enzymes (DUBs) catalyze the separation of Ub from polypeptides to reverse the ubiquitination process and to maintain Ub homeostasis[Lub et al., 2016]. Aggresome pathway (Proteosome independent pathway) also eliminate the misfolded proteins by transporting aggregated or misfolded proteins from ER to the cytosol for lysosomal degradation with the help of microtubule, HDAC6, and dynein/dynactin motor complex. Myeloma cells have a narrow index for triggering the unfolded protein response (UPR) due to the basal load of monoclonal antibody production produced by the MM cell [Bianchi et al., 2009]. Several pharmacological strategies can be applied to targeting this intrinsic vulnerability of the myeloma cell, including the blockade of proteasome activity,

inhibition of the protein deubiquitinating and chaperoning processes, and as well as modulation of the downstream UPR.

1.1: Agents that block the proteasome activity

The proteasome is a multi-subunit protease complex that plays a central role in the regulation of protein degradation of intracellular proteins and clearance of misfolded and/or unfolded proteins. MM cells have a dependency on the proteasome to remove misfolded or damaged proteins due to the high rate and a load of immunoglobulin secretion. Preclinical studies have shown that MM cells are more sensitive to proteasome inhibition compared to normal cells as well as other tumor types. Bortezomib, a dipeptide boronic acid analogue mediating selective and reversible inhibition of the 26S proteasome, has dramatically changed outcomes for MM patients. Despite the clinical success of bortezomib, majority of patient progress to a refractory stage due to the emergence of drug resistance. Second-generation proteasome inhibitors with reversible and irreversible inhibition of chymotrypsin-like (CT-L), trypsin-like, and/or caspase-like (C-L) activities have been developed. These agents showed significant activity in both bortezomib-resistant and newly diagnosed MM. Carfilzomib is a highly selective inhibitor of 20S proteasome and has chymotrypsin-like (β5) subunit activity. It is more potent than bortezomib, achieving a 24% response rate in bortezomib refractory patients. FDA recently approved carfilzomib combination therapy with dexamethasone or with lenalidomide plus dexamethasone for the treatment of patients with relapsed or refractory multiple myeloma (RRMM) who have received one to three lines of therapy. Ixazomib (MLN9708) is an orally available proteasome inhibitor recently approved in combination with lenalidomide and dexamethasone. Ixazomib demonstrated effectiveness in phase 3 trial, which was conducted in 26 countries and included 722 patients with RRMM. Clinical results showed that a combination with ixazomib, lenalidomide, and dexamethasone significantly improved progression-free survival (PFS) when compared with the doublet of lenalidomide and dexamethasone [Kumar et al., 2014].

Several other novel PIs have already established promising activity. Marizomib (NPI-0052) is an irreversible PI that non-selectively inhibits the chymotrypsin-like, trypsin-like, and caspase-like protease activities within the proteasome. Marizomib with or without dexamethasone has shown promising responses even in bortezomib- and lenalidomide-resistant MM. Marizomib in combination with pomalidomide and dexamethasone are under evaluation in RRMM [Potts et al., 2011]. Oprozomib (ONX0912) is a novel orally administered PI that is derived from carfilzomib. It binds irreversibly to CT-L subunit of the proteasome, resulting in longer duration of inhibition compared to bortezomib[Chauhan et al., 2010]. Oprozomib inhibits growth and migration of myeloma cells and activates poly (ADP) ribose polymerase (PARP), and caspase enzymes thereby induce MM cell apoptosis [Chauhan et al., 2010]. However, the challenge for proteasome inhibitors continues to be the emergence of drug resistance, and it is unlikely that the addition of more potent inhibitors will resolve emergence of clinical resistance. Perhaps, incorporation of proteasome inhibitors into combination strategies targeting additional points in the protein-recycling pathway may represent a tractable approach for effective combination strategies that delay the emergence of resistance to proteasome inhibitors. These additional potential targets are discussed below.

1.2: Agents that target enzymes of the ubiquitination cascade system

Novel strategies targeting the key components of the ubiquitination cascade system are also under evaluation to overcome PI resistance. E1 ubiquitin activating enzyme plays a major role in proteasome-mediated protein degradation in MM. Knockdown of E1 ubiquitin activating enzymes resulted in a decreased viability of MM and leukemia cells indicating that it could be an interesting target [Xu et al., 2010]. To date, two inhibitors of E1 ubiquitin activating enzyme have been examined in MM, JS-K, and PPZD-4409. JS-K is a prodrug of nitric oxide (NO) that releases NO when metabolized by glutathione S-transferase. JS-K inhibits the ubiquitin-E1 thioester formation through binding of NO to the active cysteine residue on E1[Kitagaki et al., 2009]. However, JS-K also has been shown induce DNA double-strand breaks, which may also contribute to the induction of apoptosis in human MM cells *in vitro* and *in vivo*. PYZD-4409 was also shown to inhibit the growth of MM cells *in vitro* and inhibited the growth of leukemia cells using both *in vitro* an *in vivo* models [Xu et al., 2010].

