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Bone marrow microenvironment and the identification of new targets for myeloma therapy

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Abstract

The development of multiple myeloma (MM) is a complex multi-step process involving both early and late genetic changes in the tumor cell as well as selective supportive conditions by the bone marrow (BM) microenvironment. Indeed, it is now well established that MM cell-induced disruption of the BM homeostasis between the highly organized cellular and extracellular compartments supports MM cell proliferation, survival, migration and drug resistance through activation of various signaling (for example, PI3K/Akt, JAK/Stat-, Raf/MEK/MAPK-, NFκB- and Wnt-) pathways. Based on our enhanced understanding of the functional importance of the MM BM microenvironment and its inter-relation with the MM cell resulting in homing, seeding, proliferation and survival, new molecular targets have been identified and derived treatment regimens in MM have already changed fundamentally during recent years. These agents include thalidomide, its immunomodulatory derivative lenalidomide and the proteasome inhibitor bortezomib, which mediate tumor cytotoxicity in the BM milieu. Ongoing studies are further delineating MM pathogenesis in the BM to enhance cytotoxicity, avoid drug resistance and improve patient outcome.

Keywords

multiple myeloma; bone marrow; novel therapies

Introduction

Despite the presence of a variety of chromosomal aberrations, translocations and mutations in essential growth and tumor suppressor genes in multiple myeloma (MM) cells, oncogenomic studies have identified few differences distinguishing monoclonal gammopathy of unknown significance from MM.[1-3] This finding highlights the essential role of the bone marrow (BM) microenvironment in disease maintenance and progression. Indeed, direct and indirect interactions with other cells and the extracellular matrix (ECM) within the liquid milieu of the BM environment are key requirements for MM pathogenesis, MM cell growth, survival, migration and drug resistance. Cytokines and growth factors are produced and secreted by MM and other cells within the BM microenvironment and regulated both by autocrine as well as paracrine loops and cell–cell adhesion. The

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complexity of signaling events is further enhanced by co-stimulation triggered by cell–cell contact and cytokine/growth factors.

Signaling cascades activated by cytokines, growth factors and/or adhesion in MM cells include the Ras/Raf/MEK/MAPK-pathway, PI3K/Akt-pathway, the JAK/Stat3-pathway, the NFκB-pathway and the Wnt-pathway. Promising intracellular targets for novel therapies also include protein kinase C (PKC) and heat-shock proteins (HSPs). Moreover, genomic profiling has now identified additional stage-specific intracellular targets, which are now under investigation as novel potential therapeutic targets.[2,4]

Cell surface receptors include integrins, cadherins, selectins, syndecans, and the immunoglobulin superfamily of cell adhesion molecules including syndecan-1 (CD138), H-CAM (CD44), VLA-4 (CD49d/CD29), ICAM-1 (CD54), N-CAM (CD56), LFA-3 (CD58), αvβ3, CD56, CD74, HM1.24, VLA-5 (CD49e/CD29), VLA-6 and CD51.[5,6] Most recently, a functional role in MM has been shown for cell surface glycoprotein CD2 subset-1 (CS-1), a member of the immunoglobulin gene superfamily;[7] as well as HLA-A;[8] and β2-microglobulin, which stabilizes MHC class I molecule and is a key prognostic marker in MM.[9] Several approaches to target these surface receptors are under investigation.

Initially considered to be the sole contributor of maintenance and expansion of MM cells within the BM,[10,11] the liquid milieu consists of cytokines and growth factors, including IL-6, VEGF, insulin-like growth factor-1 (IGF-1), TNF-α, SDF-1α and CD40; as well as TGF-β, bFGF, MIP-1α, SCF, HGF, IL-1β, IL-3, IL-10, IL-15 and IL-21;[10-12] as well as angiopoietin-1 and matrix metalloproteinases, for example, MMP-2 and MMP-9. Several approaches to neutralize these cytokines, growth factors or their respective receptors are under investigation.

In summary, novel biologically based treatment regimens aim to target not only MM cells alone but also MM cell–stromal cell interactions and the BM milieu. Here, we discuss three classes of new therapeutic targets and derived agents: first, those targeting pathways of MM cells; second, agents targeting the cellular BM compartment; and third, those targeting the non-cellular BM compartment (http://clinicaltrials.gov/; http://www.multiplemyeloma.org/; http://myeloma.org/).

Targeting pathways within MM cells

Ras/MEK/MAPK-pathway in MM

Several cytokines and growth factors within the MM BM microenvironment including IL-6, IGF-1 and VEGF, as well as direct cell–cell contact through integrins trigger MM cell growth, survival and drug resistance by inducing the Ras/MEK/MAPK-pathway (Figure 1). Moreover, MM patients with mutant Ras have a shortened survival and are less likely to respond to chemotherapy. The initial transfer of farnesyl groups from farnesyl diphosphate to the CAAX motif of the oncoprotein Ras is, at least in part, responsible for related downstream signal transduction, and targeted inhibition of Ras/Raf/MEK/ERK signaling using farnesyltransferase inhibitors (FTI) abrogates tumor cell growth.[6,12] However, the transfer of farnesyl groups, and therefore the effect of FTIs, on Ras may not be specific as FTI Tipifarnib (R115777) also induces MM cell apoptosis through Ras-independent pathways.[13] A clinical phase II trial demonstrated that the FTI Tipifarnib was well tolerated, induced stabilization of disease, and inhibited farnesylation and tumor survival pathways in patients with advanced MM.[14] Interestingly, the combination of bortezomib (Bort) with Tipifarnib induces synergistic MM cell apoptosis through downregulation of HDAC6 and inhibition of aggresome formation.[15] An ongoing clinical trial is testing the efficacy of Tipifarnib together with Bort.[16]

Raf-1 is a key regulator of cellular proliferation and survival within the MAPK pathway, and BAY 43-9006/sorafenib/Nexavar (Bayer Pharmaceuticals, West Haven, CT, USA) is the first oral multikinase inhibitor (PDGFR, VEGFR-1,-2,-3 and c-Kit) that targets Raf and affects tumor signaling and tumor vasculature. Several clinical studies using sorafenib, alone or in combination with novel or conventional therapies, are now ongoing in solid and hematologic malignancies including MM.

Finally, our own recent data demonstrate significant inhibitory activity of the MEK1/2 inhibitor AZD6244 (ARRY-142886) against MM cell growth and survival as well as osteoclastogenesis,[17,18] and a clinical trial with AZD6244 is planned (Table 1).

