Histone deacetylase inhibitors in multiple myeloma

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Abstract

Novel drugs such as bortezomib and high-dose chemotherapy combined with stem cell transplantation improved the outcome of multiple myeloma patients in the past decade. However, multiple myeloma often remains incurable due to the development of drug resistance governed by the bone marrow microenvironment. Therefore targeting new pathways to overcome this resistance is needed. Histone deacetylase (HDAC) inhibitors represent a new class of anti-myeloma agents. Inhibiting HDACs results in histone hyperacetylation and alterations in chromatin structure, which, in turn, cause growth arrest differentiation and/or apoptosis in several tumor cells. Here we summarize the molecular actions of HDACi as a single agent or in combination with other drugs in different in vitro and in vivo myeloma models and in (pre-)clinical trials.

Introduction

Multiple myeloma (MM) is a plasma cell malignancy, characterized by an accumulation of monoclonal plasma cells in the bone marrow (BM) and high levels of monoclonal immunoglobulins or paraprotein in blood and/or urine. Complex interactions between MM cells and the BM microenvironment are required for the growth and progression of MM and result in the development of drug resistance, angiogenesis and induction of bone disease.14 Enhanced understanding of the interactions between MM and the BM microenvironment has led to the identification of new molecular targets. Novel therapeutic approaches target growth factors [e.g. insulin-like growth factor-1 (IGF-1), interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF)], adhesion molecules and signaling cascades in the MM cells such as the mitogen-activated protein kinase kinase (MEK)/extracellular signal regulated kinase (ERK)-pathway, the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt)-pathway, the nuclear factor κ B (NFκB)-pathway and the Wnt-pathway.1,2 Moreover, cells interacting with the MM cells in the BM, such as stromal cells, endothelial cells, osteoblasts, osteoclasts and mesenchymal stem cells are also potential targets to overcome the drug resistance against conventional chemotherapy.2,14

MM represents 1% of all cancers and it is the second most commonly diagnosed hematologic malignancy. The incidence is higher with increasing age and is 4-5 per 100,000 individuals each year worldwide. The median age at diagnosis is 67 years.7 The most common clinical characteristics in MM are bone pain, anemia, recurrent infections and renal failure.19

The standard induction therapy for elderly patients with symptomatic myeloma, and who are not candidates for stem cell transplantation, used to be melphalan (M) and prednisone (P). Recently, improved effects on survival have been seen in patients receiving MP combined with lenalidomide (Revlimidi®) (MPR), bortezomib (Velcade®) (MPV) or thalidomide (MPT).11 Only the latter has been accepted as standard therapy. High-dose therapy plus autologous stem cell transplantation is considered the standard therapy for front-line treatment of MM patients aged <65 years.12,16 The most common pre-transplantation induction therapies used today are thalidomide-dexamethasone, lenalidomide-based regimes, and lenalidomide-dexamethasone.11,15 New agents such as bortezomib, thalidomide and lenalidomide in the treatment of MM do not only target the MM cells directly, but also influence the interactions of the MM cells with the BM microenvironment. Combining these new agents with conventional chemotherapy and high-dose chemotherapy with autologous stem cell transplantation increases the outcome of MM patients, although eventually all MM patients relapse. Therefore, identification of new key molecules in MM cells and in the BM microenvironment is crucial for the development of new therapeutic strategies.

There is growing evidence that not only gene defects such as deletions, mutations and chromosomal abnormalities are responsible for the onset and progression of cancer. Several studies have shown that epigenetic changes, i.e. heritable traits mediated by changes in DNA other than nucleotide sequences, play a key role in the downregulation of tumor suppressor genes and/or upregulation of oncogenes and, therefore, are also involved in the onset and progression of several malignancies.10,11 Chromatin remodeling is one of the main processes in epigenetic regulations. Nucleosomes are the repeating units of chromatin which contain 146 bp DNA wrapped around a core histone octamer. Modifications of these nucleosomes on the histone level, as well as the DNA level, can alter the chromatin state which can be open or closed. The post-translational modifications on the core histones are most common on the amino-terminal lysine rich tail which passes through and around the enveloping DNA double helix.10 These modifications, such as acetylation, methylation, ubiquitination, sumoylation, phosphorylation and glycosylation are crucial in modulating gene expression, as they affect the accessibility and interaction of DNA with other non-histone protein complexes that could contain transcriptionally co-activating or co-repressing elements.11,16 Moreover, methylation of DNA, maintained by the epigenetic enzymes, methyltransferases and demethylases, also affects the chromatin structure indirectly by recruiting protein complexes containing enzymes such as histone deacetylases (HDAC).21 HDAC and the opposite enzyme histone acetyltransferases (HAT) are the most analyzed enzymes involved in the post-translational modifications of histones. Both enzymes maintain the acetylation status of histones and non-histone proteins. HAT acetylates histones resulting in neutralizing the positive charge of histones and a more relaxed, transcriptionally active chromatin, while HDAC remove the acetyl group resulting in a more compact, transcriptionally inactive chromatin structure.22 Inhibiting HDAC leads to hyperacetylation of histones and results in gene expression alteration. In tumor cells, several HDAC inhibitors (HDACi) have shown promising anti-cancer activities with anti-proliferative, pro-apoptotic and anti-angiogenic properties.12,23

