The Role of Histone deacetylase (HDAC) Inhibitors and Cytokine-induced Killer Cell (CIK) in Multiple Myeloma

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List of abbreviations

MM	multiple myeloma
HDACs	histone deacetylases
HDACis	histone deacetylases inhibitors
NK cell	natural killer cell
CIK cell	cytokine-induced killer cell
NKT cell	natural killer T cell
IFN-γ	interferon-γ
PBMCs	blood mononuclear cells
anti-CD3 mAb	monoclonal antibody against CD3
IL-1β	interleukin-1β
IL-2	interleukin-2
MHC	major histocompatibility complex
ADCC	antibody-dependent cellular cytotoxicity
RRMM	relapsed or refractory multiple myeloma
FDA	U.S. Food and Drug Administration

1. Abstract

Multiple myeloma (MM) is a complex blood neoplasm marked by abnormal plasma cell growth due to genetic and epigenetic changes. Although treatments have improved, MM remains largely incurable, often developing resistance to drugs. Recent research focuses on histone deacetylases (HDACs), with HDAC inhibitors (HDACis) showing potential in enhancing natural killer (NK) cell effectiveness. Studies also suggest promising results with cytokine-induced killer (CIK) cell immunotherapy. The combination of HDACis and CIK cell therapy in clinical trials could potentially improve treatment outcomes for MM.

This dissertation explores the role of HDACis and CIK cell immunotherapy in MM by investigating two primary research objectives: 1) the respective roles of HDACis and CIK cell immunotherapy in the development and progression of MM; 2) the potential benefits of combining HDACis with CIK cell immunotherapy in the treatment of MM and the molecular mechanisms behind this interaction.

We examined clinically relevant HDACis (panobinostat/LBH589 and romidepsin) alongside CIK cells derived from peripheral blood mononuclear cells across diverse MM cell lines (U266, RPMI8226, OPM-2 and NCI-H929). Utilizing various *in vitro* methodologies, we investigated how HDACis enhance CIK cell lysis of myeloma cells through NKG2D/NKG2D ligand interactions.

The combination of CIK cells with HDACis significantly enhances their ability to kill MM cells, mainly by increasing apoptosis and altering immune signaling molecules like interferon-γ (IFN-γ) and granzyme B. HDACis also boost the expression of proteins MICA/B and ULBP2, vital for NKT cell-driven antitumor effects. These effects were confirmed by blocking the NKG2D receptor in CIK cells, which supports the synergistic action of HDACis and CIK cells in targeting MM.

Our analyses provide sufficient evidence to consider this clinically forgotten instance (HDACis-CIK cell combination) as a therapeutic priority for MM treatment. Furthermore, we suggest that NKG2D/NKG2D-ligand interactions activating NK/NKT cells may contribute to enhanced myeloma cell lysis in response to HDACis treatment by CIK cells.

2. Introduction

2.1 Background:

2.1.1 Multiple myeloma

Multiple myeloma (MM), a hematologic malignancy, is characterized by the proliferation of aberrant clonal plasma cells within the bone marrow, leading to significant clinical manifestations such as severe bone lesions, renal damage, anemia, and hypercalcemia (Cowan et al., 2022). This disease exhibits a higher incidence in industrialized regions, notably in Australia, Western Europe, and the United States, which report the highest prevalence rates (Padala et al., 2021). As the second most common hematologic malignancy in the United States, MM accounts for approximately 1.8% of all cancer cases and about 10% of hematologic malignancies (Rajkumar, 2022). Data from the American Cancer Society in 2022 estimate that 34,470 new cases of MM will be diagnosed in the United States, resulting in approximately 12,640 fatalities (Siegel et al., 2022). The demographic profile of MM primarily includes older adults, with a median age at diagnosis of 69 years and a median age at death of 75 years. The incidence of MM is approximately 1.5 times higher in men than in women globally (Cowan et al., 2018). Despite being incurable, recent advancements in treatment have significantly extended the five-year survival rate beyond five years and have improved the quality of life for patients (Abramson, 2021). Therefore, the development of innovative therapeutic strategies for multiple myeloma remains critically important.

2.1.2 Cytokine-induced killer cell

Cytokine-induced killer (CIK) cells represent a diverse group of immune cells that include $CD3^+CD56^-$ T cells, $CD3^-CD56^+$ natural killer (NK) cells, and $CD3^+CD56^+$ natural killer T (NKT) cells. This innovative method for generating CIK cells was first introduced by Schmidt-Wolf and colleagues in 1991. Their groundbreaking research demonstrated that CIK cells exhibit heightened cytotoxicity against lymphoma while causing minimal toxicity in a SCID mouse/human lymphoma model, offering promising therapeutic potential (Schmidt-Wolf et al., 1991).

CIK cells, derived from peripheral blood mononuclear cells (PBMCs), can be effectively expanded in a laboratory setting (Pu et al., 2024). The expansion

process begins by adding 1000 IU/ml of IFN-γ on the initial day. Subsequently, on the following day, a cocktail of 50 ng/ml monoclonal antibody against CD3 (anti-CD3 mAb), 100 IU/ml interleukin-1β (IL-1β), and 600 IU/ml interleukin-2 (IL-2) is administered sequentially. To maintain optimal growth conditions, both IL-2 and fresh culture medium are replenished every 2-3 days over a period of 2-3 weeks. The priming with IFN-γ 24 hours before introducing anti-CD3 mAb and IL-2 is crucial. This priming step enhances the activation of IL-2-responsive cells and stimulates monocytes to regulate the immunomodulatory factor IL-12, thus boosting the overall effectiveness of the cell expansion (Bonanno et al., 2010). $CD3^+$ cells form the majority of CIK cells, making up over 90%, with the potent effector subset, $CD3^+CD56^+$, varying between 7.6% and 65% in concentration (Linn et al., 2002). Notably, the $CD3^+CD8^+$ subset expands more rapidly than the $CD3^+CD56^+$ subset following stimulation with IL-2 and anti-CD3. This particular $CD3^+CD56^+$ group is characterized by a more advanced, terminally differentiated effector phenotype, typically exhibiting $CD27^+CD28^-$ or $CD27^-CD28^-$ markers, unlike its $CD3^+CD56^-$ predecessors (Linn et al., 2009). Furthermore, $CD3^+CD56^+$ cells are known for their robust antitumor properties, demonstrating major histocompatibility complex (MHC)-unrestricted cytotoxicity that effectively targets both hematological malignancies and solid tumors.

CIK cells recognize and attack tumor cells primarily through the NKG2D receptors, which interact with certain proteins highly present on tumor cells, leading to the destruction of these cells by the release of cytotoxic molecules like perforin and granzyme (Verneris et al., 2004). This process does not involve MHC molecules. Additionally, other receptors such as DNAM-1 and NKp30 also contribute to the tumor cell recognition and destruction in a manner independent of the T-cell receptor (Karimi et al., 2005).

While some reports suggest CIK cells lack CD16, which limits their ability to mediate antibody-dependent cellular cytotoxicity (ADCC), other studies indicate that CD16 expression can vary among donors. When combined with therapeutic antibodies, CIK cells can significantly enhance their anti-tumor activity through ADCC. This highlights the potential dual functionality of CIK cells, incorporating

natural killer cell like cytotoxicity with T cell receptor-driven specificity (Cappuzzello et al., 2016).

2.1.3 Histone deacetylase and its inhibitors

HDACs are key in regulating gene expression by modifying the acetylation of histones, which organize DNA in the cell nucleus (Ropero & Esteller, 2007; Falkenberg & Johnstone, 2014). In MM, HDACs affect various disease aspects, such as cell cycle regulation (Ramaiah et al. 2021), apoptosis resistance (Patra et al. 2019), and interactions with the tumor microenvironment, including proliferation, differentiation, inflammation, metastasis, and angiogenesis (Lin et al., 2023; Aventaggiato et al., 2021; Ell & Kang, 2013). Endothelial cells are vital in angiogenesis, the formation of new blood vessels crucial for tumor growth and spread (Cross et al., 2019). In MM, these cells not only change properties but also enhance angiogenesis, accelerating disease progression and drug resistance. HDACis disrupt these processes by impairing endothelial cell functions and altering the tumor's blood supply, thereby inhibiting angiogenesis. This dual strategy of targeting endothelial cells and angiogenesis is a promising way to combat drug resistance and improve treatment outcomes in MM (Xue et al., 2022). HDACs are crucial in removing acetyl groups from lysine residues in both histone and non-histone proteins, such as p53 (Kuo et al., 2016) and NF-κB (Vrabel et al., 2019). This deacetylation tightens chromatin structure, decreases gene expression, and affects cellular functions and stability (Zhao et al., 2020). In MM, HDACs influence cancer progression by promoting rapid cell growth and creating a favorable bone marrow environment (Wong et al., 2020). HDACis disrupt this by altering acetylation patterns, affecting gene activity and key pathways like NF-κB, which can slow cancer growth and enhance treatment effects (Hu & Hu, 2018). HDACis also modulate autophagy—a process critical for cellular survival under stress, which can either support cell survival or lead to cell death, impacting drug resistance and treatment efficacy (Hamedi et al., 2022). Understanding the intricate interactions between these mechanisms is vital for developing effective MM treatments.

Various HDACis, categorized into six types based on their chemical structures, have been explored for treating malignancies. These include short-chain fatty

acids, hydroxamic acids, benzamides, cyclic peptides, mercaptoketones, sirtuin inhibitors, and other compounds (Eckschlager et al., 2017; McClure et al., 2018). Non-selective HDACis, which inhibit several HDAC isoforms, mainly target HDAC 1, 2, 3, and 6. This inhibition of class I and IIb HDAC enzymes is crucial for their anti-tumor effects (Ho et al., 2020). Among these, Panobinostat, a potent oral pan-HDAC inhibitor, was approved by the U.S. Food and Drug Administration (FDA) in 2015 for treating relapsed or refractory multiple myeloma (RRMM) (Eleutherakis‐Papaiakovou et al., 2020), though it was withdrawn in the US in 2022. Other HDACis like Qusinostat, Gavinostat, and Rocilinostat, initially used for solid tumors and refractory leukemia, are showing promise in RRMM treatment (Ashjian & Redic, 2016).

Surprisingly, HDACis have never been combined with CIK cell immunotherapy in clinical trials. As pioneers in this field, we explored their compatibility in MM and conducted preclinical tests using HDACis like sodium butyrate, valproic acid, and trichostatin A with CIK cells (Stephan et al., 2017). These tests confirmed a significant impact on MM cell lines. However, unresolved issues include understanding the mechanisms of their synergistic effects and whether genetic or gender differences might limit their clinical use.

2.2 Aims

This dissertation delves into the impact of HDACis and CIK cell immunotherapy on MM. It aims to achieve two main research objectives: first, to examine the individual roles of HDACis and CIK cell therapy in influencing the onset and advancement of MM; second, to explore the synergistic effects of combining HDACis with CIK cell therapy in treating MM, along with the underlying molecular mechanisms that facilitate this interaction.

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3. Publications

3.1 Publication 1: Synergistic integration of histone deacetylase inhibitors apparently enhances the cytokine-induced killer cell efficiency in multiple myeloma via the NKG2D pathway

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ORIGINAL ARTICLE

Synergistic integration of histone deacetylase inhibitors apparently enhances the cytokine-induced killer cell efficiency in multiple myeloma via the NKG2D pathway

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Abstract

Objectives. The rapid recognition of epigenetic manipulation's potential in restricting cancer cell capabilities spurred translational initiatives, including histone deacetylase inhibitors (HDACis). Clinical trials on multiple myeloma (MM) demonstrated substantial benefits of HDACis, coupled with promising outcomes from cytokine-induced killer cell (CIK) immunotherapy. Intriguingly, the unexplored synergy of HDACis and CIK cell immunotherapy in MM prompted our study. **Methods.** We examined clinically relevant HDACis (panobinostat/LBH589 and romidepsin) alongside CIK cells derived from peripheral blood mononuclear cells across diverse MM cell lines (U266, RPMI8226, OPM-2 and NCI-H929). Utilising various *in vitro* methodologies, we investigated how HDACis enhance CIK cell lysis of myeloma cells through NKG2D/NKG2D ligand interactions. **Results.** The results of our analysis indicated several key findings. (1) Enhanced cytotoxicity of CIK cells in MM cells when combined with HDACis. (2) Significant increase in apoptosis, suggesting HDACis and CIK may together enhance apoptotic effects in specific MM cell lines. (3) Elevated IFN-γ secretion and alterations in granzyme B secretion because of the independent activity of HDACis. (4) Notably, HDACis increased the expression of MICA/B and ULBP2, crucial for inducing antitumor cytotoxicity of NKT cells. Validation through NKG2D receptor blocking in CIK cells with a purified mouse antihuman NKG2D antibody further supported our findings. **Conclusions.** Our analyses provide sufficient evidence to consider this clinically forgotten instance (HDACis-CIK cell combination) as a therapeutic priority for MM treatment. Furthermore, we suggest that NKG2D/NKG2D-ligand interactions activating NK/NKT cells may contribute to enhanced myeloma cell lysis in response to HDACis treatment by CIK cells.

Keywords: cytokine-induced killer cells, histone deacetylase inhibitors, immunotherapy, multiple myeloma, NKG2D

INTRODUCTION

Multiple myeloma (MM) is a clonal expansion of plasma cells in which genetic and epigenetic processes are significantly involved during the development and progression of this pathological condition. Despite recent advances in treatment modalities, MM remains incurable in most cases because of the drug resistance to all conventional therapies. Given that aberrant expression of epigenetic modifications has been observed in various cancers, MM is no exception to this observation.[1,2] Of particular interest in MM are variable patterns of histone deacetylase (HDACs), which have led to the exploration of several histone deacetylase inhibitors (HDACis)-based interventions *in vitro*, *in vivo* and in clinical trials over the last decades.[3–5]

Notably, the passive mechanisms underlying the involvement of epigenetic inhibitors, including HDACs, with non-oncological drugs in MM, have recently been discussed.[6] Recently, we have also revealed the mutual interactions between epigenetic machinery and non-coding genome in regulating gene expression by investigating the intriguing interactions between HDAC6-induced lncRNA and its potential sponge miRNA in MM.[7] HDACis have also been shown to sensitise NK cell-mediated killing by upregulating expression of Natural killer group 2 member D (NKG2D) ligands MICA/B or ULBP1 on cancer cells, thus suggesting that HDACis might have promising applications in cancer immunotherapy.[8,9] Like HDACis, cytokine-induced killer (CIK) cell immunotherapy has also been quite active in cancer.[10] Moreover, CIK cell immunotherapy already have a history of successful clinical trials in MM. For instance, Lin *et al*.[11] reported one case report of patient with multiple cancers (MM and lung cancer) concomitant with paraneoplastic dermatoses and demonstrated that after treatment with CIK cells, MM and lung cancer remained stable and concomitant paraneoplastic dermatoses improved markedly without side effects. Zhao *et al*.[12] also followed CIK cell therapy by examining 50 patients with MM ($n = 24$: chemotherapy, $n = 26$: combined chemotherapy with dendritic cell (DC/CIK) and confirmed a better immunomodulatory effect with the combination therapy. In 2017, Wang *et al*. published a meta-analysis including 12 trials with 594 MM patients and confirmed that adjuvant immunotherapy of dendritic cells-CIK cells enhanced the efficacy of chemotherapy for MM and further improved prognosis probably by reconstructing the immune function.[13]

Surprisingly, HDACis have never been tested in combination with CIK cell immunotherapy in the clinic. Being pioneers in CIK cell therapy, we previously raised the question whether CIK cells and HDACis would be compatible in MM. To address this, we tested several HDACis (e.g. sodium butyrate, valproic acid and trichostatin A) in combination with CIK cells (as the first preclinical model) and confirmed their significant impact on human MM cell lines (KMS-18 and U-266).[14] However, some questions remain unanswered, such as (1) what exactly are the mechanisms behind the synergistic effect of CIK cells with HDACis in MM and (2) whether genetic/gender differences could play a therapeutically restrictive role when testing HDACis-CIK combinations in clinics, because MM is known to occur more frequently in males compared with females. Considering this, herein, we extended our analysis by testing clinically applicable HDACis with CIK cells in genetically distinct MM cell lines. In addition, we investigated whether HDACis treatment could enhance CIK cell lysis of myeloma cells through NKG2D/NKG2D ligand interactions.