PI3K/Akt-pathway in MM

PI3K/Akt plays a pivotal role in IL-6, IGF-1, VEGF- and integrin-triggered pathways in MM cells (Figure 1). A novel agent that targets Akt is perifosine/KRX-0401. Specifically, perifosine: inhibits Akt phosphorylation; triggers JNK activation followed by caspase-8/9 and poly-ribose polymerase cleavage; triggers the formation of the death-inducing signaling complex as well as the recruitment of TRAIL-R1/DR4, TRAIL-R2/DR5 and Bid in lipid rafts; induces apoptosis through cytochrome *c* release as well as downregulation of survivin; and increases ERK phosphorylation.[19-21] Importantly, MEK inhibitors for example, AZD-6244 (see previous section) or exogenous TRAIL synergistically enhance perifosine-induced cytotoxicity in MM cells.[17,22] Clinical studies using perifosine in combination with Bort and/or with dexamethasone (Dex) or Len with Dex are now ongoing. Other PI3K inhibitors include SF1126 and BEZ235.[23,24]

Activated Akt phosphorylates forkhead transcription factor, glycogen synthase 3β and mammalian target of rapamycin. Mammalian target of rapamycin in turn mediates phosphorylation of P70S6 and 4E-BP1 and subsequent upregulation of D-cyclins and c-Myc.[6,12] Several preclinical studies demonstrate promising results using mammalian target of rapamycin inhibitors. A phase II clinical trial using temsirolimus alone is now completed, and a clinical phase I trial using deforolimus is ongoing. Combination clinical trials using temsirolimus with Bort or Len; sorafenib with everolimus; and sorafenib with Bort are also ongoing (Table 1).

Stat signaling pathways in MM

Dysregulated cytokine, for example, IL-6, signaling triggers constitutive Stat3 activation in at least 50% of primary MM samples,[25] as well as bone marrow stromal cells (BMSCs) from MM patients[26] (Figure 1). Inhibition of Stat3 activity by compounds including curcumin, atripimod, the JAK2 tyrosine kinase inhibitor AG490 and the pan-JAK inhibitor pyridone 6 is associated with inhibition of IL-6-induced MM cell survival.[27-31] Moreover, inhibition of constitutive Stat3 activity sensitizes MM cells to apoptosis induced by conventional chemotherapy.[32] Importantly, in the presence of BMSCs combined disruption of both the MEK/ERK and the IL-6R/Stat3 pathways is required to induce MM cell apoptosis.[33] Ongoing studies are further investigating the potential role of inhibitors of Stat3 and other members of the Stat family within MM.

NF-κB-signaling pathway in MM

Most MM cell lines and patient samples show evidence of constitutive NF-κB activation (Figure 1). Indeed, two recent studies demonstrate genetic (mutation, deletion) or epigenetic alterations in a high percentage of MM cells leading to increased expression and activation of molecules within both the canonical and the non-canonical pathway.[34-36] Furthermore, NF-κB signaling pathways not only play an important role within MM cells, but also trigger

the production of IL-6, BAFF or APRIL within several types of stromal cells.[37,38] Taken together, these studies further validate the therapeutic potential of NF-κB inhibitors.

Over 750 inhibitors of the NF-κB-pathway have been identified including antioxidants, peptides, small RNA/DNA, microbial and viral proteins, small molecules and engineered dominant-negative or constitutively active polypeptides. Some of these inhibitors appear to target multiple steps in the NF-κB pathway.[39] Importantly, anti-MM effects of Bort are strongly attributed to its inhibitory effect on NF-κB-signaling sequelae. Moreover, several recent studies strongly indicate the therapeutic potential of IKKβ inhibitors in MM: specific blockade of IKKβ decreased both growth and survival in MM cells, alone and when co-cultured with BMSCs [40-42] (Table 1).

Wnt-signaling pathways in MM

The canonical Wnt/β-catenin and the alternative Wnt/RhoA-signaling pathways have recently been implicated in MM pathogenesis (Figure 1). Specifically, N-terminally unphosphorylated β-catenin is overexpressed, and stimulation with Wnt3a additionally increases both accumulation and nuclear localization of β-catenin and MM cell proliferation.[43] Indeed, Wnt signaling is constitutively activated, correlating with hypermethylation and hence silencing of Wnt antagonist genes. Consequently, demethylation of methylated Wnt inhibitors downregulates Wnt signaling and associated MM cell proliferation.[44] Moroever, Wnt-signaling pathways induce cell adhesion-mediated drug resistance (CAM-DR) mediated through integrin α6β1 (VLA-6) and RhoA-Rho kinase-signaling sequelae;[45] and also play a key role in MM bone disease. Specifically, elevated levels of dickkopf1 (DKK1), a Wnt-signaling antagonist, in BM plasma and peripheral blood correlate with DKK1 gene expression patterns in MM patients with MRI and/or radiograph positive bone lesions but not control subjects or MM patients without bone lesions.[46]

The small molecule PKF115-584, recently identified by high-throughput ELISA screening, efficiently blocks the formation of the β-catenin/TCF transcriptional complex and thereby expression of Wnt target genes inducing cytotoxicity in both patient MM cells and MM cell lines.[47] Additional strategies to target Wnt-signaling pathways include the use of small-molecule inhibitors which block interaction of β-catenin with CREB-binding protein, siRNAs and antibodies directed against WNTs.[48]

New therapeutic approaches targeting the cellular MM BM compartment

The cellular BM compartment is composed of hematopoietic cells including hematopoietic stem cells (HSCs); hematopoietic and mesenchymal progenitor and precursor cells; BM-derived circulating endothelial precursors (CEPs); immune cells (B lymphocytes, T lymphocytes, NK cells, macrophages, monocytes, NKT cells); erythrocytes; megakaryocytes and platelets; and non-hematopoietic cells including endothelial cells (ECs); fibroblasts/BMSCs; as well as cells involved in bone homeostasis including chondroclasts, osteoclasts (OCs) and osteoblasts (OBs). The intimate functional interaction between these BM compartments is of critical importance for finely tuned physiologic functions: the regulation of hematopoiesis; the mobilization of blood cells into the blood stream; as well as the homing of mature cells to selective sites within the BM. In MM, tumor cells impact the BM and disrupt the balanced homeostasis of MM cell–stromal cell interactions and liquid factors (cytokines, growth factors). Below we give a brief overview of cells within the BM compartment, their pathophysiologic role in MM, and therapeutic implications (Figures 2 and 3, Table 1).