This review provides an overview of the anti-myeloma activity of different HDACi in pre-clinical settings and the latest clinical trials with HDACi ongoing in MM patients.
The histone deacetylase family

Eighteen HDACs have been identified in humans and are subdivided into four classes based on their homology to yeast HDACs and their enzymatic activities.\textsuperscript{2,9} Class I HDACs (1, 2, 3, and 8) are homologs to the yeast Rpd3 and can generally be detected in the nucleus. They are ubiquitously expressed in several human cell lines and tissues. Based on phylogenetic analysis, Gregoretti et al. divided class I into Ia (HDAC1 and 2), Ib (HDAC3) and Ic (HDAC8).\textsuperscript{31} Class II HDACs (4, 5, 6, 7, 9 and 10) are related to yeast Hda1 (histone deacetylase 1) and can shuttle between the nucleus and cytoplasm. This class is divided into class IIa (HDACs 4, 5, 7 and 9) and class IIb (HDAC6 and 10) which contain two deacetylase domains.\textsuperscript{32} Since HDAC6 contains a unique alpha-tubulin deacetylase (TCAD) domain, it can specifically deacetylate alpha-tubulin.\textsuperscript{32}

The third class HDACs are the sirtuins (SIRT 1, 2, 3, 4, 5, 6, 7, 9 and 10) which contain two deacetylase domains each. These enzymes require nicotine adenine dinucleotide (NAD\textsuperscript{+}) for their deacetylase activity in contrast to the zinc-catalyzed mechanism used in class I and II HDACs.\textsuperscript{33} The sirtuins appear to deacetylate non-histone proteins and transcription factors including p53. They can not be inhibited by HDACi such as suberoylanilide hydroxamic acid (SAHA) or Trichostatin A (TSA).\textsuperscript{34} HDAC11 represents class IV and contains residues in the catalytic core regions similar to both class I and II enzymes but does not have strong enough identity to be placed in either class.\textsuperscript{35}

HDAC inhibitors

Structural classification of HDAC inhibitors

Butyrate and TSA were among the first chemicals to be identified as HDAC inhibitors. Dimethylsulfoxide was used to aid superinfection of murine erythroleukemia cells with the Friend virus, whereas TSA was originally isolated as an antifungal agent from culture medium of Streptomyces hygroscopicus. Later on, it was discovered that these compounds could induce cell differentiation and a correlation with histone hyperacetylation, which was maintained by inhibiting HDACs, could be shown. \textsuperscript{36,37} It subsequently opened a new field of research. Since then, a large number of natural and synthetic HDACi have been developed by several companies and used as anti-tumor agents in pre-clinical and clinical settings (Table 1). On the basis of their chemical structure, major HDACi can be divided into four categories: short-chain fatty acids, hydroxamates, benzamides and cyclic tetrapeptides.\textsuperscript{24-30}