RESULTS

Phenotypic identification of CIK cells and the effect of HDACis on the viability of MM cell lines

In clinical trials, whether HDACis were used alone or in combination with other drugs, a plethora of knowledge exists about their relative success in MM (Figure 1 and Supplementary table 1).To advance the knowledge, we first confirmed the phenotypic identification of CIK cells and the effect of HDACis on the viability of MM cell lines. As the days went by, the proportion of $CD3^+CD56^+$ NKT cells (CIK cells) increased (Supplementary figure 1a, one representative data of three donors). To confirm this, we identified the heterogeneous population of peripheral blood mononuclear cells (PBMCs; Day 0) composed of $CD3^+CD56^+$ NKT cells ($7.0\% \pm 1.9\%$), $CD3^+CD56^-$ T cells ($56\% \pm 5.6\%$) and $CD3^-CD56^+$ NK cells ($11\% \pm 2.7\%$). After 13 days of *ex vivo* expansion, the bulk CIK cells were a heterogeneous population composed of $CD3^+CD56^+$ NKT cells ($27.0\% \pm 5.9\%$), $CD3^+CD56^-$ T cells ($71\% \pm 5.6\%$) and $CD3^-CD56^+$ NK cells

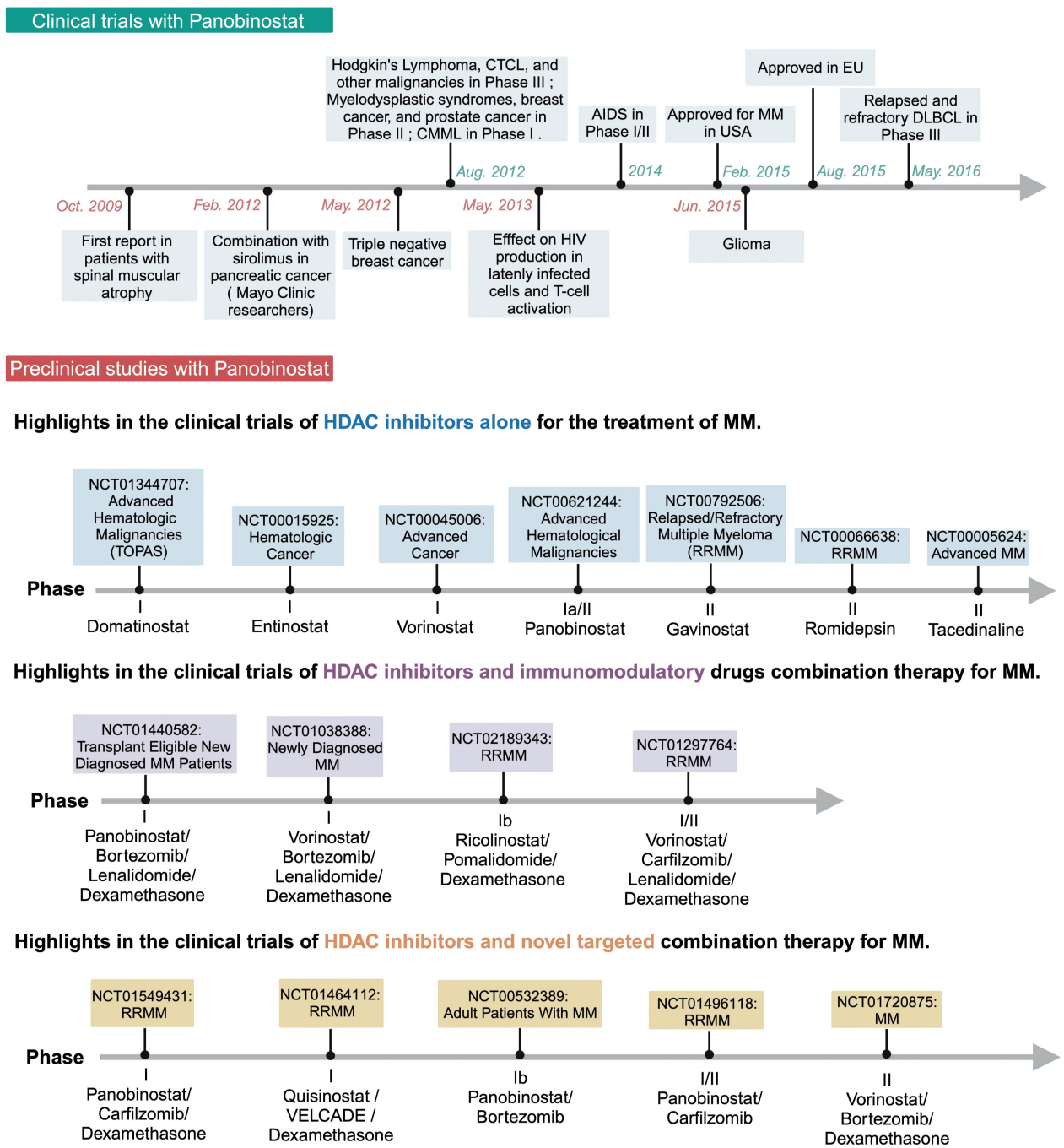

Figure 1. Histone deacetylase inhibitors (HDACis) used alone or in combination therapies in multiple myeloma. This figure was created with Biorender.com.

(0.63% ± 0.27%; Supplementary figure 1b). Likewise, the MM cell lines U266, RPMI8226, OPM-2 and NCI-H929 were cultured with gradient concentrations of panobinostat (0–250 nM) or romidepsin (0–10 000 nM) for 24 or 48 h (Supplementary figure 1c). As the concentrations of panobinostat and romidepsin increased, the viability of MM cells gradually decreased ($P < 0.05$),

and we also determined their IC_{50} values on MM cell lines. In order to avoid any background effect, we also tested effect of HDACis on viability of non-cancer cells (CCD-18Co) and CIK cells. CCD-18Co and CIK cells were treated with various concentrations of panobinostat and romidepsin. We observed that, at the appropriate concentration (for panobinostat, it is 0–50 nM; for romidepsin, it is 0–5 nM), the impact on the viability of MM cell lines was greater than that on the viability of CCD-18Co and CIK cells (Supplementary figure 1d). Overall, we established phenotypically distinct CIK cell populations and tested the cytotoxic effects of HDACis on MM cells.

Effect of CIK cells on MM cell lines and synergy of HDACis with CIK cells on MM cells

CIK cells were co-cultured with MM cell lines U266, RPMI8226, OPM-2, NCI-H929 and the control cell line CCD-18Co for 24 h (Figure 2a). CIK cells from two different buffy coats (Donor A and B) were used at different effector-to-target ratios (1:1, 5:1 and 10:1). As a result, when the ratio was 10:1, CIK cells significantly reduced the *in vitro* viability of MM cells ($P < 0.0001$). At a 5:1 ratio, CIK cells significantly reduced OPM-2, RPMI8226 and U266 *in vitro* viability ($P < 0.0001$), with the observed impact varying according to the different effector-to-target ratios. However, CIK cells also significantly decreased the viability of CCD-18Co when the ratio was 10:1 and 5:1 ($P < 0.0001$). In this context, the cytotoxic effect of CIK cells may be associated with the immune status of donors. To investigate a synergistic effect of panobinostat, romidepsin and CIK cells, myeloma cell lines NCI-H929, OPM-2 and U266 were cultured with panobinostat (0.01 μM), romidepsin (0.01 μM) and CIK cells at different effector-to-target ratios (10:1, 5:1, 1:1) for 24 h (Figure 2b). In NCI-H929, OPM-2 and U266 cell lines, we found that MM cells treated with panobinostat and CIK cells (10:1) showed more specific lysis compared with cells only treated with CIK cells (10:1) except NCI-H929 ($P < 0.0001$). In MM cells treated with romidepsin and CIK cells (10:1), a statistically significant increase in specific lysis was observed only in OPM-2 compared with cells treated only with CIK cells (10:1; $P < 0.0001$). Overall, it is reasonable to conclude the combination use of panobinostat and romidepsin with CIK cells may increase the specific lysis of MM cell lines.

Effect of HDACis and CIK cells on granzyme B and IFN-γ secretion in MM cells

Given that CIK cells are well established to release Granzyme B and IFN-γ as independent mechanisms to kill tumor cells, we evaluated their relative amounts in the supernatant using sandwich ELISA. In all three MM cell lines, Granzyme B secretion remained stable, regardless of HDACis treatment (Figure 3a). Similarly, CIK cells treated with HDACis exhibited increased total secretion. Significantly higher Granzyme B secretion was observed in the U266 cell line alone, following treatment with panobinostat or romidepsin, compared with co-culture with CIK cells alone (For panobinostat, $P < 0.05$; for romidepsin, $P < 0.01$). This suggests an increase in Granzyme B secretion in the U266 cell line following treatment with CIK cells, panobinostat and romidepsin. Likewise, MM cell lines were incubated with panobinostat (0.01 μM), romidepsin (0.01 μM) and CIK cells at the effector-to-target ratio (20:1) for 24 h. After 24 h, the supernatant was collected for a sandwich ELISA to evaluate IFN-γ secretion (Figure 3b). In all three MM cell lines, the basal secretion without any treatment was low. However, after treatment with romidepsin, there was significantly higher IFN-γ secretion in all three MM cell lines compared with only co-culture with CIK cells (U266, OPM-2: $P < 0.0001$; NCI-H929: $P < 0.05$). Meanwhile, after treatment with panobinostat, there was significantly higher IFN-γ secretion only in OPM-2 compared with only co-culture with CIK cells ($P < 0.0001$). This result suggests that after co-culture of MM cells, CIK cells, panobinostat and romidepsin treatment, IFN-γ secretion increased. Notably, after treatment with panobinostat (0.01 μM), romidepsin (0.01 μM) and CIK cells on the U266 cell line, we observed an increase in the overall number of apoptotic cells, particularly in the number of early apoptotic cells (Figure 3c). Furthermore, when compared with the untreated group (control), the proportion of early apoptotic cells and late apoptosis or necrosis cells significantly increased (Figure 3d; $P < 0.0001$). That is, CIK cells enhance the apoptosis of U266 cell line that have been treated with HDACis. This suggests that HDACis and CIK cells may together enhance apoptotic effects in specific MM cell lines.

Effect of HDACis on the NKG2D ligands in myeloma cells

As aforementioned, HDACis have been shown to sensitise NK cell-mediated killing by upregulating

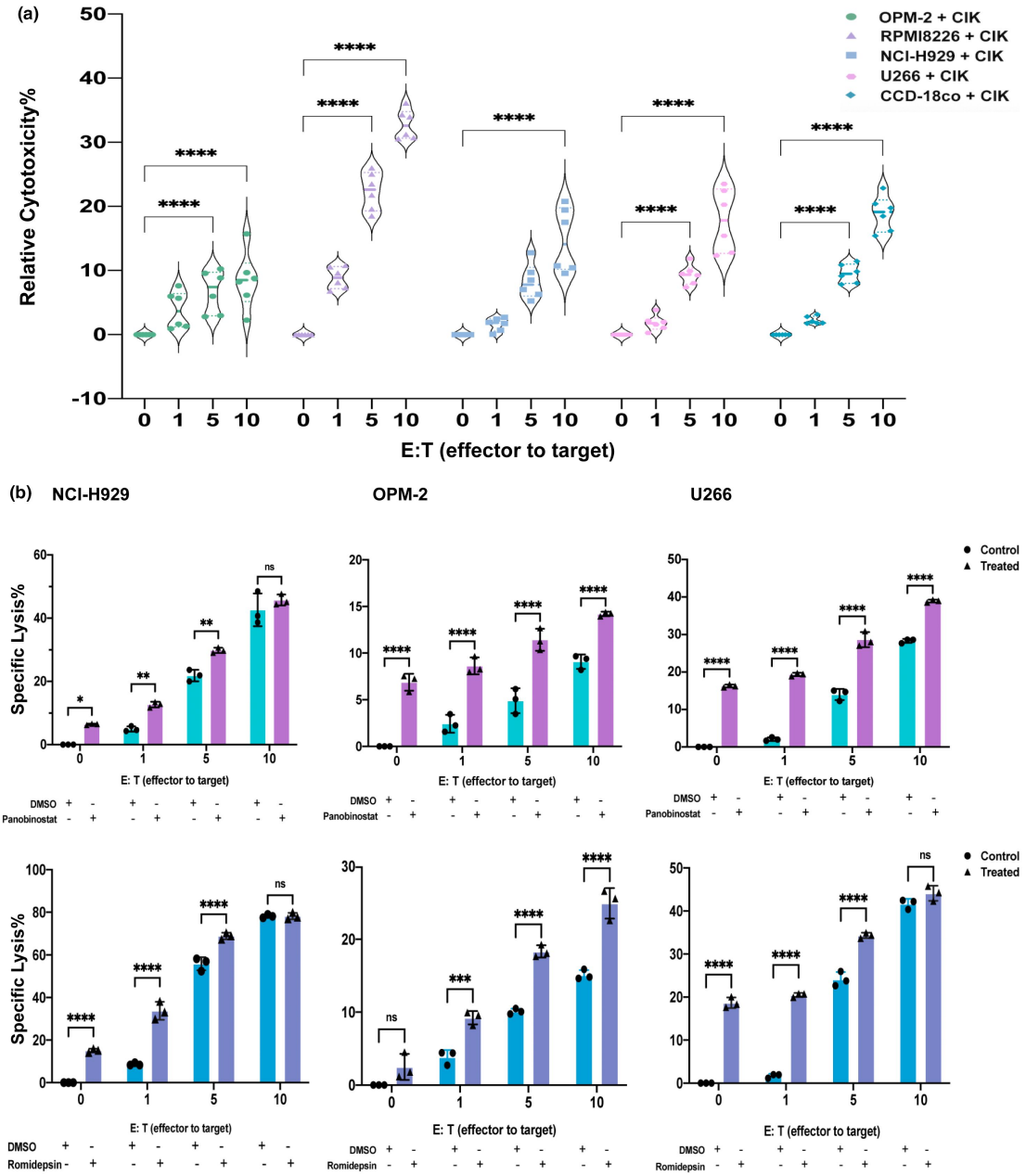

Figure 2. **(a)** Cytotoxic effect of cytokine-induced killer (CIK) cells on MM cell lines OPM-2, RPMI8226, NCI-H929, U266 and the control cell line CCD-18Co. Cells were cultured for 24 h at different effector: target ratios of 10:1, 5:1 and 1:1. The cytotoxic effect of CIK cells was measured by LDH assay. Results represent six separate experiments for U266, RPMI8226, OPM-2, NCI-H929 and CCD-18Co. **(b)** We assessed the significance among myeloma cells NCI-H929, OPM-2 and U266 in two conditions: one treated only with panobinostat (0.01 μM) and romidepsin (0.01 μM) for 24 h, and the other treated with the combination of panobinostat and romidepsin (at the same concentration). Additionally, CIK cells were treated at different effector: target ratios (10:1, 5:1 and 1:1). The results represent data from three separate experiments and are presented as mean ± SD. Significance levels were determined using two-way ANOVA with Bonferroni's post hoc test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

expression of NKG2D ligands MICA/B or ULBP1 on cancer cells. We investigated the effects of LBH589 and romidepsin on the mRNA expression of NKG2D ligands in myeloma cells. Specifically, we performed RT qPCR detection of NKG2D ligands in U266, OPM-2 and NCI-H929 before and after