BMSCs

Adhesion of tumor cells to BMSCs activates a multitude of signaling pathways (see Targeting pathways within MM cells) leading to the cytoplasmic sequestration of many transcription factors; upregulation of cell cycle regulating proteins and antiapoptotic proteins; and increased telomerase activity.[49] These molecular sequelae are triggered directly, through integrin-mediated interactions of MM cells with BMSCs; or indirectly, through cytokines and growth factors released by either tumor cells or BMSCs (see New therapeutic approaches targeting the non-cellular BM compartment). Specifically, MM cell–BMSC interaction as well as VEGF secreted by MM cells trigger NF-κB-dependent transcription and secretion of IL-6 in BMSCs, a major MM growth, survival and drug resistance factor. In turn, IL-6 enhances the production and secretion of VEGF by MM cells. Compounds directed against adhesion-mediating integrins are under investigation. Both IL-6 and VEGF secretion are also induced by CD40 activation of tumor cells, as well as by cytokines present in the BM microenvironment, including TNFα and IL-1.[50] A clinical phase II study evaluating the CD40 antibody SGN-40 [51] has now been completed, a clinical trial using SGN-40 together with Len, which augments antibody-dependent cellular cytotoxicity,[52] is ongoing.

Besides integrins and CD40/CD40 L, BMSC–MM cell interaction is also mediated through Notch, in particular Notch ligands Jagged-1, Jagged-2 and DLL-1, in particular.[53-55] Upon Notch-Notch ligand interaction, Notch-signaling pathways are activated both in MM cells as well as in BMSCs, with induction of IL-6, VEGF, and IGF-1 secretion and associated MM cell proliferation and survival.[56-58] Therefore, inhibition of Notch-1 was suggested as a new therapeutic approach to overcome resistance to chemotherapy, for example, melphalan and mitoxantrone, in MM.[56] In contrast to these data, Zweidler-McKay *et al.* reported that Notch signaling is a strong inducer of growth arrest and apoptosis in a wide range of B-cell malignancies including MM, and suggest the therapeutic potential of therapies activating Notch.[59] Further studies are required to define the potential benefits of strategies to either inhibit or promote Notch signaling.

Although suggested by some authors, the existence of MM-specific abnormal BMSCs is still controversial. For example, Zdzisinska *et al.*[60] report in a recent study that production of MMP-1, MMP-2 and TIMP-2 is significantly increased in BMSCs of MM patients versus healthy controls. Further studies are required to verify these data, to identify specific targets, and to design derived compounds.

MM stem cells

Recent data suggest the existence of MM stem cells (MMSC),[61-63] a very small subset of cells within the tumor cell population which resides in either the osteoblastic or the vascular niche and has the capacity for self-renewal and differentiation. Similar to HSCs,[64-66] it is hypothesized that the trabecular bone surface of the osteoblastic niche provides the quiescent microenvironment for MMSCs, whereas the sinusoids of the vascular niche facilitate their egress from the BM. Future research aims to identify compounds that specifically modify the stem cell niche in order to block MM stem cell engraftment while still enabling HSC development.

T and B cells

Both T and B cells show significant impairment of their functions in MM. Although T cells are hyperactive,[67] B cell-driven antibody responses are deficient. Both impairment of the global T-cell receptor diversity as well as decreased numbers and function of suppressive CD25(high)FOXP3(+)CD4(+) Treg cells in MM may, at least in part, account for the dysfunctional T-cell response.[67,68] Importantly cytokines, including IL-6 abrogate

generation of Treg cells.[69] Besides adverse immunologic effects, dysregulated T-cell response also contributes to MM bone disease (see section osteoclasts and osteoblasts). Several ongoing preclinical and clinical studies are evaluating strategies to normalize T and B cell function.

NK and NKT cells

Immunomodulatory effects are triggered by Thal/immunomodulatory drugs (IMiDs) which induce both T-cell proliferation and enhanced nature killer (NK) cell cytotoxicity.[70] Moreover, dendritic cell (DC) -induced sustained expansion and activation of glycolipid reactive innate nature killer T (NKT) cells *in vivo* in MM patients may help to boost adaptive T-cell immunity.[71] Conversely, prior studies also demonstrate that clinical progression in monoclonal gammopathy of unknown significance patients is associated with an acquired, but potentially reversible, defect in NKT cell function indicating a role in controlling the malignant growth of MM cells.[72]

DCs

DCs of MM patients are functionally defective due, at least in part, to IL-6-, VEGF- or β2-microglobulin-triggered inhibition of DC maturation.[73-76] In addition, direct MM cell–DC interaction enhances MM clonogenicity.[77] Ongoing preclinical studies are evaluating a variety of anti-MM agents for their ability to normalize DC function and inhibit their supportive role on MM cell growth. For example, Bort enhances DC-mediated induction of immunity to MM through exposure of cell surface HSP-90 on dying tumor cells;[78] but also disrupts tumor–DC interactions in MM and lymphoma.[79] HSP-90-mediated protein folding within DCs is additionally required for DC-specific function. Conversely, HSP90 inhibition disrupts the DC function.[80]

ECs

In MM, both tumor cells and stromal cells prolong survival of BM ECs both by increased secretion of EC survival factors, such as VEGF,[81-83] and by decreased secretion of anti-angiogenic factors. Triggered by this imbalance of angiogenic regulators, the ‘angiogenic switch’ accounts not only for a rapid increase of tumor vessels to support tumor growth, but also for their abnormal structure and formation of mosaic blood vessels. These mosaic vessels consist of ECs as well as highly proliferating circulating endothelial precursors/angioblasts, HSCs, progenitor cells, monocytes, macrophages and tumor cells (vasculogenic mimicry).[84-86] Moreover, recent studies suggest the existence of MM-specific ECs.[83,87,88] Functionally, tumor-associated vasculature causes chaotic and variable blood flow as well as vessel leakiness, resulting in lowered drug delivery and further selection of more malignant tumor cells. Importantly, the anti-MM activity of Thal, Bort, and Len is, at least in part, also because of their antiangiogenic effects.[89] The clinical success of antiangiogenic agents like bevacizumab (Avastin) suggests that their activity likely depends on tumor stage, prior treatment and their combination with other anticancer agents. For the treatment of MM, antiangiogenic therapies are therefore being evaluated in combination with conventional or novel anti-MM therapies.[90]

Osteoclasts and osteoblasts

Osteolytic bone (‘punch-out’) lesions associated with bone pain, pathologic fractures and diffuse osteoporosis in the central skeleton, the skull and long bones are a typical clinical feature in almost all MM patients. Functionally, these defects are triggered by increased OC formation and activity in the vicinity of MM cells, as well as lower numbers of OBs and decreased bone formation.[91,92] A variety of OC-activating factors, produced by both tumor as well as stromal cells, trigger increased OC activity. These factors include macrophage

