

## Review Article

## Action mechanisms of histone deacetylase inhibitors in the treatment of hematological malignancies

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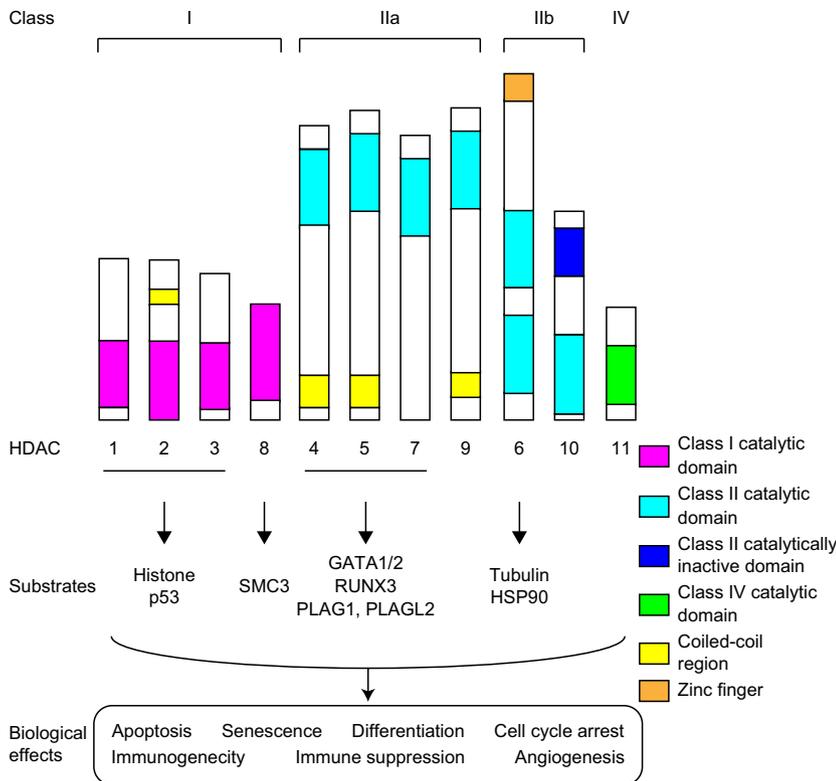
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Histone deacetylases (HDACs) critically regulate gene expression by determining the acetylation status of histones. Studies have increasingly focused on the activities of HDACs, especially involving non-histone proteins, and their various biological effects. Aberrant HDAC expression observed in several kinds of human tumors makes HDACs potential targets for cancer treatment. Several preclinical studies have suggested that HDAC inhibitors show some efficacy in the treatment of acute myelogenous leukemia with AML1-ETO, which mediates transcriptional repression through its interaction with a complex including HDAC1. Recurrent mutations in epigenetic regulators are found in T-cell lymphomas (TCLs), and HDAC inhibitors and hypomethylating agents were shown to act cooperatively in the treatment of TCLs. Preclinical modeling has suggested that persistent activation of the signal transducer and activator of transcription signaling pathway could serve as a useful biomarker of resistance to HDAC inhibitor in patients with cutaneous TCL. Panobinostat, a pan-HDAC inhibitor, in combination with bortezomib and dexamethasone, has achieved longer progression-free survival in patients with relapsed/refractory multiple myeloma (MM) than the placebo in combination with bortezomib and dexamethasone. Panobinostat inhibited MM cell growth by degrading protein phosphatase 3 catalytic subunit  $\alpha$  (PPP3CA), a catalytic subunit of calcineurin. This degradation was suggested to be mediated by the blockade of the chaperone function of heat shock protein 90 due to HDAC6 inhibition. Aberrant PPP3CA expression in advanced MM indicated a possible correlation between high PPP3CA expression and the pathogenesis of MM. Furthermore, PPP3CA was suggested as a common target of panobinostat and bortezomib.