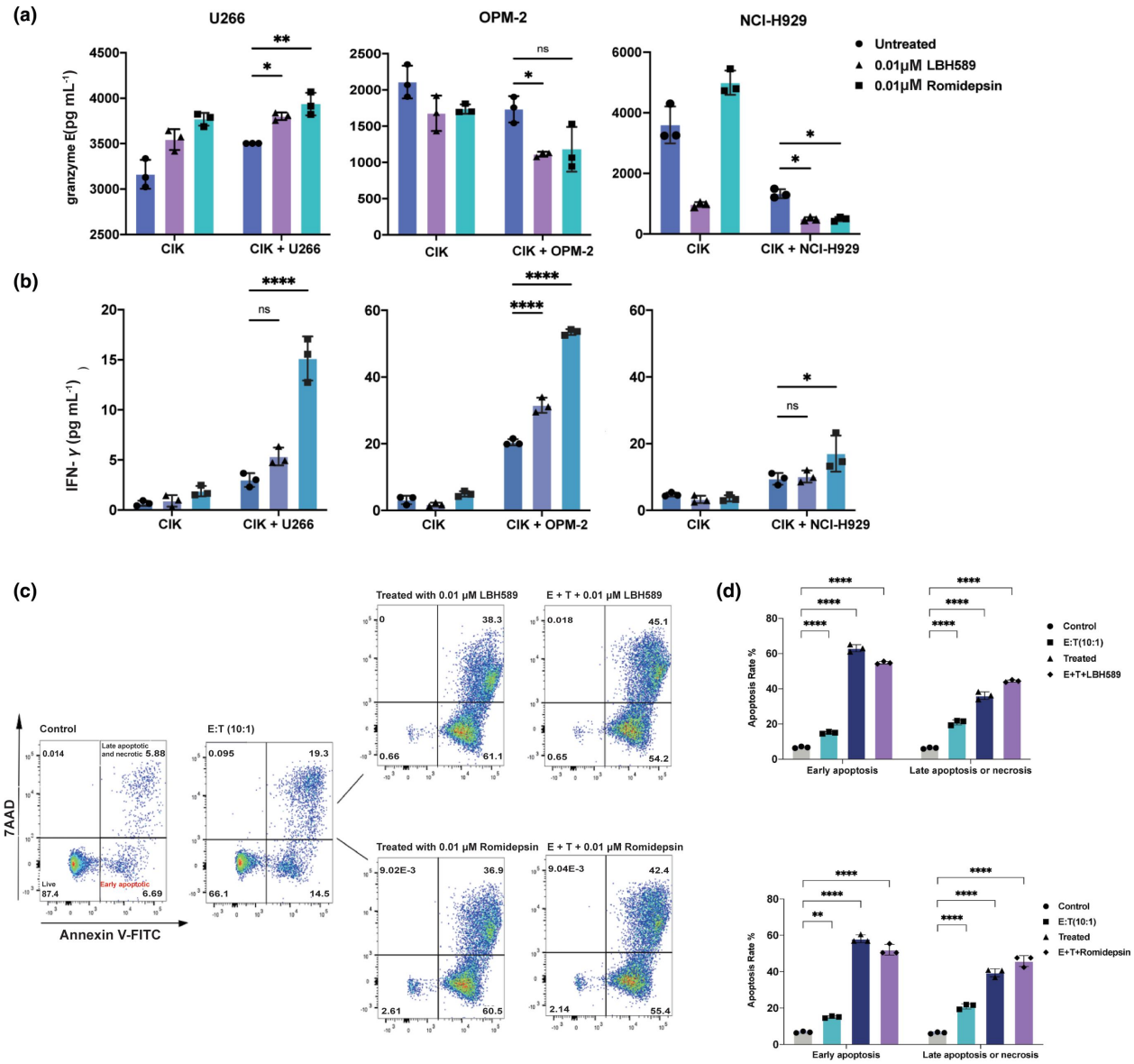

Figure 3. **(a)** Granzyme B secretion in the combination of histone deacetylase inhibitors (HDACis) and cytokine-induced killer (CIK) cells on multiple myeloma (MM) cell lines U266, OPM-2 and NCI-H929. **(b)** IFN-γ secretion in the combination of HDACis and CIK cells on MM cell lines. MM cells were cultured with panobinostat (0.01 μM), romidepsin (0.01 μM) and CIK cells at the effector-to-target ratio (20:1) for 24 h. IFN-γ and Granzyme B secretion were measured by ELISA. Results represent three different buffy coats for each cell line. **(c)** Combination of HDACis with CIK cells on the apoptosis of U266 cell line by the flow cytometry assay. Flow cytometry figure of changes in the proportion of early apoptosis cells and late apoptosis or necrosis cells. Cells were stained with FITC Annexin V and Percp 7AAD. The result is one of the representative data. **(d)** After the combination of HDACis with CIK cells, the apoptosis rate of U266 cells changed. Results represent data from three separate experiments. Data are presented as mean ± SD. (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ calculated by two-way ANOVA, Bonferroni's post hoc test).

treatment with LBH589 or romidepsin. We found that after LBH589 treatment, the relative mRNA levels of MICA, MICB and ULBP2 were significantly increased compared with the control group (Figure 4a, $P < 0.01$). Similar results were obtained after romidepsin treatment (Figure 4b, $P < 0.01$), suggesting that LBH589 and romidepsin can increase the expression of MICA, MICB and ULBP2 mRNA levels in MM cells. We further verified the effects of LBH589 and romidepsin on the surface

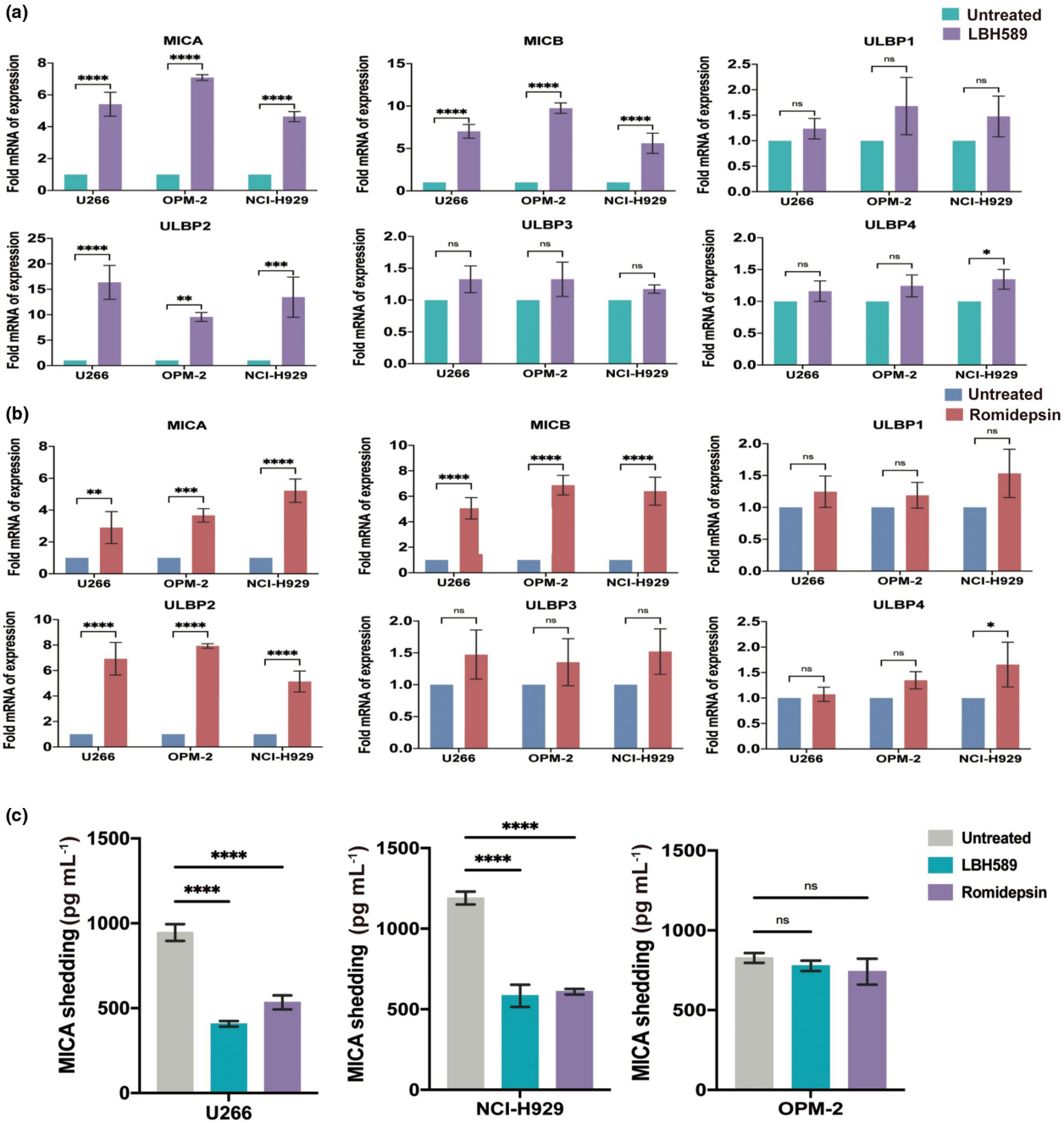

Figure 4. Relative mRNA expression of NKG2D ligands on human myeloma cell lines after treatment with 0.01 μM LBH589 or romidepsin for 24 h. **(a)** Relative mRNA expression of NKG2D ligands after LBH589 treatment on U266, OPM-2, NCI-H929 cells. **(b)** Relative mRNA expression of NKG2D ligands after romidepsin treatment on U266, OPM-2 and NCI-H929 cells. **(c)** Multiple myeloma (MM) cell lines (U266, OPM-2 and NCI-H929) were untreated or treated with 0.01 μM panobinostat or romidepsin for 48 h. Shed MICA was quantified in the supernatant by sandwich ELISA. Data are mean ± SD of triplicate measurements; data are one representative of three independent experiments. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ calculated by one-way ANOVA, Bonferroni's post hoc test).

expression of MICA/B and ULBP2 in myeloma cells by using flow cytometric analysis, gating on live U266, OPM-2 and NCI-H929 MM cells before and after treatment with LBH589 or romidepsin (Supplementary figure 2a). As depicted in Supplementary figure 2b, the treatment

significantly upregulated the expression of MICA/B ($P < 0.05$). With the exception of OPM-2 treated with LBH589, the expression of ULBP2 also significantly increased in the other cell lines after treatment ($P < 0.05$), suggesting that HDACis (LBH589 and romidepsin) can enhance the expression of NKG2D ligands (MICA/B and ULBP2). Furthermore, compared with the untreated group, the MICA shedding from U266 and NCI-H929 cells in the presence of 0.01 μM LBH589 or romidepsin was strongly inhibited (Figure 4c, $P < 0.0001$). Our findings indicate that LBH589 and romidepsin exhibit pronounced inhibitory effects on the proteolytic shedding of MICA from MICA/B-bearing tumor cells, leading to a significant augmentation in the cell surface density of MICA. Taken together, these results suggest that in MM, HDACis enhance the activation of the NKG2D pathway more efficiently.

HDACis treatment enhances CIK cell lysis of myeloma cells through NKG2D/NKG2D ligand interactions

Since the binding of MICA/B and ULBP2 ligands to NKG2D receptors causes antitumor cytotoxicity of NKT cells,[15] we investigated the functional relevance of increased MICA/B and ULBP expression in MM cells following HDACis therapy. Initially, we used a purified mouse antihuman NKG2D antibody to block the NKG2D receptor on CIK cells, employing $CD4^+$ T cells as a negative control group to assess the effectiveness of the blockade.[16] Subsequently, we observed that the NKG2D receptor on CIK cells was successfully blocked (Figure 5a). We examined CIK cells, isolated from healthy donors, against MM cells treated with or without HDACis, using the FACS cytotoxicity assay. We set up the lysis of NCI-H929 and U266 cell lines by CIK cells as the positive control (effector-to-target cell ratio – 5:1). MM cells treated with HDACis exhibited higher sensitivity to CIK cell lysis than untreated control cells. Furthermore, in the NCI-H929 cell line, only the enhancing effect of LBH589 was partially blocked by anti-NKG2D mAb pretreated CIK cells, which was statistically significant. In the U266 cell line, CIK cells pretreated with anti-NKG2D mAb significantly inhibited the potentiation by both LBH589 and romidepsin (Figure 5b). Our data suggest that the activation of NKT cells through NKG2D/NKG2D ligand interactions is a possible mechanism associated with the increased lysis of myeloma cells following HDACis treatment *in vitro* (Figure 5c).

DISCUSSION

The mechanisms of action of HDAC inhibitors, along with new data from preclinical experiments and clinical trials, have significantly broadened the range of cancers treatable with these compounds, especially MM. In clinical trials, whether HDACis were used alone or in combination therapies with other drugs, a plethora of knowledge exists about their relative success in MM. panobinostat, an orally administered HDACi, is also worth mentioning as it undoubtedly remains one of the best options for MM patients who require an additional therapeutic regimen, especially in relapsed or relapsed and refractory MM.[17] Nevertheless, the results of several preclinical evaluations with HDACis, including combination therapies in MM, suggest that they may be an interesting alternative to the established regimen.[3,18] The scenario is quite similar in case of CIK cell immunotherapy in the clinic,[10] which recently turned 30 years old and already has a history of successful clinical trials in MM. Surprisingly, HDACis have never been tested in combination with CIK cells in the clinical trials.