Although the specificity of the drug tools to probe E1 inhibition are likely not ideal, the target remains attractive for the treatment of MM. Another potential strategy to allow for more specificity in targeting the expression of the proteome is by targeting specific E3 ligases. For example, human double minute 2 (MDM2), is an E3 ubiquitin ligase accountable for degradation and inhibition of wild-type p53 (wt-p53) activation. MDM2 is overexpressed in MM cell lines; this expression has been shown to contribute to growth and survival of MM cells[Teoh et al., 1997]. Several MDM2 inhibitors were identified among them nutlin-3 was first discovered. Nutlin-3 binds to MDM2 thereby inhibits the interaction between MDM2 and p53, resulting in activation of the p53 signaling pathway [Teoh and Chng, 2014]. Nutlin-3 demonstrated the significant activity against primary MM samples and cell lines. Analogues of nutlin-3a, including MI-63, RITA, and Serdemetan, are under evaluation in preclinical models of MM.

1.2.1: Deubiquitinating enzymes (DUBs) inhibitors—The ubiquitination process reversed by a group of proteases called deubiquitinating enzymes (DUBs), which recognize ubiquitinated proteins and remove their ubiquitin tags by cleavage of the isopeptide bond at the C-terminus of ubiquitin [Colland, 2010]. Inhibition of DUBs lead to lethal ER stress and has been reported to overcome cell line models of proteasome inhibitor resistance. Several studies reported that DUBs such as ubiquitin-specific proteases (Usp) Usp9x, Usp24, and Usp7 are potential new therapeutic targets in MM. Usp9x inhibitor WP1130 shown to induce apoptosis and reduce Mcl-1 levels in human MM cells[Kapuria et al., 2010]. The novel inhibitor EOAI3402143 proved to inhibit both Usp9x and Usp24 activity and suppresses tumor growth *in vivo* [Peterson et al., 2015]. P5091, a selective inhibitor of Usp7 induced apoptosis in MM cells and shown more effective when combined with HDAC inhibitor SAHA, lenalidomide or dexamethasone[Chauhan et al., 2012].

1.3: Inhibition of Heat shock proteins

Heat shock proteins play an important role in the handling of immunoglobulin folding in myeloma. Numerous studies have shown that Hsp 70 and 90 inhibition in myeloma cells induces apoptosis. Preclinical studies have demonstrated that the inhibition of Hsp90 is

active in myeloma in vitro and in vivo. Hsp90 inhibitors 17-AAG and NVP-AUY922 are under evaluation in preclinical models of MM. Hsp70 inhibition triggers myeloma cell death via the intracellular accumulation of immunoglobulin and the generation of proteotoxic stress. HSP 70 inhibitor, Ver-155008 significantly reduced the division of myeloma cells with limited effects on normal blood cells[Zhang et al., 2014a].

1.4: HDAC 6 Inhibitors

HDAC6 plays an import role in aggresomal protein degradation because it binds to misfolded proteins on the one hand and the dynein motility complex on the other, thereby shuttling polyubiquitinated proteins to the aggresome/lysosome for degradation. Ricolinostat (ACY-1215) is a specific HDAC6 inhibitor that is cytotoxic against MM cells and synergizes with bortezomib and lenalidomide in vitro [Santo et al., 2012]. A phase 1b study of ricolinostat plus bortezomib/dexamethasone in RRMM showed a promising activity in bortezomib-refractory MM (NCT01323751).

2: Agents that target epigenetic alterations

Epigenetic modifications, such as aberrant DNA and histone methylation or abnormal microRNA (miRNA) expression, are found to contribute to the pathogenesis of MM [Chapman et al., 2011]. Histones constitute a significant level of epigenetic regulation as modifications can alter the chromatin structure, thus changing accessibility to transcription factors. Histone tails can be post-translationally reversibly modified by methylation, acetylation, phosphorylation, ubiquitination, and the addition of poly (ADP-ribose) moieties. In this section, we will discuss the agents that targeting these key modifications of histone.

2.1: Targeting the HDACs

Acetylation of histones is correlated with open chromatin and elevated transcription while deacetylated histones are often corelated with tighter backed chromatin and repression of gene transcription. Histone deacetylases (HDACs) are crucial regulators of gene expression that enzymatically remove the acetyl group from histones. Recently, expression of HDAC1-3, HDAC6 and HDAC5 and 10 were found to be significantly upregulated in MM cells compared to normal plasma cells. In this same report investigators showed that patients with increased levels of HDAC1–3, HDAC4, HDAC6, and HDAC11 had poorer outcomes [Mithraprabhu et al., 2014]. Experimental and clinical evidence indicates that targeting HDAC’s is an attractive strategy for the treatment of MM. The pan-HDAC inhibitor Panobinostat was recently approved in combination with bortezomib and dexamethasone in RRMM patients. However, pan-HDAC inhibitors have shown significant toxicity (thrombocytopenia, diarrhea, and fatigue) in phase 3 study; that limited the therapy tolerability and duration. The challenge will be to determine whether the anti-myeloma activity associated with pan-HDAC inhibitors can be achieved by targeting a specific HDAC. This strategy will hopefully lead to a decrease in overall toxicity while retaining anti-myeloma activity.