The 11 known HDACs are divided into three classes (I, II, and IV; Fig. 1),<sup>(1)</sup> and each class has different subcellular location, substrate specificity, and enzymatic activity. Class I HDACs (1, 2, 3, and 8) are generally located in the nucleus, of which the HDAC 1, 2, and 3 subtypes are gene expression-regulating substrates. Class II HDACs (4, 5, 6, 7, 9, and 10) can shuttle between the nucleus and the cytoplasm; among them, the substrates of HDAC6 are unique, and are involved in cell signaling pathways and protein stability enhancement through interaction with tubulin and HSP90.<sup>(2,3)</sup> Histone deacetylases are involved in inducing numerous biological effects such as cell apoptosis, senescence, differentiation, and angiogenesis. Immunogenicity, similar to ligand expression of immunoreceptors on NK cells, is also regulated by HDACs.<sup>(4)</sup> Aberrant HDAC expression has been reported in several kinds of human tumors including gastric, colorectal, liver, breast, and lung cancers. Therefore, HDACs have been proposed as potential targets for cancer treatment. In this study, we describe the mechanisms of action of HDAC inhibitors in treating hematological malignancies. Particularly, we discuss the novel roles of calcineurin in the pathogenicity of MM and its inhibition by HDAC inhibitors.

## Mechanisms of action of HDAC inhibitors in the treatment of AML and MDS

A transcriptional factor encoded by the *AML1* (*RUNX1*) gene, AML1, was first identified on chromosome 21, which is disrupted in t(8;21)(q22;q22). This translocation leads to the generation of the AML1-ETO fusion protein in AML.<sup>(5)</sup> AML1 not only regulates the transcription of various genes that are important for hematopoiesis<sup>(6,7)</sup> but is also indispensable for liver-derived hematopoiesis.<sup>(8)</sup> We previously reported that the co-repressor mSin3A suppresses AML1 transcriptional activity by linking AML1 to HDACs when the former is unphosphorylated.<sup>(9)</sup> AML1 is released from mSin3A following its ERK-induced phosphorylation and becomes active as a transcriptional activator. Transcriptional repression is mediated by ETO through its interaction with a complex composed of N-CoR/mSin3/HDAC1.<sup>(10)</sup> Indeed, several studies have shown HDAC inhibitors to be effective against AML with t(8;21).<sup>(11–14)</sup> However, the proposed mechanism of action is different among these studies. A previous study reported that valproic acid treatment induced cell differentiation and caspase-dependent apoptosis, which was mediated by the dissociation of the



**Fig. 1.** Classification of human histone deacetylases (HDACs) into three classes (I, II, and IV) based on their homology to yeast HDACs, and illustration of their substrates and biological effects. GATA1/2, GATA binding factor 1/2; HSP90, heat shock protein 90; PLAG1, pleomorphic adenoma gene 1; PLAGL2, PLAG-like 2; RUNX3, runt-related transcription factor 3; SMC3, structural maintenance of chromosomes 3.

AML1-ETO/HDAC1 complex from the promoter of AML1-ETO target genes.<sup>(12)</sup> Proteasomal degradation of AML1-ETO by HDAC inhibitors was reported in two different studies.<sup>(13,15)</sup> The induction of leukemic cell apoptosis by the activation of the death receptor pathway by HDAC inhibitors has also been reported.<sup>(14)</sup>

Synergistic transcriptional repression by hypermethylation and histone deacetylation at the promoter region of AML/MDS has made HDAC inhibitors potentially useful candidates for combined use with hypomethylating agents.<sup>(16)</sup> Entinostat is a benzamide HDAC inhibitor that modifies chromatin structuring through the inhibition of class I HDAC.<sup>(17)</sup> The methylation status of tumor suppressor genes including *p15<sup>INK4B</sup>*, *CDH-1*, *DAPK-1*, and *SOCS-1* was studied in patients with AML or MDS treated with 5-azacytidine, a hypomethylating agent, and entinostat.<sup>(18)</sup> No correlation was observed between their clinical response and baseline methylation and methylation reversal. Thus, it was hypothesized that the methylation reversal of tumor suppressor genes was not predictive of clinical response to these combination therapies.

Histone deacetylase inhibition induces AML cell apoptosis partly through the accumulation of DNA damage and inhibition of DNA repair.<sup>(19)</sup> MLN4924 is the first-in-class neural precursor cell expressed, developmentally downregulated 8-activating enzyme inhibitor, and its antileukemia effects are mediated through the inhibition of NF- $\kappa$ B.<sup>(20)</sup> In fact, MLN4924 and the HDAC inhibitor, belinostat, were reported to show synergistic anti-AML efficacy in diverse AML cell types.<sup>(21)</sup>