Herein, we tested clinically applicable HDACis (panobinostat/LBH589 and romidepsin) with CIK cells in genetically distinct MM cell lines (U266/male 53 years, RPMI8226/male 61 years, OPM-2/female 56 years, NCI-H929/female 62 years). The analysis showed panobinostat and CIK cells (effector-to-target cell ratio—10:1) increased specific lysis in all MM cell lines except NCI-H929. In contrast, the combination of romidepsin and CIK cells exerted this effect only in OPM-2. There was a significant increase in the proportion of cells with early apoptosis and late apoptosis or necrosis in U266 compared with the untreated group (control), suggesting that HDACis and CIK may together enhance apoptotic effects in specific MM cell lines. Romidepsin significantly increased IFN-γ secretion in all cell lines when used with CIK cells, whereas panobinostat showed this increase only in OPM-2. Likewise, changes in Granzyme B secretion were observed. Therefore, it is reasonable to speculate that intrinsic genetic/epigenetic factors may have a crucial role in a subset of patients when testing HDACis-CIK cells in the clinic. Since binding of

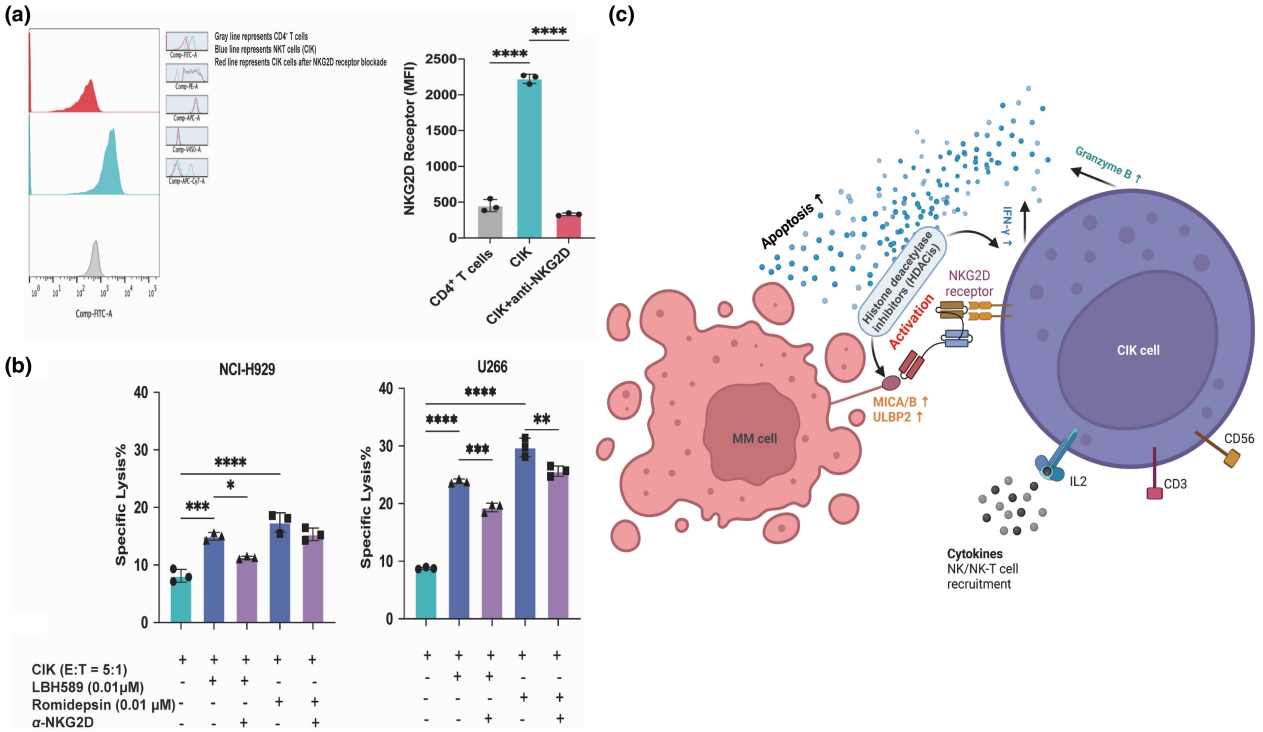

Figure 5. **(a)** Flow cytometry histogram of NKG2D receptor blocking experiment (grey line represents $CD4^+$ T cells, blue line represents cytokine-induced killer (CIK) cells, red line represents CIK cells after NKG2D receptor blockade), data are presented as mean ± SD. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ calculated by one-way ANOVA, Bonferroni's post hoc test). **(b)** CIK cells were incubated in the presence of anti-NKG2D Abs or medium alone for 60 min and then used for FACS cytotoxicity assay against histone deacetylase inhibitors (HDACis)-treated multiple myeloma (MM) cells at E/T 5:1. Data are presented as mean ± SD. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ calculated by two-way ANOVA, Bonferroni's post hoc test). **(c)** Schematic representation of a possible mechanism by which the effect of CIK cells may be enhanced by the addition of HDACis in MM. This schematic diagram was created with Biorender.com.

MICA/B and ULBP2 ligands to NKG2D receptors is well established to induce antitumor cytotoxicity of natural killer T cells,[15] we also investigated this axis and found that both HDAC inhibitors were able to significantly upregulate MICA/B and ULBP2 expression. Furthermore, we confirmed these results by blocking the NKG2D/NKG2D ligand interactions suggesting that it may be the activation of natural killer / natural killer T cells by NKG2D/NKG2D ligand interactions by which CIK cells potentiate enhanced lysis of myeloma cells in response to HDACis treatment. The evidence gleaned from these experiments strongly suggests a plausible mechanism underlying the observed phenomenon—namely the activation of natural killer and natural killer T cells via the NKG2D/NKG2D ligand interactions. This intricate cellular interplay is posited as the key contributor to the augmented lytic activity of CIK cells against myeloma cells following treatment with HDACis. The experimental design employed in this investigation not only validates the initial results but also advances our understanding of the intricate immunological processes at play during HDACis treatment. These insights contribute significantly to the broader comprehension of the cellular and molecular mechanisms orchestrating the anti-myeloma effects of CIK cells in response to HDACis, opening avenues for further exploration and therapeutic applications in the context of haematological malignancies. Since CIK cell immunotherapy has already showed promising results in MM clinical trials, in addition, approved in many countries, including Germany. Therefore, trials with CIK cells and certain inhibitors (e.g. HDACis) are quite feasible for MM patients. The same applies to HDACs for their modest efficacy as single agents in MM patients. Therefore, the

scenario for future MM research is quite straightforward. Beyond that, our supporting analyses reported here provide sufficient evidence to bring this clinically forgotten instance (HDACis-CIK cell combination) as a priority for MM treatment. The extent to which selective HDACi (e.g. ACY-1215/HDAC6) may be of benefit together with CIK cells compared with other non-selective HDACis could further help to refine treatment strategies in MM.

METHODS

Cell culture and HDACis

Genetically distinct MM cell lines (U266/ADM/male 53 years (RRID: CVCL_GZ72), RPMI8226/male 61 years (RRID: CVCL_0014), OPM-2/female 56 years (RRID: CVCL_1625), NCI-H929/female 62 years (RRID: CVCL_1600)) were used in this study. Cells were cultured in RPMI-1640 (Pan-Biotech, Aidenbach, Bavaria, Germany) medium supplemented with 10% FBS (Sigma-Aldrich Chemie GmbH, Munich, Germany) and 1% penicillin/streptomycin (Gibco, Schwerte, Germany) at 37°C, 5% CO_2, humidified atmosphere. All the cell lines were purchased from DSMZ (Braunschweig, Germany) and were mycoplasma-free, as tested by the mycoplasma detection kit (Thermo Fisher Scientific, Darmstadt, Germany). The control cell line CCD-18Co (RRID: CVCL_2379) (ATCC, Wesel, Germany; human colon fibroblasts) was cultured in Eagle's Minimum Essential Medium (ATCC) consisting of 10% FBS and 1% penicillin/streptomycin at 37°C, 5% CO_2. All the cell lines were authenticated by short tandem repeat profiling within 3 years prior to the research and regularly checked for mycoplasma contamination. Cells were cultured at 37°C, 5% CO_2.

Notably, panobinostat (LBH589, catalogue no. S1030, Selleck, Munich, Germany) and romidepsin (catalogue no. HY-15149, MedChemExpress), clinically relevant HDACis were used in the experiments. All drugs were used at different concentrations for 24–48 h.

Generation and phenotypic identification of CIK cells

For CIK cell generation, PBMCs were derived from buffy coats of healthy volunteers received from the Blutspendedienst at the University Hospital Bonn. We obtained approval from the ethics committee of the University Hospital Bonn, including signed informed consent from the volunteers. The CIK cells were cultured as described previously.[19] In brief, mononuclear cells were cultured in (10% FBS, 2.5% HEPES and 1% penicillin/streptomycin) RPMI-1640 with IFN-γ (ImmunoTools GmbH, Aidenbach) added on Day 0, 50 ng mL^{-1} of an antibody against CD3 (OKT, eBioscience, Thermo Fisher Scientific, Inc. San Diego, CA, USA), 100 U mL^{-1} IL-1β and 300 U mL^{-1} IL-2 (ImmunoTools GmbH, Aidenbach) were added 24 h later and cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and subcultured every 3 days in fresh complete medium containing IL-2 at 3×10^6 cells mL^{-1}. The CIK cells were harvested, and assays were performed at maturity after between 3 and 4 weeks of culture. At the end of expansion, CIK cells were harvested to determine phenotype by flow cytometry (FACS Canto II flow cytometer, BD Biosciences, Heidelberg, Germany). Cells were stained with FITC-CD3, FITC-NKG2D, APC-CD3, APC-CD56, PE-CD56, APC-Cy7-CD4 and corresponding isotype antibodies. The Hoechst 33258 dye (Cayman Chemical, Hamburg, Germany) was added before flow cytometry analysis to gate out intact viable cells. Samples were acquired using FACS Canto II flow cytometer.

CCK8 assay

Cells were seeded at a density of 5×10^3 to 10×10^4 per well in 96-well plates. They were then treated with varying concentrations of HDACis for 24–72 h before the addition of the Cell Counting Kit-8 (CCK-8) reagent. Following a further incubation period of 24–72 h, the plates were centrifuged for 4 min at 200 *g*, and 10 μL of CCK-8 reaction solution (product code: DJDB4000X, Dojindo Molecular Technologies, Inc.) was added. After 3–4 h, a colorimetric analysis was conducted using a microplate reader (Infinite® 200 PRO, Tecan) at 450 nm. The percentage inhibition of cell growth was calculated to determine the half-maximal inhibitory concentration (IC50) using Prism 5 (GraphPad Software, USA).

LDH assay

A commercial CyQUANT™ LDH Cytotoxicity Assay Kit (Thermo Fisher, Waltham, MA, USA) was used according to the manufacturer's instructions. Effector cells (CI K cells) were co-cultured with target cells (MM cells) in 48-well plates at effector-to-target (E:T) ratios of 1:1, 5:1, 10:1. Then, the released LDH absorbance was measured using a microplate reader at 490 and 680 nm. At the end of incubation, 25 μL of each sample was transferred to a 96-well flat-bottom plate in different wells, and 25 μL of the reaction mixture was added to each well. To calculate LDH activity, subtract the absorbance value at 680 nm from that at 490 nm. All experiments were performed in triplicates. Experiments were replicated two times with CIK cells from two different donors. In order to calculate % cytotoxicity, the following equation was applied to the corrected values:

$$\%\ \text{Relative cytotoxicity} = (\text{Experimental value} - \text{Effector cells spontaneous control} - \text{Target cells spontaneous control}) / (\text{Target cells maximum control} - \text{Target cells spontaneous control}) \times 100$$

FACS cytotoxicity assay

For *in vitro* cytotoxicity assessment, a flow cytometry-based assay was performed as described previously[20] with some modifications. 4×10^6 tumor cells were labelled with 1.25 μM CFSE (Thermo Fisher Scientific, Eugene, USA) in 1 mL PBS for

20 min at 37°C in the dark. This was followed by two washes with 5 mL culture medium (containing 10% FBS) to remove excess CFSE dye. Next, an equal number of cells (5×10^4 per well) were co-cultured with CIK cells at different E:T ratios in 48-well round-bottom plate at 37°C, 5% CO_2. 0.01 μM drug was added into each well at the time of cytotoxicity assay. For NKG2D blocking experiments, CIK cells were incubated with purified mouse antihuman NKG2D antibody (clone 1D11, IgG1, Santa Cruz Biotechnology) or normal mouse control IgG (Santa Cruz Biotechnology) at 10 μg mL^{-1} 1 h before co-culture with tumor cells. After 20 h of co-incubation, cells were stained with the Hoechst 33258 dye and measured by FACS Canto II (BD). At least 10 000 CFSE-labelled tumor cells were collected in each sample. The following formula was employed for cytotoxicity calculation:

$$\text{Specific lysis } (\%) = \left(\frac{\text{CT}-\text{TE}}{\text{CT}}\right) \text{X } 100,$$

where CT is the absolute number of live CFSE-labelled tumor cells in control tubes (target cells alone) and TE is the absolute number of live CFSE–labelled tumor cells in test tubes (target cells and effector cells).

Enzyme-linked immunosorbent assay (ELISA)

IFN-γ production by CIK cells

CIK cells were co-cultured with myeloma cells (5×10^4) at a ratio of 20:1 for 24 h in 96-well flat-bottom plates, either without treatment or in the presence of 0.01 μM panobinostat or romidepsin. At the end of the culture period, the plates were centrifuged for 5 min at 300 *g*. A volume of 100 μL of cell-free supernatant were collected for Elisa assay using the IFN-γ kit from Invitrogen (Camarillo, CA, USA), following the manufacturer's instructions. Absorbance was measured with an ELISA reader (Infinite® 200 PRO, Tecan) at 450 and 570 nm wavelengths.

Granzyme B production by CIK cells

The co-culture method of tumor cells and CIK cells was the same as before. After 24 h of culture, the cell-free supernatant was harvested, and the level of granzyme B was determined using the Human Granzyme B Elisa kit (Duoset DY2906-05 and DY008, R&D Systems, Inc., Minneapolis, MN, USA) following the manufacturer's instructions. Absorbance was measured with an ELISA reader (Infinite® 200 PRO, Tecan) at wavelengths of 450 and 570 nm.

MICA shedding by MM cells

5×10^4 MM cells per well were cultured in 48-well plates with 200 μL complete medium without treatment or in the presence of 0.01 μM panobinostat or romidepsin. After 48 h of culture, cell-free supernatant was harvested and the level of soluble MICA was determined using MICA Elisa kit (Duoset DY1300 and DY008, R&D Systems, Inc. Minneapolis, MN, USA) following the manufacturer's instruction. Absorbance was measured with an ELISA reader (Infinite® 200 PRO, Tecan) at 450 and 570 nm wavelengths.

Flow cytometry assay on apoptosis

To investigate the combination of HDACis with CIK cells on the apoptosis of the U266 cell line, 4×10^6 tumor cells were labelled with 1.25 μM Violet dye (Thermo Fisher Scientific, Eugene, USA) in 1 mL PBS for 20 min at 37°C in the dark. This was followed by two washes with 5 mL culture medium (containing 10% FBS) to remove excess Violet dye. Subsequently, a common number of cells (5×10^4 per well) were co-cultured with CIK cells at the E:T ratio of 20:1 in a 48-well round-bottom plate at 37°C with 5% CO_2. panobinostat or romidepsin at a concentration of 0.01 μM was added to each well. After 20 h of co-incubation, cells were stained with the FITC Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend, catalogue no: 640922) and measured by FACS Canto II. At least 10 000 Violet-labelled tumor cells were collected in each sample.