inflammatory protein-1α (MIP-1α)[92,93] and receptor of NFκB ligand (RANKL), also named tumor necrosis factor-related activation induced cytokine, TRANCE; as well as osteoprotegerin ligand, VEGF, TNFα, IL-1β, parathyroid hormone-related protein, HGF and IL-6. OC activity in turn modulates MM cell growth and survival.[92,93] Conversely, lower numbers of OBs and decreased bone formation in MM are associated with dysregulation of several signaling molecules including Runt-related transcription factor 2/Cbfa1, Wnt and IL-3. First, MM cells block Runt-related transcription factor 2/Cbfa1 in human BM OB progenitors triggered by VLA-4/VCAM-1-mediated contact or IL-7 secretion.[91-93] Second, the Wnt-signaling antagonist DKK1, an inhibitor of OB differentiation, is significantly overexpressed in patients with MM who present with lytic bone lesions. DKK1-induced inhibition of OB precursors shifts the balance towards OCs and blocks establishment of the BM microenvironment required for HSC differentiation. Consequently, impaired differentiation also contributes to immunosuppression and anemia in MM patients. Myeloma-derived DKK1 also disrupts Wnt-regulated OPG and RANKL production by OBs.[94] Third, IL-3 is increased in BM plasma from MM patients and triggers increased OC formation, as well as blocks differentiation of pre-OBs to mature OBs. Importantly, CD3+ T cells have been identified as the main source of IL-3 in MM patients. A potential critical role of CD3+ T cells in MM bone disease is further supported both by their increased production of RANKL; as well as their promotion of OC formation and survival.[91] Conversely, administration of a polyclonal DKK1 antibody ameliorates the bone-specific morbidity of MM;[46] and immunodepletion of secreted frizzled-related protein-2, which prevents binding of Wnt proteins to their receptors and thereby inhibits OB function, significantly restores bone mineralization *in vitro*.[95]

The main therapy used for treatment of bone disease in MM, with additional direct effects on tumor cells, is amino bisphosphonates.[96] Moreover, *in vivo* studies have demonstrated both a decrease in osteolysis, as well as an indirect anti-MM effect, using RANKL-Fc or OPG-Fc.[91-93] A phase I study with recombinant OPG construct AMGN-0007 showed rapid, sustained and profound suppression of bone resorption and was well tolerated.[97] Based on signaling pathways in bone associated cells, OCs and OBs, several additional compounds with antibone resorptive activity have now been identified including: the HDAC inhibitor PXD101;[98] the MEK inhibitor AZD6244 (ARRY-142886);[17] Resveratrol;[99] the CCR1 inhibitor MLN3897;[100] the Cox inhibitor SDX-308;[101] IMiD CC-4047[102] and Bort, which additionally increases OB activity.[93,103,104]

Mesenchymal stem cells

Mesenchymal stem/progenitor cells (MSCs), which differentiate in a context-specific manner into muscle, bone, fat and other cell types, represent another potential therapeutic target in MM. BM MSCs isolated from MM patients, as compared with normal MSCs, produce high levels of IL-6, DKK1, as well as factors associated with angiogenesis and osteogenic differentiation.[105] Moreover they have decreased ability to inhibit T-cell proliferation.[106] Bort induces MSCs to preferentially undergo osteoblastic differentiation in mice, in part by modulation of the bone-specifying transcription factor Runt-related transcription factor 2. Mice implanted with MSCs showed increased ectopic ossicle and bone formation after treatment with Bort. Bort treatment increased bone formation and rescued bone loss in a mouse model of osteoporosis.[107] These results are consistent with the therapeutic benefits of Bort on MM bone disease.[108]

Thal, Len and Bort

On the basis of the identification of the supportive role of non-tumor cells within the BM microenvironment for MM pathogenesis, several compounds have been tested which inhibit these supportive effects on MM cell growth, survival, migration and drug resistance. Most

prominently, Bort, Thal and the IMiDs have already significantly changed treatment strategies in MM during the last 5 years.

Thal/IMiDs—Based upon its antiangiogenic activity, Thal was empirically used to treat patients with refractory relapsed MM and achieved remarkable responses in one-third of cases.[109] Functionally, Thal and the more potent IMiDs overcome MM cell growth and survival advantage conferred by the BM milieu. Moreover they downregulate VEGF and thereby induce antiangiogenesis. Based upon a phase III clinical trial demonstrating increased response of Thal/Dex versus Dex, this combination was FDA approved in 2006 for treatment of patients with newly diagnosed MM.[110] Moreover, it has been combined with melphalan and prednisone and achieved prolonged extent and frequency of response, as well as prolonged progression-free and overall survival, as initial therapy for elderly patients with MM.[111,112] The IMiDs additionally co-stimulate T cells, enhance antitumor immunity mediated by IFNγ and IL-2, and augment NK cell cytotoxicity.[113] The use of Len (IMiD CC-5013, Revlimid) in a phase I dose-escalation trial in patients with relapsed and refractory MM demonstrated either response or stabilization of disease in 79% cases.[114,115] These data were confirmed by two clinical phase II trials, which achieved complete responses even in heavily pretreated patients with favorable side-effect profiles. Two clinical phase III trials demonstrated that Len plus Dex is more effective than high-dose Dex alone in relapsed or refractory MM,[116,117] and Len was FDA approved in 2006 for use in combination with Dex to treat in patients with MM who have received one prior therapy. As with Thal, Len has been combined with Dex and with melphalan and achieved promising results in newly diagnosed transplant candidates and elderly patients, respectively.[118-120] More than 100 clinical studies with Thal or Len combined with other agents are currently recruiting or ongoing.

Bort, NPI-0052, and PR171—Bort (previously denoted PS341) is a dipeptide boronic acid, which inhibits the function of the 26S proteasome complex. It is thereby leading to accumulation of misfolded/damaged proteins and inducing cell apoptosis. Inhibition of NFκB through accumulation of IκB is thought to play a key role in this process, which both inhibits MM cell growth, survival and migration and targets the MM microenvironment. Bort was FDA approved in 2003 for therapy of relapsed and refractory MM based on a phase II study.[121] This approval was later extended to patients with progressive MM after previous treatment in 2005, based upon a phase III trial. Striking results of a randomized phase III trial, which investigated MPV versus MP confirmed the superiority of MPV. Therefore bortezomib used in this combination was FDA approved for initial MM treatment in June 2008.[122] Bort induces apoptosis in drug-resistant MM cells, as well as inhibits both production and secretion of cytokines and binding of MM cells in the BM microenvironment. Specifically, Bort triggers MM cell apoptosis both by the accumulation of improperly folded proteins and subsequent ER stress; as well as blockade of the unfolded protein response by preventing stress-induced phosphorylation of IRE1 and the resultant splicing of Xbp-1.[123] Bort-mediated anti-MM activity also includes phosphorylation of both p53 protein and JNK, cleavage of DNA–PKcs and ATM and caspase-dependent downregulation of gp130. In addition, IL-6-triggered phosphorylation of ERK, but not of STAT3, is blocked by Bort. The antiangiogenic effect of Bort is another potential mechanism of its anti-MM activity. Bort:[1] downregulates caveolin-1 expression and inhibits caveolin-1 tyrosine phosphorylation, which are required for VEGF-mediated MM cell migration on fibronectin; and [2] blocks VEGF-induced tyrosine phosphorylation of caveolin-1 in HUVECs, thereby inhibiting ERK-dependent endothelial cell proliferation.[6,124,125] Moreover, Bort disrupts tumor–DC interactions and enhances DC-mediated immunity through exposure of cell surface HSP 90.[78,79]