### Mechanisms of action of HDAC inhibitors in the treatment of TCLs

T-cell lymphomas are composed of a heterogeneous subset of T-cell-derived non-Hodgkin's lymphomas and show poor prognosis following treatment with the presently available

therapeutic options.<sup>(22)</sup> Therefore, novel treatment strategies are necessary for the improvement of the prognosis of patients with TCLs. Recently, epigenetic defects due to recurrent mutations in epigenetic regulators such as the Ras homolog gene family, member A and FYN kinase have been detected in TCLs.<sup>(23)</sup> Thus, epigenetic therapies are expected to be effective for TCLs. In cell lines derived from patients with TCL, HDAC inhibitors including belinostat were synergistic in combination with decitabine, a hypomethylating agent *in vitro* and *in vivo*.<sup>(24)</sup> Gene expression profiling was different between single treatment conditions and combination therapy in a gene expression array. A greater fraction of genes was affected by the combination therapy than that affected by single-drug treatment. In addition, a significant upregulation of molecules related to the protein kinase cascade and cell cycle arrest was reported as the effects of the combination treatment on the transcriptome.

Aurora A kinase is a serine-threonine kinase, and its function is crucial in cell signaling and mitotic division.<sup>(25)</sup> Increased AAK expression is found to be related to malignant transformation, especially in TCLs.<sup>(26,27)</sup> Alisertib selectively inhibits AAK through its competitive binding to the ATP-binding site on AAK.<sup>(22)</sup> Treatment with alisertib perturbs the cell cycle, leading to the accumulation of cells in the G<sub>2</sub>/M phase and the development of polyploidization. Combination treatment with alisertib and romidepsin was synergistically cytotoxic in TLCs but not in B-cell lymphomas. This combination therapy led to the polyploidy of T cells and failure of their cytokinesis.

Cutaneous TCL is a category of TCL, and suberoylanilide hydroxamic acid (vorinostat) has been approved by the FDA for its treatment.<sup>(28)</sup> A preclinical modeling study that sought to identify biomarkers predictive of vorinostat responses in patients with CTCL revealed that persistent activation of the STAT signaling pathway is associated with lymphoma cell

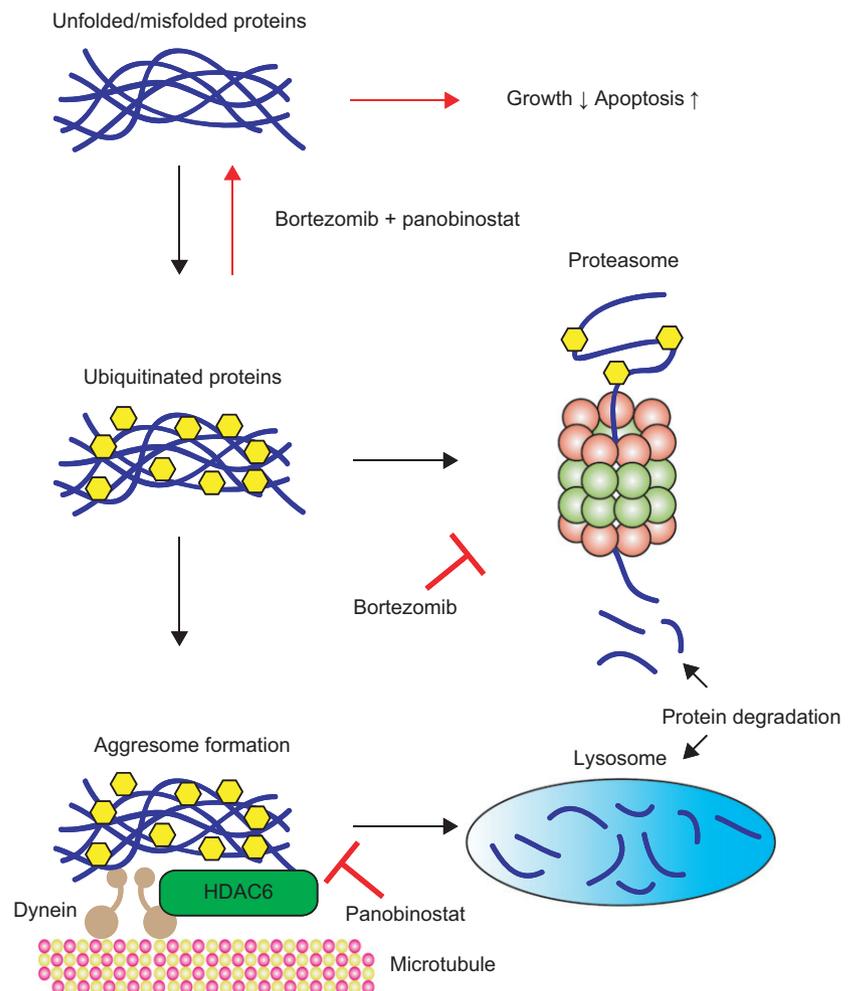
resistance to vorinostat.<sup>(29)</sup> An analysis of a phase IIB trial studying the use of vorinostat for patients with CTCL reported that increased nuclear localization of STAT1 and high levels of nuclear STAT3 phosphorylation in skin samples correlated with resistance to vorinostat therapy.<sup>(30)</sup>