2.2: Targeting the histone methyltransferases

Recent data demonstrate that changes in histone methylation may be a specific role in the pathogenesis of MM. A 15–20% subset of MM patients present with the t(4:14) chromosomal translocation, and this particular group has a significantly poorer prognosis [Kuehl and Bergsagel, 2002]. This translocation leads to the dysregulated expression of M-SET domain (MMSET), a histone methyltransferase. The MMSET gene consists of 24 exons and expression of multiple splice variants has been identified, which has led to complications understanding the function of this histone methyltransferase. The dominant histone mark generated by MSET/WHSC1/NSD2 is demethylation of H3 at lysine 36 and promotes expression of TGFA, MET, PAK1 and RRAS2 on MM cells. Moreover, reducing the expression of the gene product of WHSC1 referred to as NSD2 in KMS11 MM cells inhibited growth *in vitro* and *in vivo*[Kuo et al., 2011]. In additional methyltransferase overexpressed in MM is referred to as enhancer of Zeste Homolog 2 (EZH2) [Zhan et al., 2002]. EZH2 contributes to the methylation status of H3K27, and EZH2 dysregulation has been described to contribute to silencing of tumor suppressor genes, thus contributing to MM phenotype [Hernando et al., 2016]. Experimental evidence indicates that EZH2 upregulation in MM can be mediated by interleukin-6 (IL-6), c-Myc activation, or miR-26a downregulation [Sander et al., 2008]. In human MM cell lines, *EZH2* expression has been correlated with proliferation and growth factor independence[Croonquist and Van Ness, 2005]. Inhibition of EZH2 expression and activity is associated with myeloma cell growth inhibition and reduced tumor burden in murine models of MM [McCabe et al., 2012; Zhao et al., 2010]. Two EZH2 inhibitory molecules (EPZ005787 and GSK126) were identified independently by high-throughput screening[McCabe et al., 2012]. Future studies are warranted to define the activity of EZH2 inhibitors for the treatment of MM. Moreover, it will be important to determine the changes in histone marks as a consequence of drug selection and whether epigenetic changes contribute to the progression of MM towards drug-resistant disease.

2.3: Bromodomain-containing protein 4 (BRD4) inhibitors

BRD4 is a member of the bromodomain and extra-terminal (BET) family of proteins, which contains two bromodomains. BET family proteins bind with acetyl-modified lysine residues of histone tails and facilitate transcriptional activation. Several studies reports that oncogene MYC drives disease progression in multiple myeloma and BET inhibitor, JQ1shown antiproliferative activity by downregulating MYC transcription [Delmore et al., 2011]. Recently two BET inhibitors (I-BET151 and I-BET762) were identified and showed promising activity with acceptable off-target effects in preclinical models of multiple myeloma [Chaidos et al., 2014]. The rapid development of resistance to BET inhibitor has been reported in leukemia and breast cancer models [Rathert et al., 2015] and maybe be a concern for the development of this class of inhibitors for the treatment of MM.

3: Targeting nuclear export with CRM1/XPO1 inhibitors

Chromosome region maintenance (CRM1) the key nuclear export protein is more commonly called Exportin 1 (XPO1), is involved in transporting cargo proteins with leucine-rich nuclear export sequences from the nucleus to the cytoplasm. Recent literature suggests that

CRM1 was found to be highly expressed in MM cells, and increased activity is associated with reduce survival and increased lytic bone lesions. Experimental data indicates that CRM1 downregulation results in decreased MM cell growth and survival. Furthermore, CRM1 inhibitors KPT-251, KPT-276 and KPT-330 significantly inhibited the growth of MM cells in vivo [Tai et al., 2014]. The sensitivity of myeloma cells to CRM1 inhibitors suggests that an imbalance in nuclear/cytoplasmic trafficking contributes to survival. Moreover, myeloma cells placed in high density are known to contain a high cytoplasmic ratio of topoisomerase II alpha, and co-administration of a CRM1 inhibitor plus topoisomerase II inhibitors results in synergistic cell kill; a finding which correlated with retention of topoisomerase II in the nucleus[Turner et al., 2013]. It is attractive to think that this combination strategy may be more effective in quiescent cells, which typically have low nuclear levels of topoisomerase II [Turner et al., 2014]. Together, pre-clinical studies support the continued clinical development of CRM1 antagonists for the treatment of MM [Tai et al., 2014].

4.0: Agents that target the bone marrow microenvironment

MM cells grow and expand within the bone marrow (BM), suggesting the significance of the BM microenvironment in mediating MM cell growth and survival. The BM microenvironment consists of both a cellular compartment and a non-cellular compartment, and further complexity is provided by active crosstalk between the two compartments. The cellular compartment can be subdivided into hematopoietic cell types including myeloid cells, T-lymphocytes, B-lymphocytes, NK cells, and osteoclasts while non-hematopoietic cells include bone marrow stromal cells (BMSCs), fibroblasts, osteoblasts, endothelial cells, and blood vessels. The non-cellular compartment includes the extracellular matrix (ECM), and the soluble factors (cytokines, growth factors, and chemokines), which are produced and/or affected by the cellular compartment within the bone marrow microenvironment. These microenvironment compartments exert differential effects on MM cell progression and resistance to therapeutics and may work synergistically. In this section, we discuss the agents that target each component of BM microenvironment to prevent MM progression.

4.1: Targeting the immune microenvironment

MM patients have substantial immune cell dysregulation, which effects B-lymphocytes, T-lymphocytes, natural killer (NK) cells, and dendritic cells (DCs)[Dhodapkar et al., 2003; Raitakari et al., 2003]. The homeostasis of regulatory T cells (Treg) and T helper (Th) 17 cells is essential for maintaining anti-tumor immunity in MM [Dhodapkar et al., 2003]. Tregs play a major role in the preservation of self-tolerance and modulation of overall immune responses against infections. Immune dysregulation not only increases the risk of infections in MM patients but also affect antigen presentation and up-regulation of inhibitory antigens that advances immune escape and growth for malignant clones. Several novel approaches have been explored to enhance the immune system to fight against MM.