Despite its success, 65% of patients with relapsed or refractory MM do not respond to Bort. Several preclinical and more than 130 clinical studies which use Bort in combination with other conventional and novel therapies are ongoing and aim to further improve the activity of Bort and overcome the development of drug resistance. For example Bort-based targeted combination therapies include: Len or IMiD; Flavopiridol (NSC649890) which targets CDK; Sorafenib (BAY43-9006), a small molecule inhibitor which targets a multitude of kinase (for example, Raf, VEGFR1, VEGFR2); Bevacizumab which targets VEGF; perifosine which predominantly targets Akt; ATN-224 which targets superoxide dismutase 1; tipifarnib (R115777), a FTI; CNTO 328, which targets IL-6; Mapatumumab, which targets TRAIL receptor; 17AAG, which targets HSP90; CCI-779/temsirolimus which targets rapamycin; and HDAC inhibitors, which block aggresomal degradation of proteins.[6] It is worth noting that Bort has been combined with Dex and with melphalan and prednisone as an initial therapy for transplant candidates and elderly patients, respectively, and achieved enhanced extent and frequency of response, as well as prolonged survival.[122,126]

Importantly, besides Bort, several new proteasome inhibitors are now under clinical evaluation including NPI-0052, which blocks all three protease activities within the proteasome,[127,128] and PR-171 (carfilzomib), which binds to and inhibits the chymotrypsin-like activity of the 20S proteasome.[129] Interestingly, preclinical data demonstrate synergistic cytotoxicity of low-dose NPI-0052 and Bort, thereby strongly supporting the clinical evaluation of this combination.[130]

Other biological-based targeted therapies

Another novel therapeutic target in MM is represented by the aggresome, an alternative mechanism to the proteasome for breakdown of ubiqitinated proteins. Specifically, ubiquitinated proteins are delivered by dynein-dependent retrograde transport of microtubules to the aggresomes, whereas HDAC6 inhibitors such as Tubacin and type 2 HDAC inhibitors SAHA[131] and LBH589[132] bind both polyubiquitinated proteins and dynein motors to inhibit transport of ubiquitinated proteins to aggresomes. Importantly, Tubacin, SAHA and LBH589 combined with Bort induces synergistic cytotoxicity in MM cells, providing the preclinical rationale for clinical protocols using these combinations.[131,132] Clinical trials of SAHA and Bort in relapsed/refractory MM are promising. Specifically, Badros *et al.*[133] report in a total of 16 evaluable patients 1nCR and 7PR (overall response rate 50%), 6SD and 3PD. Similarly, Weber *et al.*[133] report in a total of 17 evaluable patients 4PR, two minimal responses, and 11SD. A clinical phase IA/II trial investigating the oral HDAC inhibitor LBH589 in advanced hematologic malignancies including MM alone and in combination with Bort is ongoing.

New therapeutic approaches targeting the non-cellular BM compartment

The non-cellular BM compartment is composed of the ECM including fibronectin, laminin, collagen, osteopontin, proteoglycans, glycosaminoglycans and the liquid milieu (Figure 2).

Key integrins mediating MM cell–ECM adhesion are β1 (CD29) and αvβ3-integrin. MM cell adhesion to fibronectin is predominantly mediated through β1-integrin and directly protects tumor cells from DNA damaging drugs (for example, anthracyclines and alkylating agents) by induction of cell adhesion-mediated drug resistance (CAM-DR), a reversible G1 arrest associated with increased p27kip1 (encoded by CDKN1B) levels.[135,136] MM cell adhesion to vitronectin and fibronectin is predominantly mediated through αvβ3-integrin-binding and triggers production and release of urokinase-type plasminogen activator, MMP-2 and MMP-9, thereby promoting tumor cell invasion and spreading.[137] Thal, Len and Bort exert their anti-MM activity, at least in part, by inhibition of MM cell binding to ECM proteins as well as stromal cells. Ongoing studies are evaluating compounds to

specifically inhibit adhesive interactions of MM cells with ECM proteins and stromal cells in the BM microenvironment to induce sensitization or overcome resistance to therapy (Figures 1-3, Table 1).

IL-6-induced signaling pathways in MM

IL-6 is a key growth and survival factor in MM[138,139] predominantly produced and secreted by BMSCs and OBs,[12,91] which correlates with MM tumor cell mass, disease stage and prognosis. Moreover, IL-6 induces expression of Xbp-1, a transcription factor involved in plasma cell/MM cell differentiation.[140-144]

IL-6 triggers activation of MEK/MAPK-, JAK/STAT3-, and PI3K/Akt-signaling pathways.[6,12] IL-6-triggered MEK/MAPK and PI3K/Akt-signaling pathways are dependent on caveolin-1/Hck-induced phosphorylation of scaffolding adapter Gab family proteins Gab1 and Gab2 followed by the recruitment of downstream signaling molecules.[145-147] IL-6 triggered JAK/STAT3 pathway induces upregulation/activation of antiapoptotic proteins Mcl-1 and Bcl_{XL}, Pim1 as well as c-Myc. Conversely, blockade of IL-6 induces upregulation of the pro-apoptotic BH3-only protein Bim concomitant with Mcl-1 downregulation and activation of Bax, thereby inducing MM cell apoptosis.[12,148]

Besides MM cell growth and survival, IL-6 also triggers drug resistance, Dex resistance in particular via activation of PI3K-Akt- and SHP2-related adhesion focal tyrosine kinase (RAFTK) and mitochondrial release of second activator of apoptosis (Smac). Smac disrupts the inhibitor of apoptosis X-linked inhibitor of apoptosis protein/caspase-9 complex and thereby allows activation of caspase-9, caspase-3 cleavage and apoptosis. Indeed, our studies demonstrate the therapeutic potential of targeting Smac/DIABLO in MM.[149,150]

Compounds targeting IL-6 signaling pathways include antibodies against IL-6 and IL-6 receptor, for example, CNTO 328, IL-6 antisense oligonucleotides and IL-6 super antagonist Sant7 [12] (Table 1). However, many MM cell lines grow independently of IL-6. Moreover, binding of MM cells to BMSCs trigger survival even after inhibition of the IL-6/gp130/STAT3 pathway, suggesting MM growth mechanisms other than IL-6. These findings may also explain why therapeutic approaches targeting IL-6 have not induced responses in phase I clinical trials.[12] Taken together, these data show that IL-6 is a crucial, but not a sole factor in MM pathogenesis. Clinical trials are now evaluating the safety and efficacy of CNTO 328, both alone or in combination, in patients with relapsed or refractory MM.