### Mechanisms of action of HDAC inhibitors in the treatment of MM

The introduction of autologous stem cell transplantation and novel drug treatments including a proteasome inhibitor (bortezomib) and immunomodulatory drugs (thalidomide, lenalidomide, and pomalidomide) has improved the survival rates of patients with MM. However, many cases of relapsed/refractory MM are reported and, therefore, new therapies to treat such individuals are needed.

Several preclinical studies have revealed the efficacy of HDAC inhibitors in treating MM.<sup>(31,32)</sup> Vorinostat induced p21<sup>WAF1</sup> by modifying the acetylation and methylation of core histones and by restricting enzyme accessibility of DNase I more strongly in the promoter region of myeloma cells.<sup>(33)</sup> Vorinostat was also capable of overcoming cell adhesion-mediated drug resistance by inhibiting interleukin-6 secretion from the bone marrow stromal cells bound by MM cells.<sup>(31)</sup> Pretreatment with bortezomib enhanced the mitochondrial dysfunction and apoptosis induced by vorinostat in MM cells. Indeed, this combination was effective in both dexamethasone- and doxorubicin-resistant MM cells.<sup>(32)</sup> Proteasomes contribute

to the maintenance of protein homeostasis by degrading the ubiquitinated misfolded and unfolded proteins that are sometimes cytotoxic.<sup>(34)</sup> Abundant proteins including immunoglobulins are produced in MM cells, and certain misfolded/unfolded proteins interfere with cell functionality.<sup>(35)</sup> Cancer cells are more sensitive to proteasome inhibition than normal cells due to their dependence on proteasomes for the clearance of cytotoxic proteins. When ubiquitinated misfolded/unfolded proteins are generated at levels beyond a proteasome's capacity for degradation, such proteins will accumulate into aggresomes. An aggresome is a pericentriolar microtubule-based structure formed by the retrograde transport of aggregate proteins. Aggresome formation generally occurs as a cellular response to the excessive accumulation of misfolded/unfolded proteins.<sup>(36,37)</sup> The formed aggresomes within the peripheries of cells travel in microtubules to microtubule organizing centers.<sup>(38)</sup> This transportation is mediated by dynein and HDAC6, a microtubule-associated deacetylase,<sup>(39)</sup> which recruits misfolded/unfolded proteins to dynein motors for transport to aggresomes. The HDAC6-inhibited cells fail to clear protein aggregates due to the failure of proper aggresome formation. Bortezomib is a reversible inhibitor of chymotrypsin that occurs in the 20S subunit of proteasomes.<sup>(40,41)</sup> Treatment of MM cells with bortezomib induces aggresome formation.<sup>(42,43)</sup> LBH589 (panobinostat) is an oral pan-HDAC inhibitor and the combination of bortezomib and panobinostat induced apoptosis by inhibiting protein degradation through a synergistic mechanism (Fig. 2).<sup>(44)</sup> This may partly explain the synergism



**Fig. 2.** Ubiquitinated unfolded/misfolded proteins degraded by proteasomes. Bortezomib induces aggresome formation, and ubiquitinated proteins are degraded by lysosomes. Bortezomib and panobinostat synergistically block protein degradation and induce growth inhibition and apoptosis of multiple myeloma cells. HDAC6, histone deacetylase 6.

induced by combined bortezomib and panobinostat therapy in MM patients.<sup>(45)</sup>