4.1.1: Immunomodulatory drugs (IMiDs)—IMiDs directly affect MM cells and bone marrow microenvironment leading to modulation of cytokines meliu present in the BME (TNFα, IL-6, and VEGF, IL-2 interferon-γ) and stimulation of NK and T-cells[LeBlanc et

al., 2004]. Recent studies suggest that IMiDs interact with cereblon, and also downregulate Ikaros (IKZF1), and Aiolos (IKZF3) proteins which was shown to improve the antitumor and host immune effects in MM [Lu et al., 2014]. Pomalidomide is currently approved to treat RRMM, and shown positive results in lenalidomide- and bortezomib-refractory disease. The positive clinical data obtained with treatment with IMiDs provided the first clinical proof that targeting the BME is a critical component for the successful management of MM.

4.1.2: Immune Checkpoint Inhibitors (PD-1/PD-L1)—Programmed cell death 1 (PD-1) is a type I transmembrane protein expressed on the surface of activated T cells, interacting with its two ligands, PD-L1, and PD-L2. The association between PD-L1 on target cells and PD-1 on T and effector cells act as an immunologic checkpoint to suppress antitumor immunity. MM cells express PD-L1, which is further upregulated in the BM microenvironment [Liu et al., 2007; Tamura et al., 2013]. In addition, the PD-1 expression is upregulated on NK or T cells in MM patients[Benson et al., 2010]. Furthermore, the growth of MM cells is inhibited in PD-1-deficient mice, and an anti-PD-1 antibody pidilizumab (CT-011) enhances NK-cell cytotoxicity against MM cells[Benson et al., 2010]. A Phase I study evaluating CT-011 in patients with advanced hematologic malignancies has shown that it is well-tolerated with clinical benefit noted in one-third of study patients. A Phase II study evaluating the combination of CT-011 and dendritic cell (DC)/myeloma vaccine is currently underway (NCT01067287). Multiple ongoing clinical studies are looking into safety and efficacy of different anti-PD-1 antibodies alone or in combination with IMiDs in the treatment of advanced MM. A phase 2 study of anti–PD-1 mAb pembrolizumab with lenalidomide (NCT02331368) and a phase 1/2 study of pembrolizumab plus pomalidomide in RRMM (NCT02289222) are ongoing. The anti–PD-1 antibody nivolumab alone or in combination with the CTLA4-blocking antibody ipilimumab or the killer cell immunoglobulin-like receptor–blocking antibody lirilumab, is being assessed in phase 1 clinical trial in relapsed or refractory hematologic malignancies, including MM (NCT01592370). Ongoing studies include combining checkpoint inhibitors, mAbs, vaccinations, and/or IMiDs to improve anti-MM memory immunity further and to accomplish durable clinical response. As all patients do not respond to checkpoint inhibitors, it will be critical to delineate the mechanism underlying primary resistance to PD1 antibodies, which may allow for rational combination strategies designed to increase the overall response rate.

4.1.3: Chimeric Antigen Receptor (CAR) T cell therapy—One emerging treatment strategy that is showing considerable promise in hematological malignancies is CAR T cell therapy. In this therapy, T-cells isolated from a patient and engineered to express a tumor-specific CAR then injected back into the patient. B-cell derived lymphoma and leukemia, express the cell surface marker CD19, allowing the design of a matching, specific CAR. Garfall et al. have observed a low expression CD19 on plasma cells and targeted this population via the use of lentivirus-transduced autologous T cells harboring CD3-zeta/CD137 based anti-CD19 chimeric receptor named as CTL019 cells, found an encouraging result in one patient [Garfall et al., 2015]. The CD19 CAR-T strategy was used in conjunction with autologous transplantation. The response was achieved despite a low percentage of MM cells presenting as CD19 positive. These data support that depletion of

the putative myeloma stem cell (CD138-/CD19+ CD20+ cells) can lead to robust clinical responses. Moreover, these data indicate that depleting the putative myeloma stem cell, which is characterized as drug resistant, using immunologic strategies, is a promising approach. Currently, additional CAR-T cells strategies are being developed directed against CD38 and SLAMF-7 and are in preclinical development in MM[Bianchi et al., 2015].

4.1.4: Vaccination therapy—Therapeutic vaccination represents another immunology based treatment option for multiple myeloma patients. Lately, dendritic cell (DC)-tumor fusion vaccines have been investigated as a promising approach for the treatment of cancer. Fusion vaccines offer several advantages that differentiate them from other DC-based vaccines. Phase II clinical trial, Rosenblatt et al. demonstrated that repeated immunization with a DC-tumor fusion vaccine after subsequent autologous stem cell transplantation induces myeloma-specific immunity and improves clinical response[Rosenblatt et al., 2013]. Another vaccine approach is established on stimulating immunity against MM antigens which are typically overexpressed such as XBP1, CD138, or HSP90, individually or in combination. For instance, a combined XBP1, CS1, and CD138 peptide vaccination strategy (PVX-410) is currently under evaluation, alone and with lenalidomide, to stimulate antitumor immunity in smoldering MM and prevent progression to active MM (NCT01718899).