RT qPCR detection of NKG2D ligands RNA expression level

RNA extraction and quantitative polymerase chain reaction (qPCR) are used for isolating RNA from biological samples and amplifying specific RNA sequences to analyze gene expression levels, respectively. RNA isolation of MM cell line (OPM-2, U266 and NCI-H929) samples was performed with RNeasy Plus Mini Kit (QIAGEN, Germany, catalogue no: 74136). Complementary DNA was synthesised by reverse transcription using HiFiScript Kit (Invitrogen, USA). Quantitative polymerase chain reaction was performed on NKG2D ligands (MICA, MICB, ULBP1, ULBP2, ULBP3 and ULBP4) using P PowerTrack™ SYBR Green Mastermix (Thermo Fisher Scientific, catalogue no: A46109). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as the internal reference gene. The primer sequences are shown in Supplementary table 2. The Δ–Δ Ct ($2^{-\Delta\Delta Ct}$) approach was used to measure the relative expression levels of target genes, which were standardised against GAPDH mRNA levels, respectively, for NKG2D ligands mRNA expression.

Surface expression of NKG2D ligands on MM cells

MM cells (1×10^5 per well) were cultured in 96-well round-bottom plates at 37°C, 5% CO_2. For co-culture, 0.01 μM panobinostat or romidepsin was added to each well. After 24 h of culture, MICA/B and ULBP2 on the cell surface were detected following staining with PE-conjugated anti-MICA/B antibody and PE-conjugated anti-ULBP2 antibody (R&D systems). Prior to the staining process, Fc receptors were blocked with human TrueStain FcX™ (BioLegend, Koblenz, Germany) at a final dilution of 1:100. The Hoechst 33258 dye was added before flow cytometry analysis for viable cells gating. Samples were acquired using FACS Canto II (a flow cytometry system).

Statistical analysis

FACS data were analysed using FlowJo V10.4 software (LLC, Ashland, Oregon, USA). Statistical analysis was performed using GraphPad Prism (version 8.0). Experimental data are presented as means ± SD. One-way or two-way analysis of variance (ANOVA) with Bonferroni's post hoc test was performed to analyse statistical significance. $P < 0.05$ was considered to be statistically significant.

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AUTHOR CONTRIBUTIONS

Jingjing Pu: Conceptualization; funding acquisition; investigation; methodology; visualization; writing – original draft; writing – review and editing. **Amit Sharma:** Conceptualization; funding acquisition; investigation; methodology; visualization; writing – original draft; writing – review and editing. **Ting Liu:** Conceptualization; investigation; methodology; visualization; writing – review and editing. **Jian Hou:** Conceptualization; resources; writing – review and editing. **Ingo GH Schmidt-Wolf:** Conceptualization; funding acquisition; resources; supervision; writing – review and editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data needed to evaluate the conclusions in the paperware present in the paper and/or the Supporting Information. The data sets generated during and/or analysed during the current study are available from the corresponding author on request.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

3.2 Publication 2: Exploring the role of histone deacetylase and histone deacetylase inhibitors in the context of multiple myeloma: mechanisms, therapeutic implications, and future perspectives

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REVIEW Open Access

Exploring the role of histone deacetylase and histone deacetylase inhibitors in the context of multiple myeloma: mechanisms, therapeutic implications, and future perspectives

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Abstract

Histone deacetylase inhibitors (HDACis) are a significant category of pharmaceuticals that have developed in the past two decades to treat multiple myeloma. Four drugs in this category have received approval from the U.S. Food and Drug Administration (FDA) for use: Panobinonstat (though canceled by the FDA in 2022), Vorinostat, Belinostat and Romidepsin. The efficacy of this group of drugs is attributed to the disruption of many processes involved in tumor growth through the inhibition of histone deacetylase, and this mode of action leads to significant anti-multiple myeloma (MM) activity. In MM, inhibition of histone deacetylase has many downstream consequences, including suppression of NF-κB signaling and HSP90, upregulation of cell cycle regulators (p21, p53), and downregulation of antiapoptotic proteins including Bcl-2. Furthermore, HDACis have a variety of direct and indirect oxidative effects on cellular DNA. HDAC inhibitors enhance normal immune function, thereby decreasing the proliferation of malignant plasma cells and promoting autophagy. The various biological effects of inhibiting histone deacetylase have a combined or additional impact when used alongside other chemotherapeutic and targeted drugs for multiple myeloma. This helps to decrease resistance to treatment. Combination treatment regimens that include HDACis have become an essential part of the therapy for multiple myeloma. These regimens incorporate drugs from other important classes of anti-myeloma agents, such as immunomodulatory drugs (IMiDs), conventional chemotherapy, monoclonal antibodies, and proteasome inhibitors. This review provides a comprehensive evaluation of the clinical efficacy and safety data pertaining to the currently approved histone deacetylase inhibitors, as well as an explanation of the crucial function of histone deacetylase in multiple myeloma and the characteristics of the different histone deacetylase inhibitors. Moreover, it provides a concise overview of the most recent developments in the use of histone deacetylase inhibitors for treating multiple myeloma, as well as potential future uses in treatment.

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Introduction

Multiple myeloma (MM) is a hematologic malignancy defined by the development of aberrant clonal plasma cells in the bone marrow, which can cause severe bone lesions, renal damage, anemia, and hypercalcemia [1]. MM is most prevalent in industrialized countries, particularly in Australia, Western Europe, and the United States, where it has the greatest prevalence [2]. It is the second most common hematologic malignancy in the United States, accounting for around 1.8% of all cancers and approximately 10% of hematologic malignancies [3]. In 2022, according to the American Cancer Society, about 34,470 new MM cases will be diagnosed in the United States, with an estimated 12,640 deaths [4]. MM is a neoplasm of older adults, with the median age of diagnosis in the United States being 69, and the median age of death is 75. Globally, men are around 1.5 times more likely than women [5]. Although recent therapies have led to a significant increase in the illness's 5-year survival rate, which now exceeds 5 years, and have improved the quality of life for patients, it is important to note that the condition is still incurable [6].

The notable enhancements in results have been correlated with the extensive utilization of autologous stem cell transplantation (ASCT) as a customary practice for eligible patients [7], along with the advancement and authorization of many innovative medications and treatment plans for managing MM [8]. In the past twenty years, various new types of drugs have been developed, including proteasome inhibitors, immunomodulatory drugs, monoclonal antibodies (mAbs), antibody–drug conjugates (ADC), bispecific T-cell engagers (BiTE), chimeric antigen-T-cell therapy (CAR-T), peptide-drug conjugates, selective inhibitors of nuclear export, and small-molecule targeted therapies [9]. With the introduction of these new treatments, treatment paradigms for MM patients have evolved as well, by employing more intricate methods, such as the use of triple therapy as opposed to dual therapy, and the increased implementation of continuous or long-term treatment, patient results can be improved. Nevertheless, the effectiveness of these treatments is frequently compromised by the emergence of resistance and the occurrence of relapse, thereby emphasising a significant deficiency in the therapy continuum [10–12]. Hence, the significance of novel therapeutic approaches for multiple myeloma cannot be overstated.

Over the last two decades, histone deacetylases (HDACs) have emerged as important therapeutic targets in cancers, particularly multiple myeloma [13, 14]. HDACis have gained significant interest as they target HDAC, which have been identified as crucial in the development of new therapy approaches for this specific condition. The fact that HDACis reduce multiple myeloma cell survival and proliferation through different mechanisms has contributed to their effectiveness. As it turns out, many HDACis have been used and evaluated in both preclinical and clinical contexts. Significantly, the FDA has granted approval to four HDACis: Vorinostat, Romidepsin, Panobinostat, and Belinostat. These HDACis are mostly utilized in clinics for hematologic tumors with less severe side effects [15]. These drugs' clinical data will be summarized later in this study.

This review provides a comprehensive analysis of the crucial role of HDACis in MM, as well as the clinical evaluation of different HDACis. It focuses on the many consequences of inhibiting histone deacetylation in MM and examines the justification for using HDACis in conjunction with medications or immunotherapies that target other pathways, with the goal of enhancing their effectiveness. Furthermore, it examines the mechanisms behind resistance to histone deacetylation inhibition and explores potential strategies to overcome this resistance through combination treatment.

In the end, it offers an in-depth review of the clinical effectiveness and safety data for treatments based on HDACis in various treatment scenarios for MM, highlighting the significance of these drugs as the primary form of treatment for MM.

Rationale for targeting HDACs in MM

Based on homology to yeast HDAC, subcellular localization, and noncellular enzymatic activity, the 18 HDAC isoforms in humans are divided into four groups, classes I (HDAC1, HDAC2, HDAC3, HDAC8), Class IIa (HDAC4, HDAC5, HDAC7, HDAC9), Class IIb (HDAC 6, DAC10), Class III (SIRT1-SIRT7), and Class IV (HDAC11) (Fig. 1a) [16–19]. Class I, II, and IV HDACs possess a deacetylase domain that relies on the presence of Zn^{2+}, while class III HDACs contain a deacetylase domain that depends on the presence of NAD^{+}. Class I members exhibit widespread expression, with nuclear localization being the predominant pattern. They also have an N-terminal catalytic domain and are made up of about 400 amino acids. Their catalytic domain is formed

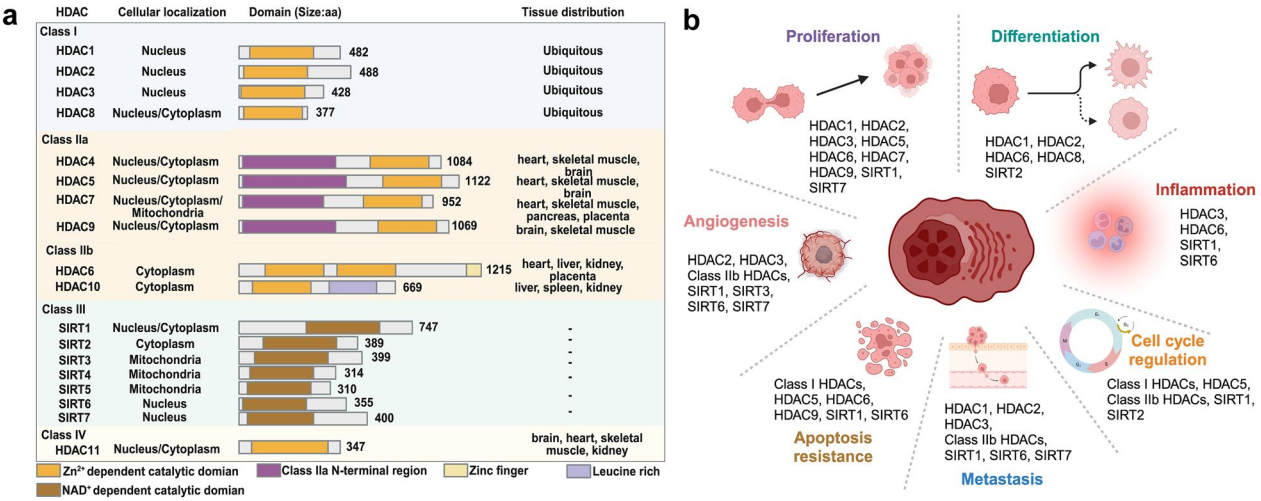

Fig. 1 **a** Classification of HDAC family; **b** The role of HDACs in MM. Figure created with BioRender.com

by two neighboring histidine residues, two aspartic acid residues, and a tyrosine residue centered on a Zn^{2+} ions [20, 21]. Class II members exhibit enhanced specificity in expression and possess the ability to actively transport between the nucleus and the cytoplasm. Class IIa HDACs consist of 600–1200 amino acids and possess an N-terminal regulatory domain that enables interactions with tissue-specific transcription factors and corepressors [22, 23]. In the C-terminal region of Class IIb HDACs, there is another catalytic domain and a ubiquitin-binding zinc finger domain, respectively [24, 25]. The sirtuin deacetylase family (SIRT1-7) belongs to class III, however they are not functionally linked to HDAC; their deacetylase activity is based on NAD^{+} rather than Zn^{2+}-dependent enzymes [26]. HDAC11, the sole member of the class IV HDAC family, is mostly found in the nucleus. The majority of its amino acid sequence is dedicated to its catalytic domain [27].

HDAC biology

HDACs have a crucial function in controlling gene expression by altering the acetylation state of histones, which are proteins involved in the packaging and organization of DNA in the cell nucleus [28, 29]. In the context of MM, HDACs have been associated with several facets of the disease (Fig. 1b), including cell cycle regulation [30], apoptosis resistance [31], and interactions with the tumor microenvironment (Proliferation, differentiation, inflammation, metastasis, angiogenesis) [32–34]. Notably, endothelial cells play a crucial role in the process of angiogenesis, which involves the development of new blood vessels. This process is essential for the growth and dissemination of tumors. In the microenvironment of MM, these cells undergo alterations in their properties and concurrently promote angiogenesis, thereby expediting the advancement of the disease and the development of medication resistance. HDACis have become a prominent inhibitory factor in this process by compromising the activities of endothelial cells and affecting the blood supply network of the tumor [35, 36]. Their mechanism of action involves the inhibition of HDACs, which induces alterations in gene expression in endothelial cells, ultimately leading to anti-angiogenic effects [37]. The integration of endothelial cell targeting and angiogenesis in the treatment of MM is a promising approach to overcome drug resistance and improve therapeutic results.