### Histone deacetylase inhibitors induce calcineurin degradation in MM

Recent studies suggest that HDAC inhibition by panobinostat might induce antileukemia activity by blocking the chaperone function of HSP90. C-X-C-chemokine receptor type 4 is a receptor for stromal cell-derived factor 1, and its elevated expression in AML cells is associated with poor prognoses of AML.<sup>(46)</sup> C-X-C-chemokine receptor type 4 is a client protein of HSP90 and is protected from degradation by the 20S proteasome.<sup>(47)</sup> Treatment of AML cells with panobinostat not only induced acetylation of HSP90, but also reduced CXCR4 protein levels by reducing interactions between CXCR4 and HSP90. In an AML mouse model harboring t(8;21), treatment with panobinostat resulted in a robust antileukemic response through proteasomal degradation of AML1-ETO9a, the fusion protein generated by t(8;21).<sup>(15)</sup>

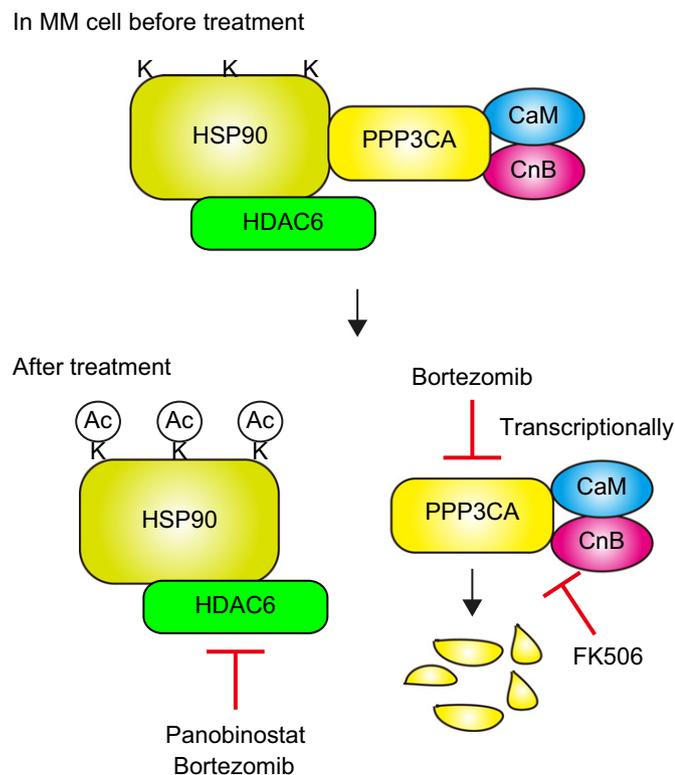
We discovered that panobinostat induces degradation of PPP3CA, a catalytic subunit of calcineurin, in MM cells (Fig. 3).<sup>(48)</sup> Protein phosphatase 3 catalytic subunit  $\alpha$  is a serine/threonine protein phosphatase, and NFATc1 is one of its dephosphorylation targets.<sup>(49)</sup> The translocation of dephosphorylated NFATc1 from the cytoplasm to the nucleus is indispensable for T cell activation induced by T-cell receptor activation. Calcineurin inhibitors such as FK506 and

cyclosporine A are widely used as immunosuppressive agents to inhibit the interaction between PPP3CA and its heterodimeric partner calcineurin B. In mouse models of T-cell acute lymphoblastic leukemia, calcineurin activation has been shown to play an important role in the maintenance of tumor cells.<sup>(50)</sup> However, certain studies have highlighted the importance of calcineurin in B cell functionality. This includes a study that addressed defective B cell activation stemming from calcineurin inactivation.<sup>(51)</sup> We found that PPP3CA was highly expressed in CD138-positive bone marrow cells from patients with advanced MM.<sup>(48)</sup> These results indicate a possible correlation between high PPP3CA expression and the pathogenesis of MM. In this study, PPP3CA acted as a client protein of HSP90 in MM cells. Treatment with ACY-1215, a selective HDAC6 inhibitor, resulted in PPP3CA degradation through its release from HSP90.<sup>(52)</sup> Therefore, panobinostat may induce protein degradation of PPP3CA by blocking the chaperone function of HSP90.<sup>(48)</sup> PPP3CA has been shown to be indispensable to the maintenance of MM cell growth via NF- $\kappa$ B signaling. Moreover, MM cell growth was inhibited by panobinostat treatment. Although FK506 itself did not affect PPP3CA expression or MM cell growth, its combined use with panobinostat enhanced the inhibition of PPP3CA and cell growth induced by panobinostat *in vitro* and *in vivo*. Calcineurin B has been shown to protect PPP3CA from degradation and the additive effect of FK506 is thought to enhance the degradation of unstable PPP3CA in panobinostat-treated MM cells.<sup>(53)</sup>