VEGF-induced signaling pathways in MM

VEGF is secreted by several MM cell lines and present in patient MM BM plasma. VEGFR-1 is highly expressed by MM cells, consistent with autocrine signaling.[83] Specifically, VEGF induces: (1) a caveolin-1/PI3K/PKCα-dependent cascade mediating MM cell migration on fibronectin; (2) a MEK/ERK-pathway mediating MM cell proliferation; and (3) survival signaling through upregulation of Mcl-1 and survivin.[83,87,88] In addition to MM cells and ECs, VEGF also affects OBs, NK cells, monocytes and endothelial progenitors.[83,90]

The most successful approach to date to therapeutically target VEGF in cancer is the use of a humanized monoclonal antibody against VEGF, bevacizumab (Avastin) (Genentech Inc., CA, USA), especially in combination with conventional chemotherapies.[151,152] Several ongoing clinical trials in MM are testing the effects of bevacizumab used in combination with other agents including lenalidomide, dexamethasone or bortezomib. In addition to bevacizumab a multitude of other VEGF-targeting compounds are under preclinical and clinical investigation in MM including pazopanib, sorafenib and sunitinib (Table 1). For example, the VEGF receptor tyrosine kinase inhibitor PTK787/ZK222584 [153] is now

undergoing evaluation in a clinical phase II trial as post-transplant maintenance therapy. Agents that indirectly target VEGF-signaling sequelae include not only Thal, Len and Bort; but also 2-methoxyestradiol-2, resveratrol, lysophosphatidic acid acyltransferase-β inhibitors, and CD40 inhibitors.

TNF-α superfamily-induced signaling pathways in MM

The TNF-α superfamily includes SDF-1α, CD40, BAFF and APRIL. In MM, SDF-1α and its G-protein-linked cognate receptor CXCR4 (CD184) are expressed in the BM of MM patients. SDF-1α is primarily produced by BMSCs, but also by MM cells. Functionally, SDF-1α rapidly and transiently upregulates VLA-4-mediated MM cell adhesion to both the CS-1 region of fibronectin and VCAM-1. Furthermore, SDF-1α promotes proliferation, induces migration and protects against Dex-induced apoptosis in MM cells through MAPK, as well as NF-κB and Akt-mediated pathways. In addition, SDF-1α upregulates secretion of IL-6 and VEGF in BM stromal cells,[154] and is a critical regulator of MM cell migration and homing.[155,156] The CXCR4 inhibitor AMD3100 (Genzyme, Cambridge, MA, USA) [157,158] and bicyclam molecule reversibly block SDF-1 binding to CXCR4 and inhibit MM cell homing.[155] The role of AMD3100 to enhance stem cell mobilization has been tested in several independent clinical trials. Specifically, AMD3100 induced a rapid and statistically significant mobilization of CD34+ cells in patients who have received prior chemotherapy[159] (http://clinicaltrials.gov/). Moreover, AMD3100 together with G-CSF is superior to G-CSF alone for a HCS mobilization in MM patients.[160] Its clinical role in the inhibition of MM cell homing in the patient is under investigation.

CD40 is expressed by antigen-presenting cells, T cells, as well as B-cell malignancies including MM. Functionally, it mediates p53-dependent increases in MM cell growth, PI3 K/Akt/NFκB-dependent MM cell migration, triggers VEGF secretion and induces membrane translocation of Ku86 and Ku70 proteins involved in IgH class switching. Moreover, CD40-activated MM cells adhere to fibronectin and are protected against apoptosis triggered by irradiation and doxorubicin. Therefore, targeting CD40-signaling sequelae in MM cells both inhibits monoclonal Ig secretion and overcomes cell adhesion-mediated drug resistance (CAM-DR).[161] A clinical phase I trial using SGN, the humanized anti-CD40 monoclonal antibody, in patients with refractory and relapsed MM has now been completed [51] and a combination trial with Len, which augments antibody-dependent cellular cytotoxicity,[162] is ongoing.

TNFα, secreted mainly by macrophages, triggers only modest MM cell proliferation, survival and drug resistance. However, it markedly upregulates (5-fold) secretion of IL-6 in BMSCs and induces NF-κB-dependent expression of CD11a/LFA-1, CD54/ICAM-1, CD106/VCAM-1, CD49d/VLA-4 and/or MUC-1 on MM cell lines; as well as CD106/ VCAM-1 and CD54/ICAM-1 expression on BMSCs. Expression of these molecules results in increased (2-to 4-fold) specific binding of MM cells to BMSCs, with related induction of IL-6 transcription and secretion, as well as CAM-DR. Agents that target TNFα (that is, Bort, Thal and IMiDs) therefore, at least in part, abrogate the paracrine growth and survival advantage conferred by MM cell adhesion in the BM microenvironment.[163]

BAFF or B lymphocyte stimulator is normally expressed by monocytes, macrophages, DCs, T cells and BMSCs, and exists both as a membrane-bound and a cleaved soluble protein. In addition, high levels of BAFF and APRIL are also produced by OCs. In MM, both tumor cells and BMSCs express high levels of BAFF and APRIL, as well as levels of their receptors.[164,165] BAFF secretion by BMSCs is further augmented upon adhesion to MM cells.[38] Functionally, BAFF and APRIL protect MM cells from apoptosis induced by IL-6 deprivation and Dex and promote MM cell growth as well as adhesion to BMSCs. These processes are mediated through NFκB-, PI3K/Akt-, and MAPK-pathways. Furthermore,

both BAFF and APRIL induce strong upregulation of Mcl-1 and Bcl-2, as well as regulate TACI- and c-Maf-dependent expression of both cyclin D2 and integrin β7.[38,164-166] Microarray analysis defined two patient groups based on TACI expression: MM patients with high TACI expression (TACI (hi)) display mature plasma cell gene signature indicating dependence on the BM environment and MM patients with low TACI expression (TACI (lo)) display a gene signature of plasma blasts, suggesting an attenuated dependence on the BM microenvironment. These data identify a group of patients who may benefit most from treatment with BAFF/APRIL inhibitors [167] and strongly suggest the therapeutic value of antibodies or small-molecule inhibitors which target BAFF/APRIL-induced signaling pathways.