4.2: Targeting the other cellular components of bone marrow microenvironment

Apart from hematopoietic cells, bone marrow microenvironment also contains bone marrow stromal cells (BMSC), Osteoblasts, and Osteoclasts.

4.2.1: Targeting BMSCs—MM cells adhere to BMSCs and ECM into the BM. Adhesion of myeloma cells to BMSCs triggers many pathways resulting that promote survival and drug resistance. The anti-apoptotic phenotype that occurs in the MM BME niche is a complex interplay between soluble and matrix derived factors. For example, the interaction between MM cells and BMSCs activates NFkB signaling pathway and IL-6 secretion in BMSCs. IL-6 increases the production and secretion of vascular endothelial growth factor (VEGF) by MM cells[Kumar et al., 2003]. BMSCs from MM patients expresses several pro-angiogenic molecules such as VEGF, basic-fibroblast growth factor (bFGF), angiopoietin 1 (Ang-1), TGF-b, platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), and IL-1 [Giuliani et al., 2011]. Neutralizing antibodies is one approach to disrupt the impact of soluble factors on the progression of MM. To this end monoclonal antibodies (Mabs) that target IL-6 (Siltuximab) and VEGF (Bevacizumab) are showing promising results in early clinical studies in MM patients [Orlowski et al., 2015; White et al., 2013]. It has been established by our laboratory as well as others that cell adhesion is sufficient to induce a multi-drug resistant phenotype commonly referred to as CAM-DR[Damiano et al., 1999; Hazlehurst et al., 2007; Hazlehurst et al., 2003]. Thus, experimental data indicates that drug discovery must consider not only intrinsic vulnerabilities of MM but identify and target vulnerabilities in the context of bone marrow microenvironment models. To this end Silva and colleagues have developed a non-destructive assay that allows for assessment of drug response using a high-throughput assay that includes bone marrow stroma cells and collagen [Khin et al., 2014; Silva et al., 2015]. Importantly these investigators showed that this assay

system could be used for primary MM cells to test drug sensitivity reproducibly. It will be critical to consider high content screening of novel agents in systems that allow for crosstalk with the tumor microenvironment.

4.2.2: Targeting Osteoclasts—The equilibrium between bone resorption and ossification is disrupted during the progression of MM, leading to bone destruction and the development of painful osteolytic lesions [Bataille et al., 1989]. Clinical evidence indicates that MM is associated with excessive tumor induced, osteoclast-mediated bone destruction. Osteoclast activation is mediated by several key factors, such as receptor activator of NF-κB ligand (RANKL), interleukin-3 (IL-3), macrophage inflammatory protein-1a (MIP-1a), and IL-6. RANKL is a member of the tumor necrosis factor (TNF) family and plays a vital role in the increased osteoclastogenesis in MM. Osteoclast cells express RANK receptor; interaction between MM cell and BMSC within the bone marrow results in the elevated release of RANKL in BM microenvironment. In turn, elevated RANKL levels leads to stimulating osteoclast activity through the binding of RANKL to its receptor, on osteoclast precursor cells. RANKL is also intricated in inhibition of osteoclast apoptosis. Blocking the interaction of RANKL with RANK has been shown to regulate bone loss and tumor burden in myeloma SCID-Hu animal model [Yaccoby et al., 2002]. Recently, a monoclonal antibody, denosumab blocking the RANKL was proven to be effective in relapsed or plateau-phase multiple myeloma patients [Vij et al., 2009]. Phase III studies are underway for use of denosumab in myeloma patients.

4.2.3: Targeting Osteoblasts—Experimental evidence indicates that suppression of osteoblast activity is accountable for both bone destructive process and progression of myeloma tumor burden[Yaccoby, 2010]. DKK 1(Dickkopf-1) plays a vital role in inhibiting osteoblast activity in MM [Tian et al., 2003]. DKK1 is a Wnt-signaling antagonist produced by MM cells, and it inhibits osteoblast differentiation and disrupts Wnt-regulated OPG and RANKL production by osteoblasts [Tian et al., 2003]. The DKK1 expression is significantly higher in myeloma patients with lytic bone lesions [Dun et al., 2009]. Studies have shown that antagonizing DKK1 and activating Wnt signaling prevents bone disease in MM [Tian et al., 2003]. Recent studies suggest that the anti-Dkk1 antibody (BHQ880) increases osteoblast differentiation in vitro and increases osteoblast number and trabecular bone in vivo [Fulciniti et al., 2009]. Ongoing clinical studies will determine whether agents that inhibit MM-induced lytic lesions and disruption of the MM bone interface niche will have an impact on MM progression.

4.3: Targeting cell adhesion mediated drug resistance associated with the bone marrow microenvironment

MM homes to the bone and molecules that mediate homing to the bone include VLA-4 (α4β1), and CD44. Targeting these key molecules as well as other adhesion receptors that contribute to survival such as VLA-7 (α4β7) remains a promising approach in the treatment of MM.