Among the various classes of HDACi, short chain fatty acids such as phenylbutyrate, the anti-epileptic drug valproic acid (VPA) and sodium butyrate are only effective at mM concentrations and thereby form the less potent class of HDACi.\textsuperscript{31} Clinical evaluations have been performed with these compounds either alone or in combination and are well tolerated in patients. However due to the short plasma half-life, high doses are needed to obtain a therapeutic effect.\textsuperscript{41} The first natural hydroxamate was TSA and is now considered as the reference compound of hydroxamate based inhibitors. Most of the synthetic hydroxamate based HDACi target class I and class II with high potency. SAHA has a potency at pM range and has recently been approved for the treatment of cutaneous T-cell lymphoma. M-carboxycinnamic acid bishydroxamide (CBHA) is another potent second generation inhibitor which is the structural basis for example LAQ824 and PXD101, both effective at nM range towards classes I and II. Two of the newest hydroxamate based HDACi are LBH589 and ITF2357 with very low IC\textsubscript{50} values at nM concentrations.\textsuperscript{42,43} Benzamides include MS-275 and CI-994 and are generally less potent than the hydroxamates and cyclic tetrapeptides. Cyclic tetrapeptides, include the natural product depsipeptide (FK 228 or FR 901228) and apicidin. Depsipeptide is a produg and has to be metabolically activated via reduction of the disulfite binding.\textsuperscript{44} Recently KD5170, a novel mercapto ketone-based histone deacetylase inhibitor, has been developed. KD5170 showed significant anti-proliferative activity against a variety of human tumor cell lines, including human MM cell lines.\textsuperscript{45}

Isoenzyme-selectivity of pan-HDACi and mechanism of HDAC inhibition

In general, none of these inhibitors, except tubacin, exhibit specificity towards one isoenzyme. However, they inhibit the enzyme activity of HDACs with varying efficiency (Table 1). For example, depsipeptide preferentially inhibits HDAC1 and 2 compared to HDAC4 and 6, whereas the potency of MS-275 to inhibit HDAC1 is 26 times higher compared to HDAC3 and appears to lack the ability to inhibit the HDAC6 and 8.\textsuperscript{46,47} Tubacin, the HDAC6 selective inhibitor, induces hyperacetylation of \( \alpha \)-tubulin and has no effect on the histone acetylation status, while other hydroxamate inhibitors like TSA, SAHA and LBH589 induce histone – \( \alpha \)-tubulin hyperacetylation.\textsuperscript{48-51}

X-ray crystallographic analyses clarify the structure of an HDAC enzyme using an HDAC-like protein (HDLP) isolated from an anaerobic bacterium, on the one hand and on the other hand how inhibitors such as SAHA and TSA mediate HDAC inhibition. The HDAC catalytic domain consists of a tube like pocket whereby a Zn\textsuperscript{2+} cation is positioned near the bottom of this narrow pocket. The basic structure of the HDACi contains a cap group, an aliphatic chain for a spacer, and a functional group (except depsipeptide). The cap group may be necessary for packing the inhibitor at the rim of the tube-like active site pocket, while the aliphatic group forms interactions with the residues of the lining pocket. For TSA, the hydroxamic acid group (the functional group) coordinates the zinc through its carboxyl and hydroxyl groups, resulting in the formation of a penta-coordinated zinc and thereby altering the activity of the enzyme.\textsuperscript{52,53}

Pre-clinical observations of HDACi in multiple myeloma

Anti-myeloma activity of HDACi as a single agent in vitro

HDACi modulate the gene expression profile of multiple myeloma cells

Microarray based studies showed that HDACi induce transcriptional modulations of 7-10 % of the genes in malignant cell lines by acetylation of histones and non-histone proteins.\textsuperscript{54,55} The patterns of the HDACi induced gene expression alterations are quite similar for different HDAC inhibitors. Definite differences, however, could be observed by different agents in different cancer cell lines.\textsuperscript{56,57}

In MM, the first cDNA array using SAHA in the human MM1S cell line was performed by Mitsiades et al. SAHA caused selective gene expression alterations of oncogenes, proliferative/anti-apoptotic transcription factors, cell cycle regulators and members of the IGF-1R and IL-6R signaling cascades.\textsuperscript{58,59} Recently, gene expression profiling of MM1S cells exposed to VPA have also been performed and showed that VPA also targeted genes involved in the cellular pathways crucial for the survival of the MM cells as seen for SAHA. Furthermore, they could demonstrate modulation of genes that contribute to RNA splicing/transcription and DNA replication, indicating that HDACi could affect cell growth differently from apoptosis or cell cycle regulation.\textsuperscript{55}