HDACs, as a whole, facilitate the elimination of acetylation from lysine residues in target proteins [30, 38]. They play a critical role in regulating cell function, not only by removing acetyl groups from lysine residues on core histones, leading to tighter chromatin and reduced gene expression [14], but also by deacetylating non-histone proteins such as the tumor suppressor p53 [39–41], STAT3 [42], HSP90 [43], and NF-κB [44]. This action significantly affects these proteins' function, interactions, and stability, influencing various cellular activities [45] (Fig. 2). In MM, this regulation becomes particularly important. The constant activation of the NF-κB pathway [46] and other cancer-promoting mechanisms leads to fast cell growth and a supportive environment in the bone marrow [47]. This creates a cycle that helps MM cells survive and multiply. HDACis can break this cycle. They change the acetylation pattern of both histone and non-histone proteins, which impacts chromatin structure, gene activity, and critical signaling pathways, such as NF-κB, PI3K/AKT/mTOR, and MAPK [48, 49]. As we mentioned before, by also affecting the tumor environment and promoting cell

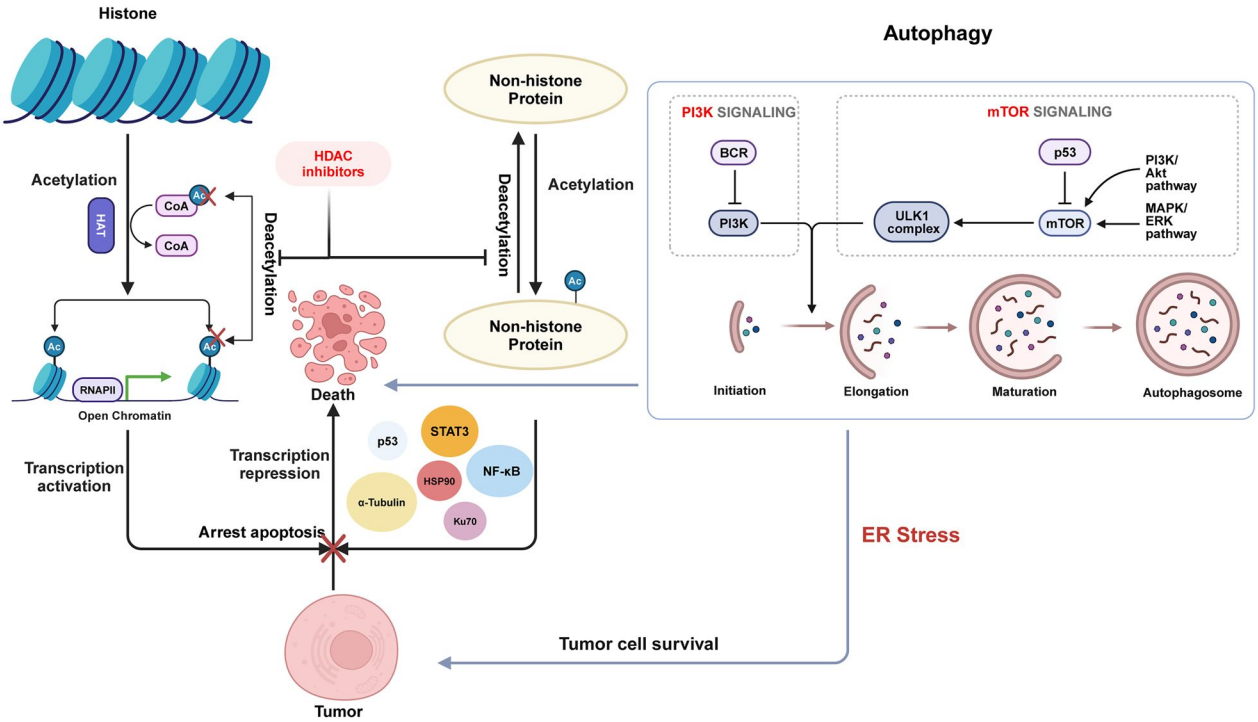

Fig. 2 Acetylation of lysine in histone and non-histone proteins. Histone acetylation causes a loose chromatin structure, which causes gene expression. Additionally, the double-edged sword role of autophagy in tumor development and progression. Figure created with BioRender.com

death and cell cycle arrest, HDACis show strong potential against MM. Their ability to target both epigenetic and non-epigenetic factors highlights their promise in MM treatment, especially when used alongside other therapies [30]. Moreover, autophagy, an essential cellular mechanism responsible for the degradation and recycling of impaired organelles and proteins, assumes a multifaceted and ambivalent role in the pathology of MM [40, 50, 51]. This process facilitates cellular survival under conditions of stress by provisioning vital nutrients and energy, thereby contributing to the development of drug resistance. Conversely, aberrant or excessive autophagy may precipitate cellular demise, potentially amplifying the efficacy of anti-cancer therapeutics [52] (Fig. 2). HDACis are observed to modulate autophagy within MM cells through a bifurcated mechanism: initiating protective autophagy that favors cellular survival or provoking cytotoxic autophagy, culminating in cellular mortality [53, 54]. This comprehensive approach aims to disrupt the key cellular processes that MM cells rely on to survive and grow. In conclusion, factors such as autophagy, drug resistance, and endothelial cells are interrelated factors that influence the efficacy of MM treatment [55], especially in the context of HDAC inhibition. Understanding the complex interplay between these factors can help guide the development of new treatment strategies and improve outcomes for patients with MM.

HDAC inhibitors

A range of HDACis have been investigated in the context of malignancies. HDACis are categorized into six types based on their chemical structure. Short-chain fatty acid, hydroxamic acid, benzamide, cyclic peptide, mercaptoketone, sirtuin inhibitors, and other compounds [56, 57] (Table 1). Non-selective HDACis have the ability to inhibit various HDAC isoforms. However, previous research has shown that the primary focus of clinically important HDACis are HDAC 1, 2, 3, and 6. These findings indicate that the primary mechanism behind the anti-tumor properties of non-selective HDACis is the inhibition of class I and class IIb HDAC enzymes [58]. Clinical development of HDACis continues benefiting a growing number of patients with RRMM. Among them, Panobinostat (LBH589) is a strong non-selective oral pan-histone deacetylase inhibitor with efficacy in myeloma patients [59]. Panobinostat was approved by the FDA in 2015 to treat RRMM based on promising preclinical and clinical research. However, it was withdrawn in the United States in March 2022 (Fig. 3). As clinical studies progress, an increasing number of HDACis are becoming viable options for treating RRMM.

Table 1 Characteristics of HDACis in MM (selected)

Chemical class	Drug name	Approved by the FDA	In Phase I/II/III clinical trials	Reported targets (HDAC)	Type of cancer targeted against
Short-chain fatty acid	Valproic acid (VPA)		III	Class I, IIa	Cervical and ovarian
	Sodium butyrate		II	Class I, IIa	Colonic cancer
	Penyl butyrate		II	Class I, IIa	Urea cycle disorders
Hydroxamic acid	LBH589 (Panobinostat)	Approved for MM in 2015 (withdrawn in 2022)	III	Class I, II, IV	MM and CTCL
	Trichostain-A (TSA)		Preclinical	Class I, II, IV	Cervical and hepatoma
	SAHA (Vorinostat)	Yes (USA) Approved for CTCL	I/II	Class I, II, IV	CTCL
	JNJ-26481585 (Qusinostat)		I/II	Class I, II, IV	RRMM and solid tumors
	ITF2357 (Gavinostat)		II	Class I, II	Refractory leukemia and RRMM
	PXD101 (Belinostat)	Yes (USA) Approved for PTCL		Class I, II, IV	PTCL and RRMM
	NVP-LAQ824 (Dacinostat)		I	Class I, II	NSCC and colonic cancer
	Suberoylanilide bis-hydroxamic acid (SBHA)			Class I	Melanoma and sarcoma
	RAS2410 (Resminostat)		I/II	Class I, II	Hodgkin lymphoma and HCC
	ACY-1215 (Rocilinostat)		I	HDAC6	RRMM
	CR-2408			Class I, II, IV	MM
	Practinostat		II	Class I, II, IV	Prostate cancer
	CHR-3996 (Nanatinostat)		I	Class I	Refractory metastatic solid tumors
Benzamide	MGCD-0103 (Mocetinostat)		II	Class I, IV	Hodgkin lymphoma
	SNDX-275 (MS-275, Entinostat)		II/III	Class I	Leukemia, colorectal, gastric, pancreatic, lung, ovarian, MM
	CI-994 (Tacedinaline)		III	Class I	Pancreatic cancer, NSCC, MM, leukemia
	4SC-202 (Domatinostat)		I	Class I	Advanced hematological malignancies
	Chidamide (Tucidinostat)	Yes (China)	II	Class I, IIb	Solid tumors, PTCL, MM and ATLL
Cyclic peptide	Depsipeptide (FR901228, FK228, Romidepsin)	Yes (USA) Approved for CTCL	II	Class I	CTCL and RRMM
	Apicidin			Class I	Melanoma and leukemia
Mercaptoketone	KD5170			Class I, II	MM
Sirtuins inhibitors	Nicotinamide		III	Class III	Laryngeal cancer
	Sirtinol		Preclinical	SIRT1, II	
	Cambinol		Preclinical	SIRT1, II	
	EX-527		Preclinical/I/II	SIRT1, II	Huntington disease, glaucoma
Others	Tubacin			HDAC6	MM
	ACY-241 (Citarinostat)		Ib	HDAC6	MM

MM: multiple myeloma, RRMM: relapsed/refractory multiple myeloma, CTCL: cutaneous T-cell lymphoma, PTCL: peripheral T-cell lymphoma, NSCC: non-small cell lung carcinoma, HCC: hepatocellular carcinoma, ATLL: adult T-cell leukemia-lymphoma

For instance, Qusinostat, Gavinostat and Rocilinostat employed exclusively in the management of solid tumors and refractory leukemia, demonstrate potential efficacy in the treatment of RRMM [60, 61].

Mechanisms of action of HDACis

HDACis work in several ways to prevent myeloma cell survival and growth. Cancer cells, particularly MM cells, exhibit cell cycle disruption, resulting in accelerated cell proliferation. Non-selective HDACis or class I HDACis cause G0/G1 cell cycle arrest by upregulating cell cycle regulators, such as p21 (WAF1) [62, 63] and p53 [64, 65], or downregulation of antiapoptotic proteins such as Bcl-2 [66]. HDACis facilitate the restoration of regular immunological function, leading to a reduction in the excessive growth of malignant plasma cells. Furthermore, HDACis

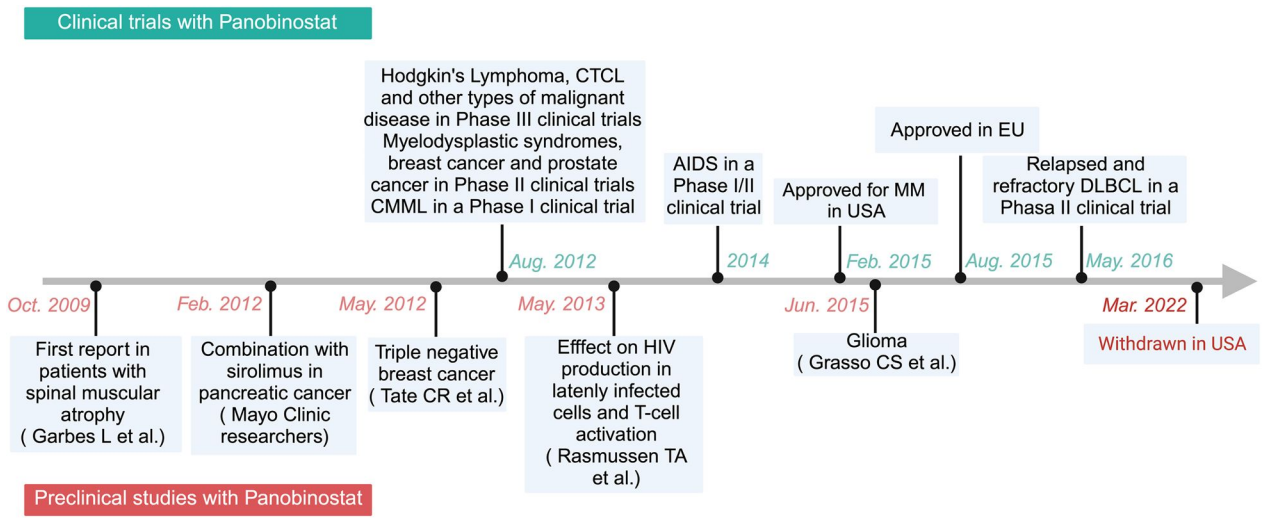

Fig. 3 Highlights in the development of panobinostat which was firstly approved by the FDA to treat RRMM. Figure created with BioRender.com

exert various direct and indirect effects on cellular DNA, resulting in oxidative damage [67]. They induce mitotic delays by bypassing the spindle assembly checkpoint. In our recent exploration, we uncovered the reciprocal relationships between the epigenetic machinery and the non-coding genome in the control of gene expression. This involved delving into the fascinating connections between HDAC6-induced lncRNA and its prospective sponge miRNA in the context of MM [68]. Simultaneously, as discussed in Section "HDAC biology", Heat-shock protein 90 (HSP90), a cellular chaperone essential for proteins involved in intracellular signaling (Her2/neu, Raf, ERK, NF-κB), is likewise inhibited by HDACis [69, 70]. For instance, the protein Hsp90, which acts as a molecular chaperone, is affected by the process of deacetylation carried out by HDAC6. Various pieces of evidence indicate that inhibiting both HDAC6 and Hsp90 at the same time leads to enhanced anti-tumor effects on various cancer cell lines. This emphasizes the advantages of creating a single compound that can target multiple molecules simultaneously [71]. As such, dual-targeting strategies against histone deacetylase are designed to enhance therapeutic efficacy while minimizing the side effects associated with broad-spectrum HDAC inhibition.

Synergy with and resistance to HDAC Inhibition

Suppressing histone deacetylase has several effects that result in increased efficacy when combined with other chemotherapeutic and targeted therapies in MM, either via synergy or addition. Previous studies have shown that either panobinostat or vorinostat anticancer effects were increased in preclinical trials in patients with RRMM when combined with proteasome inhibitors such as bortezomib [72–76]. Both of them exhibit a synergistic impact in restraining cell proliferation and enhancing programmed cell death in MM cells [77]. The investigation further revealed that the co-administration of tubacin, a selective inhibitor of HDAC6, with bortezomib elicited a comparable outcome, concomitant with a notable augmentation in polyubiquitinated proteins [78]. In addition, the synergistic effect of panobinostat and romidepsin combined with proteasome inhibitors was also found in the MM cell mouse xenograft models in vivo [79, 80].

The strategy of combining therapies to overcome resistance to HDACis has been demonstrated to occur through multiple mechanisms [72]. The concurrent suppression of the proteasome and aggresome pathways is the most extensively studied manifestation of synergy between proteasome inhibitors and HDACis [81, 82] (Fig. 4). The convergence of bortezomib, a proteasome-targeting agent, with an HDAC6 inhibitor, specifically directed at aggregates within tumor cells, engenders heightened accumulation of polyubiquitinated proteins, consequently inducing increased cellular stress and death [81, 82]. In particular, proteasome inhibition promotes aggregation formation, which is dependent on HDAC6 interactions with tubulin and dynein complexes. Furthermore, both proteasome inhibitors (bortezomib) and HDAC6 inhibitors (tubacin or panobinostat) enhance tubulin hyperacetylation and polyubiquitinated protein synthesis, which increases cellular stress responses and leads to autophagy and apoptosis. This is partly determined by caspase activity [81, 82]. The potential overcoming of resistance mechanisms in multiple myeloma may be achieved through the synergistic combination

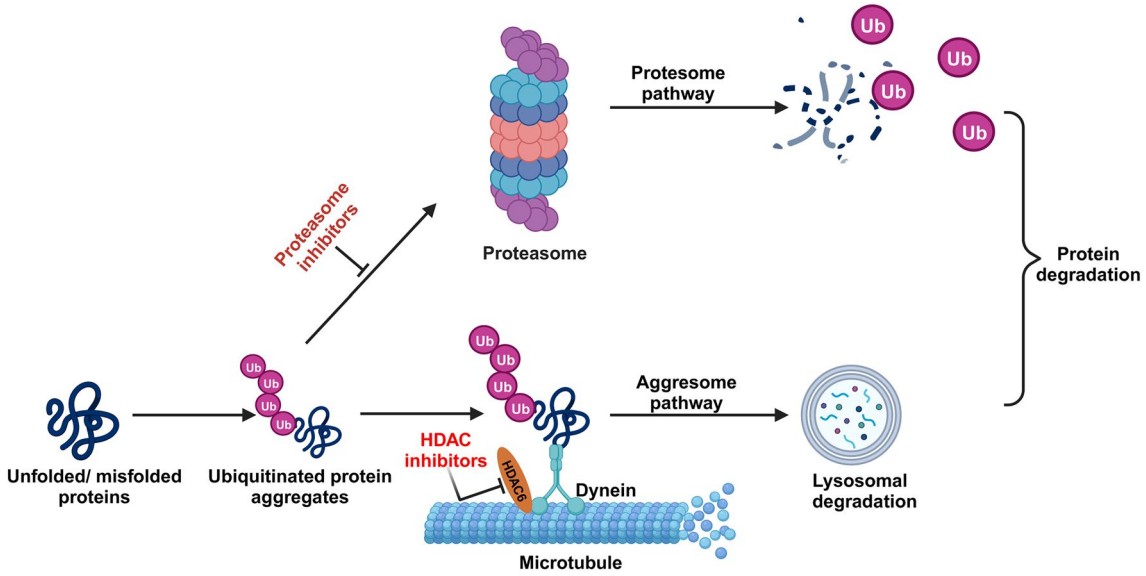

Fig. 4 Aggresome pathway and synergy with proteasome inhibitors. Ubiquitin targets unfolded and/or misfolded proteins for destruction via the proteasome and aggresome pathways. Inhibiting proteasome pathways with inhibitors like bortezomib or carfilzomib results in the formation of ubiquitin protein aggregates, which are subsequently shuttled to the lysosome and destroyed via the aggresome pathway. Protein aggregates migrate across microtubules utilizing the dynein motor protein in the aggregation process. HDAC-6 promotes protein aggregate/microtubule complexes. If histone deacetylase (HDAC) is inhibited (together with proteasome inhibitors) at this moment, ubiquitin protein aggregates would develop further, resulting to apoptosis. If histone deacetylase (HDAC) is inhibited (together with proteasome inhibitors) at this moment, ubiquitin protein aggregates would develop further, resulting to apoptosis. Figure created with BioRender.com

of HDAC inhibitors with other active agents possessing diverse mechanisms of action within the context of MM, or by incorporating novel targeted agents specifically designed to address resistance pathways, allowing the persistent use of histone deacetylase inhibition as the mainstay of the entire course of treatment.