4.3.1: Targeting the cell adhesion molecules—MM cells demonstrate adhesion towards various ECM constituents, including laminin, the microfibrillar collagen type-VI,

and fibronectin (FN), via β1 integrin-mediated adhesion (Kibler, Schermutzki et al. 1998). In addition to FN, VLA-4 can bind vascular cell adhesion molecule-1 (VCAM-1), which is constitutively expressed on bone marrow stromal cells. The interaction between VCAM-1 and VLA-4 promotes myeloma cell colonization in bone marrow[Okada et al., 1999]. Moreover, cell adhesion via VLA-4 integrin was shown to be sufficient to cause a multi-drug resistant phenotype[Damiano et al., 1999]. Indeed, antibodies that target α4 integrins have been shown to have success in decreasing tumor burden. Targeting α4β1 integrin using antibody (Natalizumab), suppresses the MM cell growth in BM microenvironment[Mori et al., 2004; Podar et al., 2011]. In addition, to antibodies peptides are an attractive approach for inhibition of cell adhesion. HYD1 was identified using a decapeptide all D-amino acid library using a phenotypic screen of cell adhesion as an endpoint[Sroka et al., 2006]. The lead candidate identified using this high-content screening approach was termed HYD-1. Our laboratory subsequently determined that HYD-1 blocked adhesion of MM cells to extracellular matrixes but also induced caspase-independent necrotic cell death as a single using both in-vitro and in vivo MM models[Emmons et al., 2011; Nair et al., 2009]. Another unique feature of this class of compounds is that ex-vivo testing of primary patient specimens indicated that this class is more potent in specimens obtained from patients which had relapsed on standard of care agents. These data suggest that targeting alternative cell death pathways maybe an important strategy for RRMM. Standard of care agents typically all converge mechanistically at activation of caspase and subsequent apoptotic cell death, and targeting alternative cell death pathways has not been explored for the treatment of RRMM. To increase the bioavailability and potency of HYD-1, cyclization strategies were used to constrain the minimal active sequence of MVISW. The cyclized HYD1 molecule referred to as MTI-101 binds to a CD44/VLA-4 containing complex in MM cells and was shown to have robust *in vitro* and *in vivo* activity [Gebhard et al., 2013]. Interestingly MTI-101 appears to have agnostic properties as treatment resulted in activation of Pyk2 and Erk1/2. Mechanistic studies revealed that cell death was only partially dependent on the RIPK mediated necrotic pathway, and further studies are required to fully understand the mechanism of cell death of this class of molecules.

CD44 is a cell surface transmembrane glycoprotein encoded by the single gene, and is a receptor for hyaluronic acid (HA). Both CD44 and VLA-4 are both thought to be critical for homing of normal hematopoietic stem cells as well as malignant leukemias[Cao et al., 2016; Krause et al., 2006]. Reducing the expression of CD44 was shown to sensitize MM cell lines to lenalidomide[Bjorklund et al., 2014]. Finally, CD44 was recently shown to be highly expressed in extracellular vesicles obtained from the serum of primary MM patients and high expression was found to be a poor prognostic indicator[Harshman et al., 2016]. Extracellular vesicles or exosomes have gained much attention for a mechanism whereby tumors can condition the tumor niche. Targeting cell adhesion molecules maybe an attractive approach for inhibiting the trafficking of exosomes to the bone marrow niche for the treatment of MM [Hoshino et al., 2015].

4.3.3: Proline-rich tyrosine kinase 2 (Pyk2) and focal adhesion kinase (FAK) inhibitors—Due to the redundancy of signaling of integrin and cell adhesion receptors, it is attractive to consider blocking common or convergent downstream signaling pathway. Pyk2

is a member of the focal adhesion kinase(FAK) family and plays a major role in tumor development of various cancers. Zhang et al reported that MM patients have increased expression of Pyk2 when compared to healthy individuals. Reduing Pyk2 levels results in reduced in vivo MM cell growth as well as decreased MM cell proliferation in vitro by suppressing Wnt/β-catenin signaling[Zhang et al., 2014b]. Furthermore, FAK/Pyk2 inhibitor VS-4718 effectively inhibits MM cell growth both in vitro and in vivo. Recent studies indicate that interaction between β1 integrin, fibronectin, and interleukin-6 in bone marrow microenvironment results in increased activation of Pyk2, resulting in amplification of signal transducer and activator of transcription 3 (STAT3) activation. Molecular and pharmacological targeting of Pyk2 results in attenuated MM progression in vivo [Meads et al., 2016]. Interestingly the dependency of Pyk2 in mediating survival in these studies was more notable when MM cells were placed in co-culture with bone marrow stroma cells compared to unicellular MM model. Again these studies point to the need for exploring cancer cell dependencies in the context of the tumor microenvironment.

5: Monoclonal antibodies (mAbs)