HDACi inhibit the proliferation of multiple myeloma cells

Before investigating the molecular effect of HDACi in certain human MM cell lines, assays such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)- or 3H-thymidine incorporation assays were performed to study the anti-proliferative effect of the HDACi. Table 2 shows an overview of different HDACi and their concentration range needed to inhibit the proliferation of the human MM cell lines and/or primary human MM cells. HDACi such as VPA, FK228 and ITF2357 affected the viability of IL-6 dependent as well as IL-6 independent MM cell lines, indicating
that the anti-myeloma activity of the HDACi is not influenced by one of the key growth factors in MM.\textsuperscript{78}\textsuperscript{88} Furthermore, co-culturing the MM cells with bone marrow stromal cells (BMSC) does not protect the cells from cell death induced by the HDACi LAQ824, IFT2357, LBH589 or KD5170.\textsuperscript{65,66,68,71} These data suggest that HDACi could overcome the protective effect of the BM micro-environment. The MM1S cells were resistant to KD5170 and showed no increase in histone acetylation, whereas KD5170 sensitive cell lines exhibited histone hyperacetylation after KD5170 treatment.\textsuperscript{65,89,90} This finding indicates that inhibition of the HDAC enzymes is necessary for the anti-tumor effects of the HDACi.

JNJ-26481585, a recently developed novel hydroxamate based HDACi with prolonged pharmacodynamic properties, has anti-proliferative effect at mM concentrations in the murine 5T33MM model.\textsuperscript{72} This murine MM cell line is derived from the 5T33 mouse model which mimics the human disease closely at the molecular, cellular and clinical level.\textsuperscript{72,74} HDACi induce cell death in multiple myeloma

Besides inhibition of proliferation, HDACi induced cell death is one of the major mechanisms to inhibit the survival of the myeloma cells. Extrinsic and intrinsic apoptotic pathways as well as non-apoptotic cell death such as autophagy have been reported in myeloma cells treated with an HDACi. Figure 1 demonstrates effects of the HDACi on the compounds of the intrinsic and extrinsic apoptotic pathway.

The extrinsic apoptotic pathway is activated by ligand binding to death receptors such as Fas (Apo-1 or CD95), tumor necrosis factor receptor-1 (TNFR-1) and TNF-related apoptosis-inducing ligand (TRAIL or Apo2L) receptors (DR4 and -5), resulting in activation of caspase-8 and caspase-10. Apo2L/TRAIL interacts with two death receptors (DR4 and DR5) and potently induces apoptosis in various tumors, including primary MM cells and MM cell lines, while exerting minimal or no toxicity in normal cells.\textsuperscript{91,92} Several studies have demonstrated that HDACi can upregulate the expression of both death receptors and their ligands and are posed to occur selectively in tumor cells.\textsuperscript{73} The U266 human MM cell line, although expressing significant levels of DR4 and caspase-8, is resistant to Apo2L/TRAIL and this resistance could be overcome with VPA. This sensitizing effect of VPA is mediated by the redistribution of DR4 to lipid rafts followed by an improved DR4 signaling.\textsuperscript{92} However, opposite results have been obtained by Schwartz \textit{et al}. who have demonstrated that VPA activated caspase-3 but not caspase-9 and caspase-8 in the U266, OPM2 and RPMI human MM cell lines.\textsuperscript{93} In the MM1S line, treated with LBH589, no upregulation of death receptors and their ligands could be observed. Caspase-8, however, was activated and the gene expression of the TOS4 gene, negative regulator of the Fas ligand (FasL) or TRAIL induced apoptosis was downregulated.\textsuperscript{94} SAHA sensitized MM1S cells to a Fas-activating monoclonal antibody CH-11 and to recombinant TRAIL. This sensitizing effect was associated with decreased expression of the anti-apoptotic protein FLICE-like inhibitory protein (FLIP) and members of the inhibitors of apoptosis (IAP) family such as X-linked IAP (XIAP).\textsuperscript{95}

Despite these results showing that HDACi affect the extrinsic pathway, in MM and other malignant cells it is still not clear how important the death-receptor pathway is for the therapeutic effects of HDACi.