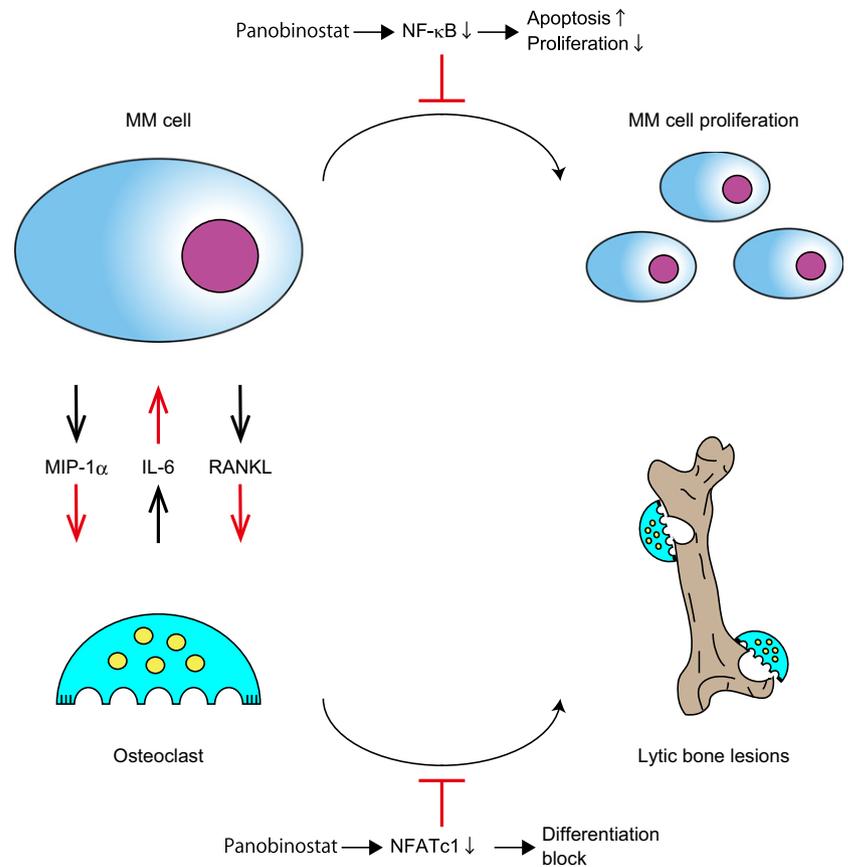
### Clinical significance of targeting PPP3CA in MM treatment

FK506 is one of the several immunosuppressive drugs widely used in allogeneic stem cell transplantation, including RIST. The latter is both feasible and effective in certain heavily treated patients with relapsed/refractory MM including those who experienced a relapse following autologous stem cell transplantation, by reducing the intensity of other conditioning therapies.<sup>(54)</sup> The introduction of panobinostat in combination with FK506 as a consolidation therapy following RIST may be effective in patients with residual disease due to the reduced intensity of the conditioning therapies. Combined bortezomib and an HSP90 inhibitor therapy has shown promising results in relapsed/refractory MM.<sup>(55)</sup> However, HSP90 inhibitors such as 17-AAG induce protein expression of HSP70, which protects cancer cells from apoptosis and may reduce the antimyeloma effects of this combination therapy.<sup>(56)</sup> However, increases in HSP70 expression by panobinostat alone or in combination with FK506 proved that the loss of the antimyeloma effects due to HSP70 expression were less pronounced with this combination therapy than they were with direct inhibition of HSP90.<sup>(48)</sup>

Combined panobinostat with bortezomib and dexamethasone therapy is expected to be effective even in patients who are resistant to bortezomib.<sup>(57)</sup> The analyses of our patients revealed that PPP3CA expression was significantly higher in patients who were bortezomib-resistant than in those who were sensitive.<sup>(48)</sup> Bortezomib reduced PPP3CA expression through HDAC6 inhibition and direct transcriptional suppression of PPP3CA. Combined bortezomib and panobinostat therapy synergistically inhibited MM cell viability by reducing PPP3CA (Fig. 3). These results show that PPP3CA is a common target of panobinostat and bortezomib. Therefore, the antimyeloma effects of this combination therapy may be explained by the synergistic PPP3CA reduction as well as inhibition of aggregate formation induced by bortezomib.