Myeloma cells can be targeted by cell surface receptors which are enriched on the plasma cell or B-cell lineage. mAbs induce cytotoxicity by several mechanisms. For example, mAbs can cause antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), antibody-dependent phagocytosis (ADCP) and they can cause apoptosis/growth arrest via blocking intracellular signaling pathways. FDA recently approved two monoclonal antibodies daratumumab and elotuzumab for the treatment of MM. Targeting antibodies take advantage of cell surface receptors that are present on plasma cells and/or B-cells. Daratumumab is a human CD38 IgG1 mAb that was generated by immunizing transgenic mice possessing human immunoglobulin genes. Daratumumab can cause cytotoxicity in myeloma cells by ADCC, ADCP, and CDC. Recent clinical trial (NCT01985126) results reported that Daratumumab monotherapy showed promising efficacy in PIs and IMDs refractory patients with multiple myeloma[Lonial et al., 2016]. Elotuzumab has been approved for the combination therapy with lenalidomide and dexamethasone for RRMM. Elotuzumab binds to the cell surface receptor signaling lymphocytic activation molecule F7 (SLAMF7). SLAMF7 is selectively expressed on myeloma cells and natural killer cells, induce antibody-dependent cellular cytotoxicity and direct natural killer cell activation. Elotuzumab does not show cytotoxicity by CDC in myeloma cells. In a phase III clinical trial (NCT01239797), the addition of elotuzumab to lenalidomide and dexamethasone therapy in patients with RRMM was associated with a significant improvement in progression-free survival and overall response rate. Several new mAbs are under development for various cell membrane targets such as CD20, CD40, CD56, CD74, CD138 and CD200(See Table 1). Additionally, Mabs are in development that is designed to neutralize soluble factors within the tumor microenvironment that promote myeloma-induced bone destruction such as RANKL and DKK or promote progression of myeloma cells such as IGF-1R, IL-6, VEGF, and BAFF(see Table 2).

Conclusion and future directions

Therapy for MM has significantly improved in the past decade with the introduction of novel therapies and survival outcomes in MM patients markedly changed. The number of agents available to treat MM has increased dramatically, suggesting that multiple pathways are required to inhibit the progression of MM. Pathways discussed in this review may not be sufficient to cause transformation yet are critical to facilitate disease progression and emergence of drug resistance. Continued challenges include developing rational combination strategies based on patient-specific MM tumor cell and BME vulnerabilities. As such, it is critical to continue to understand the emergence of drug resistance and newer technologies such as RNA-SEQ, whole exome sequencing and CHIP-SEQ will allow for unbiased molecular monitoring of disease progression. These initiatives will likely provide insight into the evolution of drug resistance and will hopefully allow for the direct discovery of novel druggable targets from specimens derived from patients progressing on therapy. It is likely that identification of target discovery directly from drug resistant patient specimens will be a powerful approach to expedite drug discovery.

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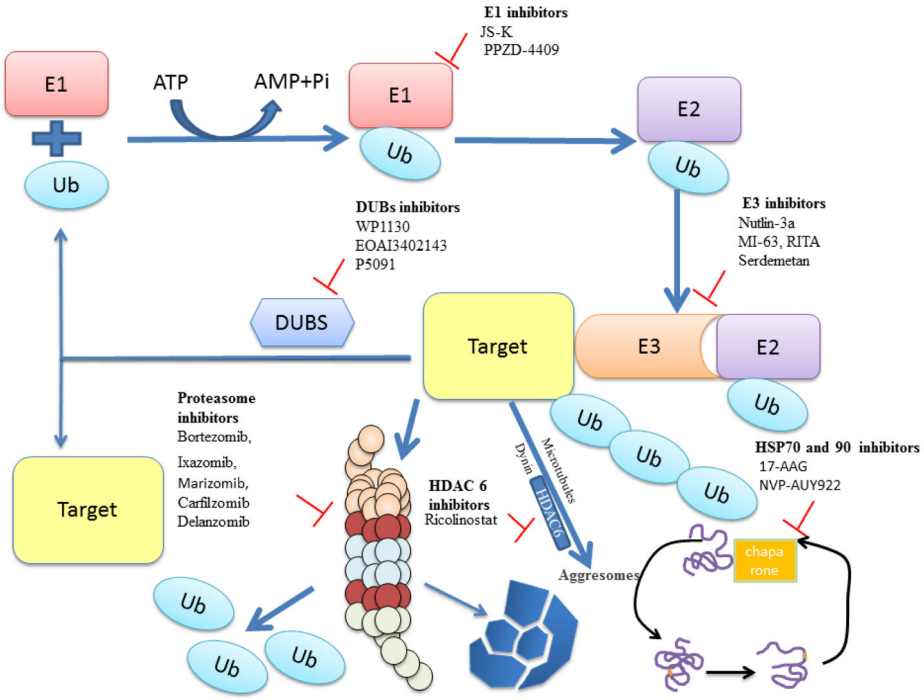

Figure 1. Therapeutic approaches to target protein homeostasis in multiple myeloma (MM)

MM cells depend on tight control of protein homeostasis to prevent terminal unfolded protein response and apoptosis. Inhibition of proteasome is a successful strategy in MM. In addition, modulation of enzymes that involved in ubiquitin proteasome systems (UPS) such as E1, E2, and DUBs can effect the growth of myeloma cells. Moreover, HSP70 and HSP90 inhibitors can induce proteotoxic stress. Furthermore, HDAC6 inhibitors can induce apoptosis in MM. DUB, deubiquitinating enzyme; HDAC6, histone deacetylase 6; HSP, heat shock protein; Ub, ubiquitin.