The intrinsic apoptotic pathway is mediated by the mitochondria whereby proapoptotic signals result in the release of mitochondrial intermembrane proteins, such as cytochrome c (cyto-c), apoptosis inducing factors (AIF) and second mitochondria-derived activator of caspase (Smac). Cytosolic cyto-c binds to apoptotic protease activating factor (Apaf-1), resulting in Apaf-1 oligomerization and subsequent caspase-9 activation while cytosolic Smac binds to XIAP and thereby eliminates its inhibitory effect on caspase-9. Cytosolic AIF induces caspase-independent apoptosis.\textsuperscript{96} Members from the BCL2 family partially regulating this pathway, contain the pro-apoptotic (e.g. Bax, Bak, Bid and Bim) and anti-apoptotic (e.g. Bcl2, BclXL and Mcl1) proteins. The BCL2 protein Bid, can be cleaved by caspase-8 after death-receptor ligation, and truncated Bid (tBid) localizes to the mitochondria to initiate the intrinsic apoptotic pathway.\textsuperscript{97}

MM cells contain higher levels of the anti-apoptotic proteins Bcl2 and Mcl1 and lower levels of the pro-apoptotic protein Bax compared to normal plasma cells.\textsuperscript{98,99} These findings could play a role in the survival of the MM cells and the resistance to chemotherapeutic agents.

How HDACi activate the intrinsic apoptotic cascade is cell context dependent and is still not completely understood. Treatment of the U266 MM human cell line and primary MM human cells with depsipeptide resulted in a decrease of the anti-apoptotic proteins Mcl1,
cell death is calpain-independent and partially
strate in M M 1S cells that the LB H 589 induced
m e  thyl ketone (Z-V AD-fm k ), they could dem on -
treatm ent w ith LB H 589. 69 These data represent
novel apoptosis and caspase activation
respectively. U sing the calpain inhibitor,
calpeptin, and the caspase-3 inhibitor, benzy -
more, gene expression profiling revealed a
two different enzym es, caspase-3 and calpain,
polym erase (P AR P ) cleavage in M M  cells by
age of B id, caspase-9 and caspase-3. F urther -
LB H 589 and SAH A induced poly (ADP -ribose)
through m itochondrial perturbations. B oth
to the cytosol, upregulation of Apaf-1 and cleav -
since no cleavage of caspase-8 nor upregula -
tion of DR -4 have been found, whereas cleav -
ence of caspase-3 and -9 and dow nregulation of
intrinsic pathw ay rather than through the
extrinsic pathw ay in the K M S18 M M  cell line
and cleavage of caspase-9 and caspase-3, caus -
ing loss of m itochondrial m em brane potential
through m itochondrial perturbation in the
U266 cells. KD5170 provoked Bax activation
and cleavage of caspase-9 and caspase-3, caus -
ing loss of m itochondrial membrane potential
and subsequent pro-apoptotic factor release.
The fact that AIF  w as released, and that the
nuclear condensation w as partially block ed in
some for degradation of polyubquitinated mis -
represents an alternative system  to the protea-
some for degradation of polyubquitinated mis-
folded/unfolded proteins (Figure 2). 6 When
degradation of misfolded proteins exceeds the
proteasomal degradation through e.g. protea-
in accumulation of ubiquitinated proteins, organized into perinuclear structures termed “aggresomes”\textsuperscript{32,90}. Aggresomes are formed by the retrograde transport of the aggregated proteins on microtubules (MT) and travel to the MT organizing center (MTOC) region, where they are sequestered as a single structure susceptible for lysosomal degradation. Movement of aggresomes requires intact microtubules and association with motor dynein.

HDAC6 deacetylates alpha-tubulin and plays a key role in the aggresomal pathway since it can bind polyubiquitinated proteins and dynein, facilitating the transport of aggresomes along the MTs\textsuperscript{32,90}. Targeting HDAC6 with tubacin or pan HDAC inhibitors such as SAHA or LBH589, results in hyperacetylation of alpha-tubulin, accumulation of polyubiquitinated proteins and apoptosis\textsuperscript{71,91}. It has been shown that tubacin inhibits MM cell growth in drug-sensitive (MM1S, U266, INA-6 and RPMI8226) and drug-resistant cell lines (RPMI-LRS and RPMI-Dox40) with an IC\textsubscript{50} between 5-20 µM, whereas no cytotoxicity in peripheral blood mononuclear cells (PBMCs) could be observed at µM levels\textsuperscript{98}. This indicates that tubacin sensitivity is independent of drug resistance and that tubacin selectively targets malignant cells.