Clinical outcomes of HDACis in MM

Numerous studies have established the applicability of histone deacetylation inhibitors in the treatment of MM during the course of more than a decade of continuous development of HDACis. Since the FDA approved some nonselective HDACis for the treatment of MM, a growing number of HDACis have become the cornerstone of overall MM treatment and are now or are being studied as an option for induction, consolidation, and maintenance therapy, as well as a single agent or in multiple highly effective combination regimens in RRMM. Here, we summarized clinical trials involving HDACis used alone, combined with dexamethasone, immunomodulatory drugs (IMiDs), traditional chemotherapy, and novel targeted agents. It is worth noting that recent advancements in the development of HDAC inhibitors for cancer treatment are geared towards specificity and improved outcomes. Innovations include the development of class I HDAC inhibitors [83], targeting enzymes frequently overexpressed in tumors to reduce growth and offer better therapeutic options. CN133, a promising HDAC inhibitor, showcases high selectivity for class I HDACs and improved penetration into prostate tissue, hinting at enhanced efficacy in prostate cancer treatment, particularly in combination therapies [84]. Additionally, research into HDAC10 targeting has led to the creation of specific inhibitors, like Tubastatin A and its analogues, aiming for precise action against HDAC10, which is linked to poor prognosis in neuroblastoma [85]. These efforts represent a move towards more targeted cancer therapies with the potential for fewer side effects in treating MM.

Monotherapy in MM

Wolf et al. [86] conducted a Phase II research (NCT00445068) with 38 patients with RRMM. The study used a dose of Panobinostat at 20 mg, administered three times a week, on a weekly basis within 21-day cycles. Prior to this, patients had undergone a minimum of two therapy regimens, which involved the use of an IMiD (thalidomide or lenalidomide) and bortezomib. The overall activity was deemed to be low, as seen by one partial reaction and one minimum response. Both of these responses exhibited excellent durability, lasting for 19 and 28 months, respectively. However, the trial was ended owing to insufficient efficacy. More than 80% of patients had gastrointestinal adverse events (AEs), with the bulk of these occurrences classified as

grade 1–2. The most common grade 3–4 occurrences were related to blood disorders, including neutropenia, thrombocytopenia, and anaemia. Additionally, 26% of the patients reported experiencing fatigue. A Phase Ia/II dose-escalation study of oral Panobinostat was conducted on 176 patients with hematologic malignancies, including 12 with RRMM, as part of another clinical trial (NCT00621244) [87]. The doses of Panobinostat ranged from 20 to 80 mg in two different dose-escalation regimens, either administered three times per week or once every two weeks. In Phase II, the prescribed dosage for MM was 40 mg administered on a weekly basis. The maximum acceptable dose, on the other hand, was Panobinostat 60 mg given every two weeks. Coincidentally, one RRMM patient responded somewhat like adverse events, particularly gastrointestinal and hematologic AEs, were similar with those found in earlier studies. This trial confirmed overall safety and guided dosage for further monotherapy and combo treatment. In addition to Panobinostat, Vorinostat (NCT00045006), ITF2357 (NCT00792506), Entinostat (NCT00015925), Tacedinaline (NCT00005624), Domatinostat (NCT01344707) and Romidepsin (NCT00066638) were also used in monotherapy clinical trials. In summary, while Panobinostat has shown some efficacy as a monotherapy in treating MM, its clinical benefits are more pronounced and better supported when used in combination with other therapies. The management of multiple myeloma remains complex, requiring a multidisciplinary approach to optimize patient outcomes (Table 2).

Doublet combination therapy with dexamethasone

The preclinical research demonstrated the synergistic effects of HDACis in combination with bortezomib and dexamethasone in MM cell lines. Additionally, the safety data from monotherapy provided a foundation for conducting combination studies (Table 2). These trials ultimately resulted in the accelerated approval of the treatment regimen [82]. In a phase II study (NCT01720875) [88], 16 MM patients, previously treated once, received a regimen of bortezomib, dexamethasone, and vorinostat, showing an 81.3% overall response rate with 100% clinical benefit. Despite a median progression-free survival of 11.9 months and maintenance treatment with vorinostat, 75% of the participants required dose adjustments or discontinued treatment due to side effects. The findings reveal that, although toxicity and dosage reductions were challenges, this combination therapy is effective in treating relapsed myeloma. This success underscores the importance of continuing to refine HDAC inhibitor-based combinations, aiming to improve both their tolerability and efficacy for myeloma treatment. Between July 2012 and August 2015, a study (NCT01583283) enrolled 38 patients to test ricolinostat [89]. Yee et al. found ricolinostat to be mostly safe, with the best dose determined as 160 mg once daily for 21 days in a 28 day cycle, combined with two other medications. The most common side effects were mild to moderate fatigue and diarrhea. The drug effectively inhibited its target enzyme without significantly affecting other enzymes, and its effectiveness wasn't compromised when taken with the other medications. In early assessments, 55% of patients showed a positive response to the treatment, suggesting ricolinostat could be a promising option for patients with RRMM. The studies (NCT01502085 and NCT00642954) explored a new combination therapy of vorinostat, lenalidomide, and dexamethasone for treating MM, based on promising lab research. It was a phase I trial involving patients with RRMM, aiming to find the highest dose patients could tolerate without severe side effects. The maximum dose tested was well-tolerated, with drug-related adverse events in 90% of patients and serious ones in 45%. About 47% of participants showed a partial or better response to the treatment, indicating the combination's potential effectiveness with manageable side effects [90, 91]. In a study (NCT01023308) conducted between January 2010 and February 2012 involving 768 patients with RRMM, participants were divided into two groups: one received a combination of panobinostat, bortezomib, and dexamethasone (387 patients), and the other received a placebo with bortezomib and dexamethasone (381 patients). The panobinostat group showed a significantly longer median progression-free survival of nearly 12 months compared to 8 months in the placebo group. Although overall survival rates were not conclusive, the panobinostat group had a slightly higher median overall survival at the time of analysis. The study also found a higher rate of complete or near complete response in the panobinostat group compared to the placebo group, though overall response rates (ORR) were similar. The panobinostat group experienced more serious adverse events and grade 3–4 laboratory abnormalities. The findings suggest panobinostat could be beneficial for treating this patient population, but longer follow-up is needed to assess the impact on overall survival [92]. Furthermore, more and more clinical trials show that Doublet combination therapy with dexamethasone can improve the efficacy of treatment in RRMM [93–97].

Combination therapy with IMiDs

Due to encouraging preclinical anti-MM action, the effectiveness of HDACis has been investigated in combination with other treatments, such as IMiDs (Table 2). Specifically, panobinostat has been used in combination with lenalidomide and dexamethasone. The Phase

Table 2 Clinical studies evaluating HDACis for the treatment of MM

NCT number	Phase	Patients	Regimens	HDACis dose/Schedule	Response rates	Grade 3/4 toxicities	References
NCT00445068	II	n = 38	Panobinostat	20 mg, Days 1, 3, 5, 8, 10, 12, every 21 days	ORR: 2.63%	Thrombocytopeni (50%), anemia (11.2%), neutropenia (8%), fatigue (26%)	[86]
NCT00621244	Ia/II	n = 176	Panobinostat	From 20-80 mg three times a week or once every 14 days	Not found	Thrombocytopenia (41.5%), neutropenia (21%), fatigue (21%)	[87]
NCT01720875	II	n = 16	Vorinostat/ Bortezomib/ Dexamethasone	400 mg, Days 1–14, every 21 days	ORR: 81.3%	Thrombocytopenia (50%), diarrhea (6.3%), fatigue (6.3%), anemia (6.3%)	[88]
NCT01583283	I	n = 38	ACY-1215/ Lenalidomide/ Dexamethasone	From 40–240 mg once daily to 160 mg twice daily	ORR: 55%	Fatigue (18%), diarrhea (5%)	[89]
NCT01502085	I/II	n = 25	Vorinostat/ Lenalinomide/ Dexamethasone	Vorinostat: 400 mg once a week; Lenalidomide: 25 mg once two weeks; Dexamethasone: 40 mg, Days 1, 8, 15 and 22	ORR: 24%	Thrombocytopenia (56%), fatigue (72%), diarrhea (72%), neutropenia (68%), vomiting (12%)	[90]
NCT00642954	I	n = 31	Vorinostat/ Lenalidomide/ Dexamethasone	Vorinostat: 400 m, days 1–7 and 15–21; Lenalidomide: 25 mg, days 1–21; Dexamethasone: 40 mg, Days 1, 8, 15, 22, every 28 days	ORR: 47%	Anemia (58%), thrombocytopenia (58%), diarrhea (55%), fatigue (55%), cough (45%)	[91]
NCT02290431	II	n = 31	Panobinostat/ Bortezomib/ Dexamethasone	20 mg, Days 1, 3, 5, 8, 10, 12, every 21 days	ORR: 80.6%	Thrombocytopenia (48.4%), fatigue (25.8%), diarrhea (22.6%), neutropenia (22.6%), lymphopenia (22.6%)	[93]
NCT01440582	I	n = 55	Panobinostat/ Bortezomib/ Lenalidomide/ Dexamethasone	Bortezomib: (1.3 mg/m^2), Days 1, 4, 8, 11; Lenalidomide: 25 mg, Days 1–14; Dexamethasone: 20 mg, Days 1, 2, 4, 5, 8, 9, 11, 12	Not found	Thrombocytopenia (17%), diarrhea (17%)	[94]
NCT02654990	II	n = 248	Panobinostat/ Bortezomib/ Dexamethasone	82 to panobinostat 20 mg thrice weekly, 83 to panobinostat 20 mg twice weekly, and 83 to 10 mg panobinostat three times weekly	ORR: 62·2% (20 mg three times weekly group); 65·1% (20 mg twice weekly group), 50·6% (10 mg three times weekly group)	Thrombocytopenia (32%), neutropenia (15.2%), pneumonia (11.6%)	[95]
NCT01083602	II	n = 55	Panobinostat/ Bortezomib/ Dexamethasone	20 mg, Days 1, 3, 5, 8, 10, 12, every 21 days	ORR: 34.5%	Thrombocytopenia (63.6%), diarrhea (20%), fatigue (20%), anemia (14.5%), neutropenia (14.5%), pneumonia (14.5%)	[96]
NCT00773838	II	n = 143	Vorinostat/ Bortezomib/ Dexamethasone	400 mg, Days 1–14, every 21 days	ORR: 11.3%	Diarrhea (4.2%), asthenia (2.8%), thrombocytopenia (2.8%), pneumonia (2.1%), neuralgia (1.4%)	[97]
NCT00858234	I	n = 9	Vorinostat/ Bortezomib	400 mg, Days 1–14, every 21 days	ORR: 44%	Thrombocytopenia (100%), lymphopenia (43%), neutropenia (29%), anemia (29%), nausea (29%), dehydration (29%), pneumonia (29%), diarrhea (14%), decreased appetite (14%), fatigue (14%), hypokalemia (14%)	[101]

Table 2 (continued)

NCT number	Phase	Patients	Regimens	HDACis dose/Schedule	Response rates	Grade 3/4 toxicities	References
NCT01023308	III	n = 768	Panobinostat/ Bortezomib/ Dexamethasone/ Placebo	Panobinostat: 20 mg (hard gelatin capsules); Bortezomib: 1.3 mg/m2 as a 3 to 5 s bolus intravenous injection; Dexamethasone: 20 mg every day	ORR: 60.7%	Pneumonia (14.7%), thrombocytopenia (7.35%), diarrhea (11.29%) anemia (3.67%), vomiting (3.15%), asthenia (3.94%), fatigue (2.89%) pyrexia (4.20%),	[102]
NCT01549431	I	n = 32	Panobinostat/ Carfilzomib	20 mg, Days 1, 3, 5, 15, 17, 19, every 28 days	ORR: 63%	Thrombocytopenia (41%), fatigue (17%), nausea (12%), vomiting (12%)	[103]
NCT01496118	I/II	n = 80	Panobinostat/ Carfilzomib	20 mg, 30 mg, Days 1, 3, 5, 15, 17, 19, every 28 days	ORR: 84.4%	Thrombocytopenia (60.6%), fatigue (18.2%), anemia (12.1%), dyspnea (12.1%), diarrhea (9.1%), neutropenia (9.1%), nausea (6.1%), vomiting (6.1%), peripheral neuropathy (3%)	[104, 105]
NCT01464112	I	n = 18	JNJ-2641585 / VELCADE / Dexamethasone	JNJ-2641585: 10 mg three times weekly oral dose with VELCADE + Dexamethasone	ORR: 88.2%	Thrombocytopenia (61.1%), asthenia (55.6%), diarrhea (66.7%)	[110]
NCT00773747	III	n = 637	Vorinostat/ Bortezomib	400 mg, Days 1–14, every 21 days	ORR: 11.3%	Thrombocytopenia (45%), neutropenia (28%), anaemia (17%)	[111]
NCT00532389	Ib	n = 62	Panobinostat/ Bortezomib	Panobinostat 20 mg thrice weekly every week + Bortezomib 1.3 mg/m^2, every 21 days	ORR: 73.3%	Thrombocytopenia (85.1%), neutropenia (63.8%), asthenia (29.8%), fatigue (20.0%)	[112]
NCT00742027	II	n = 27	Panobinostat/ Lenalidomide/ Dexamethasone	20 mg, Days 1, 3, 5, 15, 17, 19, every 28 days	ORR: 41%	Neutropenia (59%), thrombocytopenia (31%), fatigue (12.5%), infection (15.6%), diarrhea (9.4%) anemia (5%)	[113]

ORR: overall response rate, data sourced from the clinicaltrials.gov site

I clinical trial (NCT01440582) demonstrates the safety and efficacy of combining VRd (Bortezomib plus lenalidomide and dexamethasone) with a 10 mg dose of panobinostat in newly diagnosed multiple myeloma patients who are eligible for transplantation. In early testing, the lowest dose did not cause serious side effects, while a higher dose did in two patients, indicating it was too strong. Therefore, the study established the lower dose as the safest and most tolerable for patients. This combination therapy shows promise for treating newly diagnosed multiple myeloma in patients eligible for a transplant, but more extensive research is needed to confirm these findings [94]. Between July 2012 and August 2015, a study (NCT01583283) enrolled 38 patients to assess the safety and efficacy of ricolinostat in treating MM. The study identified a recommended dose of ricolinostat at 160 mg daily for future research, following two cases of significant adverse effects at a higher dosage. Common side effects included fatigue and diarrhea, but the drug demonstrated a promising ability to selectively inhibit HDAC6 without significantly impacting HDAC1, suggesting it could enhance treatments with lenalidomide and dexamethasone. Preliminary results showed a 55% response rate among participants, indicating that ricolinostat could be a safe and effective option for RRMM [89]. Moreover, the Phase I/II clinical trial (NCT01502085), and the Phase I clinical trial (NCT02569320) demonstrate that vorinostat, and AR-42 have the potential to synergize with lenalidomide and dexamethasone, hence improving their effectiveness in RRMM [89, 90, 98].