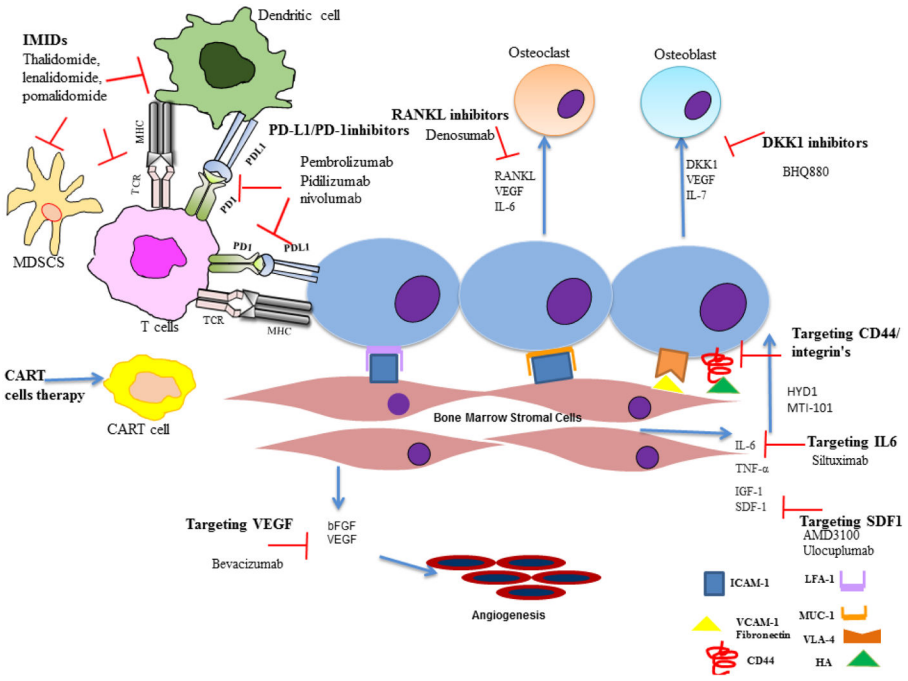

Figure 2. Therapeutic approaches to target the elements of bone marrow microenvironment

MM cells interact with many cellular and non-cellular components of the BM such as hematopoietic cells, osteoclasts, osteoblasts, stromal cells, endothelial cells and ECM. Several cytokines and chemokines are secreted in response to these cell-cell and cell- ECM interactions, leading to enhanced tumor growth, inhibition of osteoblasts, and increased osteoclast activity. IMIDs and PDL1-PD1 inhibitors increase the anti-tumor immunity; CART cell therapy directly targets Myeloma cells. RANKL, DKK1 inhibitors decrease the osteoclast activity and promote osteoblasts activity respectively. Targeting the IL6, VEGF and SDF 1 inhibits myeloma cells proliferation and survival. MM cells interact with many cellular and non-cellular components of the BM such as hematopoietic cells, osteoclasts, osteoblasts, stromal cells, endothelial cells and ECM. Several cytokines and chemokines are secreted in response to these cell-cell and cell- ECM interactions, leading to enhanced tumor growth, inhibition of osteoblasts, and increased osteoclast activity. IMDs and PDL1-PD1 inhibitors increase the anti-tumor immunity; CART cell therapy directly targets Myeloma cells. RANKL, DKK1 inhibitors decrease the osteoclast activity and promote osteoblasts activity respectively. Targeting the IL6, VEGF and SDF 1 inhibits myeloma cells proliferation and survival. Targeting the CD44 and integrin's reverses the cell adhesion mediated drug resistance and inhibit the myeloma cell survival.

bFGF, basic fibroblast growth factor; CD44, cell adhesion molecule; CAR, chimeric antigen receptor; Dkk1, Dickkopf-related protein 1; HA, hyaluronic acid; IMiD, immunomodulatory drug; IL-6, interleukin 6; IL-7, interleukin 7; IGF-1, Insulin-like growth factor 1; ICAM1, intercellular Adhesion Molecule 1; LFA-1, Lymphocyte function-associated antigen 1; MUC-1, mucin1; MHC, major histocompatibility complex; MDSC, myeloid-derived suppressor cell; PD-1, programmed death 1; PD-L1, programmed death ligand 1; RANKL, receptor activator of nuclear factor κ B ligand; SDF1, stromal cell-derived factor 1;TCR, T cell receptor; TNF-α, Tumor necrosis factor; TGF-β, Transforming growth factor beta;

VEGF, vascular endothelial growth factor; VLA-4, Very Late Antigen-4; VCAM-1, vascular cell adhesion molecule 1

Table 1

mAbs that target myeloma cell surface.

Target	Antibody	Phase	Status	Identifier
CD38	Daratumumab	Approved	----	----
SLAMF7	Elotuzumab	Approved	----	----
CD20	Rituximab	II	C	NCT00003554
CD40	Dacetuzumab	II	C	NCT00435916
CD56	Lorvotuzumab	II	R	NCT02420873
CD74	Milatuzumab	I	C	NCT00421525
CD138	BT062	II	ONR	NCT01638936
CD200	Samalizumab	I	C	----

R: Recruiting; C: Completed; ONR: Ongoing, not recruiting;

Table 2

mAbs that target the components of bone marrow microenvironment.

Target	Antibody	Phase	Status	Identifier
IL-6	Siltuximab	II	C	NCT00911859
VEGF	Bevacizumab	II	C	N01-CM-62209
BAFF	LY2127399	I	C	-
RANKL	Denosumab	III	C	NCT00330759
DKK1	BHQ880	I	C	NCT20050244
IL-6	Siltuximab	II	C	NCT00911859

R: Recruiting; C: Completed; ONR: Ongoing, not recruiting; BAFF: B-cell activating factor;