**HDACi affect cytokines and proteins implicated in multiple myeloma survival, progression and immune escape**

Mitsiades et al. showed that SAHA suppresses the expression of receptor genes involved in MM cell proliferation, survival and/or migration such as IGF-1R, IL-6R and its key signal transducer gp130, TNF-R, CD138 (syndecan-1) and CXCR-4\textsuperscript{88,89}. Furthermore, in MM1S cells they could demonstrate that SAHA suppressed autocrine IGF-1 production and paracrine IL-6 secretion of BMSC by triggering MM cell binding. This suggests that SAHA can overcome cell adhesion-mediated drug resistance\textsuperscript{20,94}. OPM-2 cells treated with NaB decreased IL-6R but when cells were transfected with an expression vector of IL-6R no decrease of the receptor could be observed. Increased p21 expression and apoptosis could be observed in both transfected and untransfected cell lines, indicating that downregulation of the IL-6R is not required for the induction of p21 or apoptosis\textsuperscript{98}. This observation again confirms that HDACi act on multiple cellular pathways.

Several studies provide evidence that HDACi suppress angiogenesis through a direct effect on the growth and differentiation of endothelial cells on one hand and by down-regulating the expression of pro-angiogenic genes in tumor cells on the other hand\textsuperscript{86,100}. The anti-angiogenic effect of HDACi in myeloma has been demonstrated using OPM-2 and KM3 cells treated with VPA. VPA decreases VEGF secretion and VEGF receptor expression, resulting in inhibition of the vascular tubule formation of endothelial cells in co-cultures with myeloma cells. These data confirm the anti-angiogenic effect of HDACi on myeloma which is important to suppress spread of the MM cells\textsuperscript{101-103}.

Recently, De Bruyne et al. showed that the tetraspanin CD9 which shows an inverse correlation between its expression level and tumor metastasis in solid tumors, is epigenetically down-regulated in MM and could be up-regulated by treating myeloma cells with LBH589. Myeloma cells expressing CD9 become more susceptible for natural killer mediated cytolsis and the expression correlates with non-active MM disease. These observations suggest that the immune escape of the tumor cells and molecules, correlating with the MM disease status, can be affected by HDACi\textsuperscript{100}.

**Anti-myeloma activity of HDACi in combination therapy in vitro**

**Bortezomib**

Bortezomib, a first-in-class, potent and reversible proteasome inhibitor, has been successfully introduced in clinical practice and represents the standard of care in symptomatic MM patients\textsuperscript{104}. The anti-myeloma activity of bortezomib is a result of NF-κB inhibition, upregulation of various apoptotic pathways, and effects on the tumor micro-environment\textsuperscript{105-107}. Pei et al. were the first to demonstrate in vitro that HDACi in combination with bortezomib resulted in an improved cytotoxic effect compared to their effect as single agent. Sequential exposure of U266 and MM1S cells to bortezomib and SAHA or NaB potent induced caspase-3, -8 and -9 activation and release of the pro-apoptotic mitochondrial proteins cyto-c and Smac, resulting in a synergistic induction of apoptosis. This effect was associated with a reduction in NF-κB DNA binding activity, modulation of JNK activation and a reactive oxygen species (ROS)-dependent downregulation of Cyclin D1, Mcl-1 and XIAP. Combining bortezomib with PXD101 caused oxidative stress accompanied by an

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**Figure 2.** The aggresome pathway prevents accumulation of misfolded proteins. Unfolded or misfolded proteins, that exceed proteasomal degradation, form aggregates and are transported to the microtubule organizing center (MTOC) for degradation. This transport requires HDAC6 which deacetylates alpha-tubulin and binds both polyubiquitinated proteins and dynein. Inhibiting HDAC6 with tubacin, whether or not combined with the proteasome inhibitor bortezomib, accumulates misfolded or unfolded proteins and leads to apoptosis.
enhanced effect on Bim expression, DNA damage, MAPK p38 activation and p53 phosphorylation. These observations indicate that there are several molecular mechanisms that may contribute to the synergy between bortezomib and HDACi.\textsuperscript{71,69,91} Specific inhibition of the aggresomal pathway by tubacin together with proteasome inhibition by bortezomib also resulted in an accumulation of ubiquitinated proteins followed by a synergistic anti-MM activity, mediated by stress-induced JNK activation, followed by caspase/PARP cleavage.\textsuperscript{69} In addition, further investigations on cytoskeletal events showed that bortezomib alone lead to aggresome formation and, combining it with LBHS89 or SAHA, both inhibiting HDACi, resulted in a disruption of aggresome formation leading to apoptosis.\textsuperscript{71,69} Nawrocki et al. demonstrated that the oncogenic Myc regulates the sensitivity of MM cells to bortezomib in combination with SAHA. Oncogenic activation of Myc is a hallmark of nearly all rapidly dividing malignant cells. In MM, the Myc expression is directly correlated with intracellular endoplasmatic reticulum (ER) content and protein synthesis rate. Bortezomib in combination with SAHA resulted in an induction of the pro-apoptotic BH3-only protein Noxa and ER stress indicated by a disruption of calcium homeostasis and activation of caspase-4. Further knock-down studies demonstrated that caspase-4 and Noxa play significant roles in Myc-driven sensitivity to the combination of bortezomib and SAHA.\textsuperscript{69}