Insulin-like growth factor-1-induced signaling pathways in MM

In MM, IGF-1 induces tumor cell growth, survival and migration. Moreover, a positive relation of baseline body mass index and MM was recently reported on two large prospective cohorts, the Nurses' Health Study and the Health Professionals follow-up Study,[168] further supporting an important role of IGF-1 in MM pathophysiology. Functionally, IGF-1 promotes proliferation and drug resistance in MM cells through activation of MAPK and PI3K/Akt-signaling cascades;[6,12] and MM cell migration and invasion through a PI3K-dependent Akt-independent protein kinase D/PKCμ/RhoA/β1-integrin-associated pathway.[6,12] As for IL-6 and VEGF-signaling pathways, caveolae are also required for IGF-1-signaling sequelae;[125] and cross-activation of IGF-1 and IL-6 receptors is facilitated by the close proximity of these two receptors at lipid rafts on the plasma membrane.[169] Consequently, inhibition of IGF-1 receptor using NVP-ADW742 also blocks the IL-6-triggered response in MM cells.[170]

PKC-signaling pathways in MM

Recent studies have also identified members of the intracellular PKC family of serine/threonine kinases as potential therapeutic targets in MM. Functionally, PKCs are: (1) involved in MM cell apoptosis; (2) required for VEGF- and Wnt-induced MM cell migration; and (3) associated with the control of IL-6 receptor α shedding. Importantly, the unique gene signature of MM patients with the adverse prognostic t(4;14)(p16;q32) translocation shows marked upregulation of PKCβ.[171] Preclinical and clinical studies using the macrocyclic bisindolylmaleimide Enzastaurin or the *N*-benzylstaurosporine Midostaurin/PKC412 to target PKC pathways demonstrate promising activity in a variety of tumors including MM and Waldenstrom's Macroglobulinemia [171-174] (Table 1).

Directly and indirectly targeting heat-shock proteins

Promising intracellular therapeutic targets in MM also include HSPs, Hsp27, Hsp70 and Hsp90 in particular. Specifically, overexpression of Hsp27 is associated with resistance to Dex, which is mediated through inhibition of Smac release. In contrast, significant upregulation of Hsp27, Hsp70 and Hsp90 is also observed in MM cells treated with Bort, indicative of a stress and protective effect. Importantly, blocking both Hsp27 and Hsp90 enhances Bort-induced death of MM cells and provides the preclinical rationale for treatment strategies combining agents to target heat-shock protein sequelae (that is, p38 inhibitors and Smac mimetics) with Bort.[6,12,149] Pleiotropic effects of Hsp90 inhibitors were observed on key elements of MM pathogenesis, including suppression of IGF-1 and IL-6-signaling sequelae, as well as proteasome, telomerase and HIF-1α activities.[6,175] Clinical trials evaluating the efficacy of Bort, both alone and combined with Bort, are ongoing (Table 1).

Conclusion

Advances in biochemistry, molecular biology and cytogenetics have markedly enhanced our knowledge of MM cell biology within the BM microenvironment. These studies defined the supportive role of stromal cells, immune cells and ECM proteins to trigger various signaling sequelae leading to MM cell growth, survival, migration and drug resistance. Based on these studies, new therapeutic targets and derived agents were identified. Moreover, significant therapeutic benefits of some of these drugs were achieved when combined with conventional or novel therapies to treat patients at earlier disease stages or when used together with stem cell transplantation strategies. Nevertheless MM still remains incurable, and in the next generation more potent and selective drugs targeting tumor cells within the BM microenvironment are urgently needed to both overcome drug resistance and improve patient outcome.

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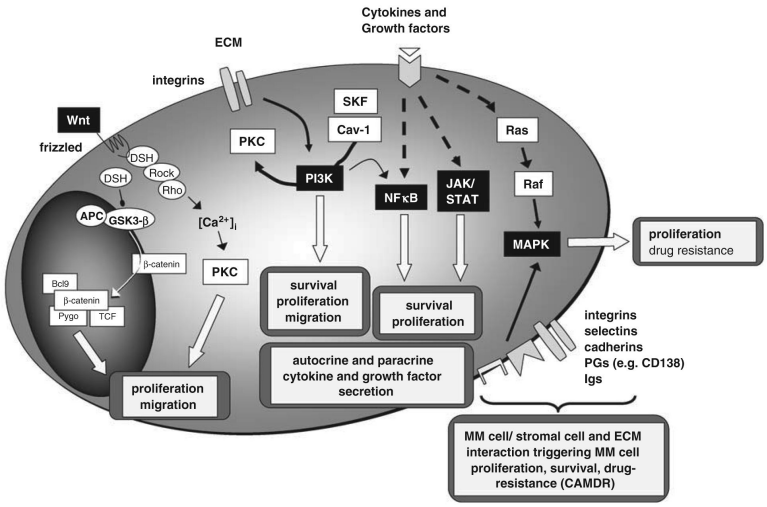

Figure 1.
Signaling cascades in MM.

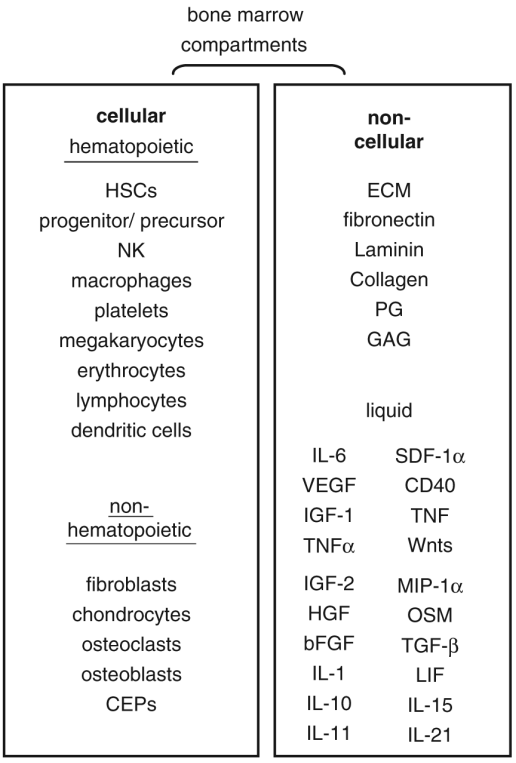

Figure 2.
The MM bone marrow microenvironment and its compartments.

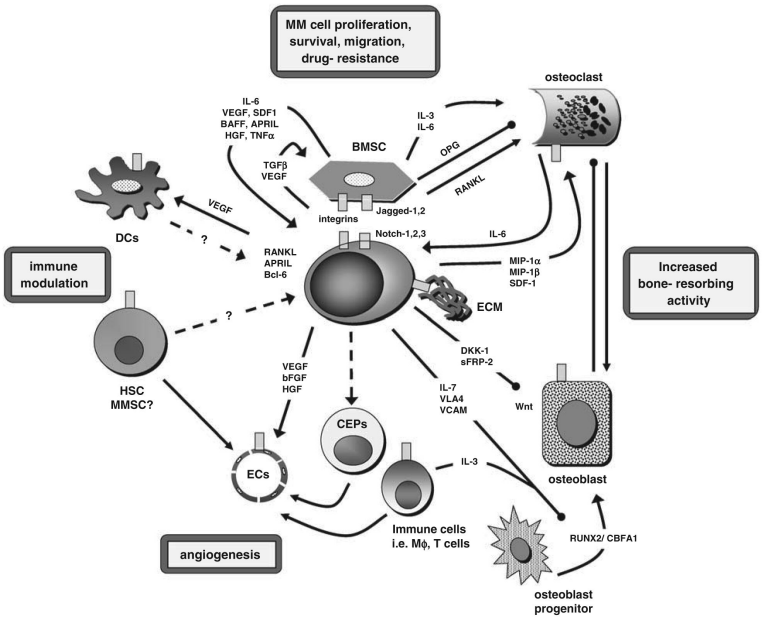

Figure 3.
The MM cell and its interactions with the cellular and non-cellular bone marrow compartments.