**Fig. 3.** Protein phosphatase 3 catalytic subunit  $\alpha$  (PPP3CA) is a client protein of heat shock protein (HSP90) deacetylated by histone deacetylase 6 (HDAC6). HDAC6 inhibition by panobinostat and bortezomib blocks HSP90 function through its acetylation and degradation of PPP3CA. Bortezomib also transcriptionally suppresses PPP3CA. Ac, acetylation; CaM, calmodulin; CnB, calcineurin B; K, lysine; MM, multiple myeloma.



**Fig. 4.** Cytokine production like macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), interleukin-6 (IL-6), and receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) by multiple myeloma (MM) cells and osteoclasts creates a vicious cycle of MM cell proliferation and induces bone lysis. Blockade of MM cell proliferation and bone lysis by panobinostat would be useful in stopping this cycle. NFATc1, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1.

Proper balancing of the activation and inactivation of adhesion molecules  $\alpha_4$  integrins are essential for the efficient migration and homeostasis of lymphocyte distribution.<sup>(58)</sup> In MM, ITGA4 are associated with cell adhesion-mediated drug resistance.<sup>(59)</sup> The correlation between  $\alpha_4$  integrin and *PPP3CA* expression in patients with MM may explain why patients with high *PPP3CA* expression respond so poorly to bortezomib-containing chemotherapies.<sup>(48)</sup>

Lytic bone lesions generated by osteoclast formation are serious complications often observed in patients with MM.<sup>(60)</sup> The induction of NFATc1 is necessary for osteoclast differentiation, which is inhibited by FK506 treatment.<sup>(61)</sup> Panobinostat-inhibited osteoclast differentiation was believed to be mediated by *PPP3CA* protein degradation.<sup>(48)</sup> The addition of FK506 strengthened the blockade of osteoclast formation by panobinostat alone. The inhibition of MM cell proliferation and osteoclast formation by panobinostat and FK506 should prove useful for MM treatment by stopping the vicious cycle that occurs between the proliferation of MM cells and bone lysis (Fig. 4).<sup>(60)</sup>

## Conclusion

In this review, we interpreted the underlying mechanisms of action of HDAC inhibitors used in the treatment of hematological malignancies including AML/MDS, TCLs, and MM. The fusion partner of AML1 in t(8;21)(q22;q22), ETO, mediates transcriptional repression through its interaction with the complex N-CoR/mSin3/HDAC1. In fact, HDAC inhibitors have been proposed as effective treatment agents for patients with AML associated with t(8;21). In TCL cell lines, HDAC

inhibitors including belinostat showed synergism with decitabine, a hypomethylating agent *in vitro* and *in vivo*. The usefulness of monitoring the persistent STAT signaling pathway activation as a biomarker in patients with CTCL resistant to vorinostat treatment was suggested. Panobinostat in combination with bortezomib and dexamethasone can be prescribed to patients with relapsed/refractory MM. Indeed, this combination therapy appears to be effective in such patients, including those resistant to bortezomib treatment. We discovered that panobinostat induces *PPP3CA* degradation in MM. Bortezomib also reduced *PPP3CA* expression, and the latter was shown to be a common target of panobinostat and bortezomib. The anti-myeloma effects of panobinostat combined with bortezomib and dexamethasone therapy may be explained by the synergistic *PPP3CA* reduction in tandem with panobinostat-induced blockade of aggresome formation enhanced by bortezomib.

## Disclosure statement

The authors have no conflict of interest.

## Abbreviations

AAK	aurora A kinase
AML	acute myelogenous leukemia
CTCL	cutaneous T-cell lymphoma
CXCR4	C-X-C-chemokine receptor type 4
ETO	eight twenty-one oncoprotein
HDAC	histone deacetylase
HSP	heat shock protein
MDS	myelodysplastic syndrome
MM	multiple myeloma

mSin3A	mammalian SIN3 transcription regulator family member A	PPP3CA	protein phosphatase 3 catalytic subunit $\alpha$
N-CoR	nuclear receptor co-repressor	RIST	reduced intensity-conditioned allogeneic stem cell transplantation
NFATc1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	STAT	signal transducer and activator of transcription
NF- $\kappa$ B	nuclear factor- $\kappa$ B	TCL	T-cell lymphoma

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