Enhanced anti-MM activity of the combination therapy could not only be observed in primary human MM cells but also in co-culture conditions and conditions with exogenous growth factors IL-6 or IGF-1. Taken together, bortezomib in combination with HDACi may represent a promising therapeutic strategy that can overcome drug-resistance.\textsuperscript{71,69,67,69,73}

Death receptor ligands
In several tumor cells, an enhanced apoptotic effect can be observed using HDACi and activators of the TRAIL and Fas pathway. However, the molecular mechanism underlying this synergism is still unclear and is cell-type specific. Fandy et al. demonstrated that TSA, as well as SAHA, in combination with TRAIL have potent synergistic effect in the ARO-1 MM cells.\textsuperscript{67} Similar apoptotic effects have been observed in MM1S, U266 and H929 cell lines treated with KD5170 and TRAIL.\textsuperscript{73} The fact that SAHA and TSA could up-regulate the two death receptors DR4 and DR5 in the MM cells, coupled with a downregulation of anti-apoptotic proteins (Bcl-2 and XIAP) could explain the synergistic effect of combination therapy.\textsuperscript{67} However, it has been shown that HDACi could also achieve synergy with TRAIL without changing the TRAIL receptors or anti-apoptotic proteins, by simultaneously activating the intrinsic and extrinsic pathways.\textsuperscript{67,68}

**DNA methyltransferase inhibitors**

5-azacitidine is a DNA methyltransferase inhibitor and shows activity against MM.\textsuperscript{106} 5-azacitidine and analogs such as 5-azacytidine (decitabine) are interesting tools to investigate hypermethylation in tumorigenesis and the clinical efficacy is currently being assessed in phase II trials.\textsuperscript{106,107} Several investigations have already shown that hypermethylated tumor suppressor genes can be most efficiently reactivated by combining DNA demethylating agents with HDACi, this could thereby result in an enhanced reduction of tumor cell growth.\textsuperscript{106,108}

**Clinical observations of HDACi in multiple myeloma**
Several clinical trials with HDACi alone or in combination with other antimyeloma agents are ongoing (Table 3).\textsuperscript{108-112} Phase I clinical trials showed that HDACi, such as SAHA, LBHS89 and depsipeptide are well tolerated in myeloma patients. In phase II clinical trials, the activity of the HDACi as single agent was limited. However, combining HDACi with dexamethasone and/or bortezomib resulted in a more promising therapeutic setting in the treatment of MM, even in patients with refractory and relapsed MM.

**Future directions**
It has become clear that pan-HDACi have anti-neoplastic activities by affecting multiple pathways involved in cell growth, survival, immune response and tumor vasculature. However, the precise underlying mechanism of the inhibition of the different HDACs by pan-HDACi and their biological role in MM pathogenesis remain to be clarified. A greater

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understanding of the molecular effects of the HDACi and the role of HDACs is essential in selecting patients who are potential candidates for HDACi therapy and in designing combination studies. The development of isoform-specific inhibitors would be a valuable tool to investigate the biological role of specific HDACs. However, it is still not clear whether selective inhibition of HDACs has therapeutic advantages over a pan-HDACi. Clinical trials demonstrated promising anti-tumor responses to HDACi, mainly in combination with other agents such as bortezomib or dexamethasone which are already in clinical use. Therefore, the development of new and improved HDACi should be encouraged together with their use in combination therapy to improve the outcome for MM patients.

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