Table 1

Novel targeted therapies

Target	*Agent*	*Company*	*Administration*	*Phase*
Targeting signaling events in tumor cell development				
FGFR3	SU5402	Sugen Inc.		
	SU10991	Sugen Inc.		
	PD173074	Pfizer		
	CHIR-258	Novartis/Chiron	p.o.	I/II
	PRO-001 (hu-anti-FGFR3 Fab)	ProChon Biotech Ltd.		
HDAC	SAHA (Vorinostat)	Merck	i.v.	I
	NVP-LAQ824	Novartis	p.o.	
	LBH 589	Novartis	p.o.	II
	romidepsin (depsipeptide, FK228)	Gloucester Pharm.	i.v.	I/II
	ITF2357	Italfarmaco	p.o.	
	PXD101	CuraGen	i.v.	II
	belinostat	TopoTarget AIS	p.o.	II
	MS-275/SNDX-275	Bayer Schering, Syndax	p.o.	I
HDAC6	tubacin	Broad Institute		
telomerase	GRN163	Geron Corp.	i.v.	I
	TMPyP4			
	Telomestatin			
microtubuli	Epothilone B (KOS-862)	Novartis	i.v.	
CDKs	Flavopiridol/alvocidib	NCI	i.v.	II
	PD 0332991	Pfizer	p.o.	I/II
Targeting the cell membrane				
HMG-CoA	Statins:	Merck	p.o.	I/II
	Lovastatin			
	Fluvastatin			
	Simvastatin			
Targeting cytokines, growth factors and their receptors				
IL-6	CNT328	Centocor	i.v.	I/II
	Sant7	Sigma-Tau	i.v.	
VEGF	PTK787/ZK222584 (vatalanib)	Novartis	p.o.	II
	Pazopanib (GW786034B)	GlaxoSmithKline	p.o.	II
	Sorafenib (BAY43-9006/nexavar)	Bayer and Onyx	p.o.	I
	Avastin	Genentech	i.v.	I/II
	ZD6474	Astra Zeneca	p.o.	II
	SU5416	Sugen Inc.	i.v.	II
	Sunitinib (SU011248)	Pfizer	p.o.	II
	PI-88	Progen	s.c.	
	XL999	Exelixis	i.v.	II
	Neovastat (AE-941)	Aeterna Zentaris	p.o.	II

Target	*Agent*	*Company*	*Administration*	*Phase*
FGF, VEGF	BIBF100	Boehringer Ingelheim	p.o.	
SDF-1	AMD3100/perixafor/JM3100	Genzyme	i.v.	II/III
CD40	SGN-40	Seattle Genetics	i.v.	I
	Chir12-12	Chiron	i.v.	
TRAIL-R1	HGS-ETR1 (mapatumumab)	Human Genoma	s.c.	II
TACI-Ig	Atacicept	ZymoGenetics	i.v.	
IGF-1	NVP-ADW742	Novartis	p.o.	
	CP-571871	Pfizer	i.v.	
	JB-1	Chiron	i.v.	
CD56	BB-10901	ImmunoGen	I.v.	I
Targeting downstream signaling pathways				
FT	R115777 (tipifarnib/zarnestra)	J&J	p.o.	I/II
	SCH66336 (lonafarnib)	Schering- Plough	p.o.	
Raf-1	Sorafenib (BAY43-9006/nexavar)	Bayer	p.o.	I/II
MEK1/2	AZD6244 (ARRY-14886)	Astra Zeneca	p.o.	
Akt	Perifosine (KRX-0401)	Keryx	p.o.	I/II
mTOR	Rapamycin, P70S6	Genentech	p.o.	
	CCI-779 (temsirolimus)	Wyeth	i.v.	II
	RAD001 (everolimus)	Novartis	p.o.	II
	AP23573 (deforolimus)	Ariad	i.v.	II
JAK/Stat	Atiprimod	Callisto Pharm.	p.o.	I/II
SAPK/JNK	Aplidin (plitidepsin)	PharmaMar	i.v.	II
p38	SCIO-469	SCIOS Inc.	p.o.	II
NFkB, IkK	PS-1145	Millenium	p.o.	
	BAY11-7082	Biomol		
	RTA 402 (CDDO-Me)	Reata	p.o.	I
	AS602868	Merck	p.o.	
	MLN120B	Millenium		
	ACHP	Bayer		
Wnt	PKF115-584			
PKC	Enzastaurin	Eli Lilly	p.o.	I/II
	Midostaurin (PKC412)	Novartis	p.o.	
HSP90	Tanespimycin (KOS-953)	Kosan Biosciences	p.o.	II
	Geldanamycin (17-AAG)	Kosan	i.v.	II
Inducing MM cell apoptosis				
Smac agonists	LBW242	Novartis		
SOD	2ME2	EntreMed	p.o.	II
Bcl2	B3139 (Genasense)/oblimersen	Genta Incorp.	i.v.	III
Targeting MM cells and the MM BM microenvironment				
proteasome	NPI-0052	Nereus	i.v.	I
	PR-171	Cephalon	i.v.	I
	CEP-18770	Cephalon	i.v.	

Target	*Agent*	*Company*	*Administration*	*Phase*
Thal	Thalidomide	Celgene	p.o.	
IMiD	Lenalidomide	Celgene	p.o.	
Treating MM bone disease				
RANKL: Fc	RANKL: Fc	J&J	p.o.	I/II
OPG: Fc	OPG: Fc	Schering- Plough	p.o.	
OPG	AMGN-0007	Bayer	p.o.	
HDAC	PXD101 (belinostat)	Astra Zeneca	p.o.	II
MEK	AZD6244/ARRY- 142886	Keryx	p.o.	
-	Resveratrol	Genentech	p.o.	
CCR1	MLN3897	Wyeth	i.v.	
COX	SDX-308	Novartis	p.o.	
IMiDs	IMiDs: CC-4047, Lenalidomide	Callisto Pharm.	i.v.	
Proteasome	Bortezomib	PharmaMar	i.v.	
DKK-1				