Combination therapy with conventional chemotherapy

In the 1980s, the primary therapeutic choices for MM were induction therapy utilizing alkylating agents such anthracyclines and steroids, as well as high-dose chemotherapy followed by autologous stem cell transplantation. As previously stated, the introduction of advanced medicines, including proteasome inhibitors, immunomodulatory drugs, monoclonal antibodies, and histone deacetylase inhibitors, has led to a notable enhancement in prognosis through the use of a new therapy strategy. Multiple treatment protocols including these innovative medications in different combinations have been formulated and assessed in clinical trials. Annually, the outcomes of these novel therapeutic regimens are disseminated through publication. In the context of this multifaceted contemporary landscape, conventional chemotherapeutic agents persist in retaining prominence, particularly when integrated with emerging therapeutic modalities [99]. We reviewed clinical trials of HDACis in combination with conventional chemotherapy, among them, we found only two (NCT00744354 and NCT01394354) and were unable to track the results.

Combination therapy with novel targeted agents

As elucidated in Section "Synergy with and resistance to HDAC Inhibition", proteasome inhibitors exhibit synergistic effects, concurrently impeding cellular proliferation and augmenting apoptosis in MM cells [77]. We found that Bortezomib, Carfilzomib, and Ixazomib were predominantly used in clinical trials (Table 2). Bortezomib is a specific and reversible inhibitor of proteasomes. It works by directly attaching to the β1 and β5 subunits of the catalytic 20S complex, hence preventing chymotrypsin-like activity85. Treatment with bortezomib enhances the bone marrow microenvironment by stimulating the development of osteoblasts and decreasing the activity of osteoclasts that depend on the receptor activator of NF-κB (RANKL). This effect is achieved through the activation of NF-κB, p38, and AP-1 pathways, and is influenced by the dosage of bortezomib [100]. The Phase I clinical trial study (NCT00858234) revealed that the most predominant adverse events were thrombocytopenia, leukopenia, neutropenia, diarrhea, nausea, decreased appetite, and vomiting [101]. Another Phase II clinical trial study (NCT01720875) showed that despite observed toxicity and dose reductions, which demonstrated that the combination of vorinostat, bortezomib, and dexamethasone was effective and had good response rates in relapsed myeloma, suggesting further optimization of HDAC inhibitor-based combination therapy for myeloid Tumor to improve tolerance and enhance efficacy [88]. However, the findings from the Phase III clinical trial study (NCT01023308) revealed that panobinostat was linked to a marginal improvement in overall survival when juxtaposed with the combination of bortezomib and dexamethasone placebo. Optimized regimens have the potential to prolong therapeutic duration and enhance patient outcomes; however, additional trials are requisite to corroborate these observations [102]. Carfilzomib is a second-generation drug that inhibits proteasomes and is mostly used for patients with multiple myeloma who have not responded to previous treatments or have experienced a relapse. Carfilzomib inhibits chymotrypsin-like activity by attaching to the catalytic 20S proteasome. Unlike bortezomib, this interaction is permanent and more specific, which accounts for certain side effects that are absent in bortezomib therapy. The usual route of administration for carfilzomib is intravenous, with a frequency of twice per week for a period of three weeks. The recommended dose is 27 mg/m^2. Carfilzomib's molecular mode of action is similar to that of bortezomib, which includes inducing apoptosis and improving bone injury. Carfilzomib side effects may include hypertension, cardiotoxicity, thrombocytopenia, hypocalcemia, and gastrointestinal problems [103–105]. Ixazomib is an innovative proteasome inhibitor used

orally at a dosage of 4 mg once per week. It functions by obstructing the enzyme in MM cells, impeding their capacity to proliferate and endure [106], nevertheless, only one clinical trial (NCT02057640) has been completed so far, but no definite results can be obtained. Common adverse effects of ixazomib encompass thrombocytopenia, edoema in the lower extremities, peripheral neuropathy (resulting in weakness, numbness, and pain in the hands and feet), gastrointestinal disturbances such as diarrhoea, constipation, nausea, vomiting, and back pain [107].

The clinical safety of HDAC inhibitors in MM

There is an overexpression of HDAC in cancer cells, and the use of HDACis has been shown to enhance the outcomes of individuals who have been diagnosed with haematological malignancies include T-cell lymphomas and multiple myeloma. Five drugs were previously approved in different national jurisdictions, namely belinostat, chidamide, romidepsin, vorinostat and Panobinostat. It is worth noting that Secura Bio, Inc. requested the withdrawal of FDA approval for Panobinostat in 2021, citing the impracticality of conducting necessary postmarketing trials. Subsequently, in March 2022, the FDA withdrew panobinostat from the US market [108]. However, despite its removal from the US market, panobinostat continues to be employed in Europe as a viable treatment option for patients whose diseases have advanced after undergoing standard therapies. These drugs have been linked to a variety of severe and/or significant side responses, including myelosuppression, diarrhea, hepatic effects and various cardiac effects [109]. In this section, we have selected the most important side effects for review (Table 2, Fig. 5a).

Myelosuppression

From Fig. 5b, we can see five medication clinical studies revealed 3 common side effects including thrombocytopenia, neutropenia and anemia. Thrombocytopenia is common and can result in bleeding, although neutropenia is frequently a sign of infection. These side effects may be sufficiently serious to necessitate the transfusions of blood and/or the administration of granulocyte colony-stimulating agents. To reduce the clinical effects, blood counts should be checked on a frequent basis and dose modifications done as needed; nonetheless, if toxicities of grade 3 or 4 return after reducing the dosage, treatment should be discontinued. In the aggregate, the majority of clinical trials have demonstrated myelosuppression as a noteworthy side effect, warranting careful consideration.

Cardiac effects

The ether-a-go-go (hERG) channel in humans is responsible for controlling the duration of ventricular repolarization, which is visually represented as the QT interval on the surface electrocardiogram (ECG). Drugs

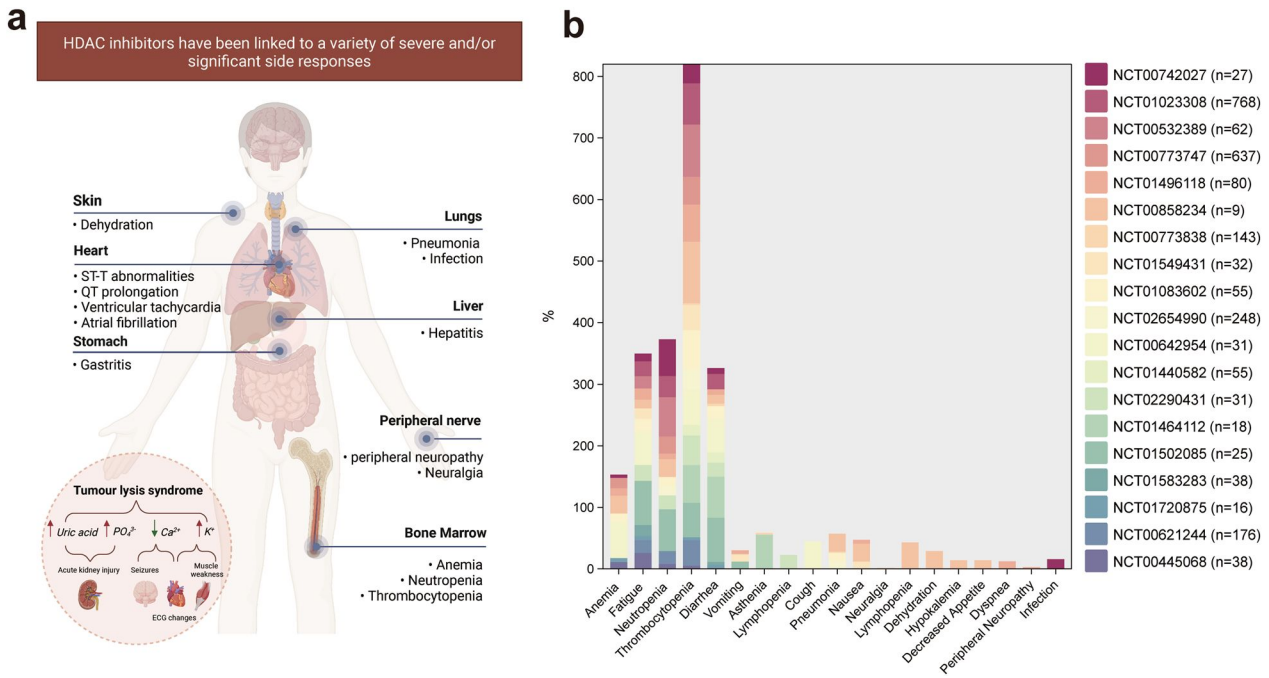

Fig. 5 **a** HDAC inhibitors have been linked to a variety of severe and/or significant side responses; **b** Distribution of grade 3/4 toxicities in clinical trials (Table 2) of histone deacetylase inhibitors. Figure created with BioRender.com

that inhibit or reduce the function or expression of hERG channels lead to an elongation of the QT interval. Torsades de pointes (TdP), a potentially fatal ventricular tachyarrhythmia, can occur when the QTc interval is extended due to excessive duration or the presence of risk factors. Schiattarella et al. [114] discovered that HDACis elicit typical albeit insignificant cardiac side effects, mostly manifesting as ECG abnormalities such as ST-T abnormalities and QT prolongation. This conclusion was drawn after analysing 62 trials with a collective patient population of 3268 individuals. The most common electrocardiographic abnormalities seen in patients treated with romidepsin (25.3%) and panobinostat (22.3%) were ST depression and/or T wave inversion, which accounted for 14.5% of the patients. QTc prolongation was observed in 4.4% of the total 3268 individuals. This percentage was lower than the rates reported for belinostat (12.2%), panobinostat (4.3%), vorinostat (3.4%), and romidepsin (3.3%). Ventricular tachycardia was observed in 0.6% (21/3268) of the entire study group, with the majority of cases occurring after the administration of romidepsin (19/944, 2.0%) or panobinostat (2/1047, 0.2%). Treated persons exhibited atrial fibrillation, whereas 13 individuals (0.4%) reported experiencing atrial fibrillation. This was mostly detected in vorinostat (8/888) and belinostat (2/221) patients. [109].

Gastrointestinal effects

From Fig. 5b, It is readily apparent that gastrointestinal side effects are also one of the main side effects. A comprehensive analysis of clinical studies has indicated that the use of antiemetic and antidiarrheal medications, together with fluid and electrolyte supplements, may be necessary to manage symptoms of nausea, vomiting, and diarrhoea following therapy with any of the five treatments (Belinostat, Panobinostat, Romidepsin, Vorinostat, Chidamide). Panobinostat has the potential to induce severe diarrhoea (grade 3 or 4) in 25% of people on therapy, which may necessitate a decrease in dosage or complete cessation of the treatment.

Hepatic effects

Complications arising from therapeutic interventions with romidepsin, panobinostat, belinostat, and chidamide have been systematically documented, frequently manifesting as elevated blood transaminases and/or bilirubin levels. Notably, vorinostat has not been correlated with any hepatic side effects. Despite a comprehensive literature search yielding no reports of clinically significant hepatotoxicity associated with these agents, a pivotal event in a belinostat clinical study, marked by a treatment-related fatality linked to hepatic failure, prompted the FDA to modify the approved label for belinostat. The revised label underscores the potential for fatal toxicity and advocates for pre-treatment and cyclical liver function test monitoring, it is particularly important. In the event of discernible hepatic impairment, a judicious course of action involves either dose adjustment or discontinuation, contingent upon the severity of the observed hepatotoxicity [109].

Agent-specific adverse effects

Table 3 concisely summarizes distinct extra adverse effects linked to various HDACis that set them apart from the wider class. The infections observed with belinostat and romidepsin are most likely caused by neutropenia, while cases of hemorrhage associated with panobinostat and pericardial effusion after chidamide therapy are coupled with thrombocytopenia created by these drugs. Increased levels of creatine phosphokinase in conjunction with chidamide and the presence of cardiac ischemia with panobinostat may indicate the potential of these particular drugs to cause harm to the myocardium.

Tumor lysis syndrome, a phenomenon that often occurs in the early stages of treatment and is frequently associated with belinostat and romidepsin, is commonly seen in patients with advanced-stage disease and/or high levels of hematological tumor burden. This syndrome is a metabolic disorder that can be life-threatening. It is characterized by high levels of uric acid, potassium, and phosphate, and low levels of calcium. This condition not only causes gastrointestinal symptoms like nausea and vomiting, but also leads to serious complications such as acute uric acid nephropathy, acute kidney failure, seizures, cardiac arrhythmias, and even death.

However, the clarification of prothrombotic and hyperglycemic effects associated with vorinostat poses challenges, as these phenomena may be attributed to factors such as the investigational drug itself, the characteristics of the patient population under scrutiny, or concurrent therapeutic interventions.

HDACis are a hopeful treatment for MM, aiming to correct cancer-specific gene patterns. Yet, their effectiveness is complicated by the fact that MM patients differ greatly in their genetic makeup, leading to varied

Table 3 The agent-specific reported adverse effects associated with HDACis

HDACi	Specific adverse effects reported in clinical trials
Panobinostat	Cardiac ischaemia, haemorrhage
Vorinostat	Hyper-glycaemia, pulmonary embolism, deep vein thrombosis
Belinostat	Infections, tumour lysis syndrome
Romidepsin	Infections, tumour lysis syndrome
Chidamide	Raised creatine phosphokinase levels, pericardial effusion

responses to these drugs. This variation highlights the need for identifying markers that can predict who will benefit most from these treatments. Additionally, the side effects of HDAC inhibitors can vary from mild to severe, making it crucial to manage these carefully to ensure patients truly benefit from the treatment. Looking ahead, research is zeroing in on finding these predictive markers, creating drug combinations that work better and have fewer side effects, understanding why some patients develop resistance, and paying closer attention to how treatments impact patients' quality of life. This approach aims to make HDACis treatment more personalized, maximizing benefits while reducing drawbacks for MM patients.

Challenges in the combined use of HDACis and immunotherapy

Immunological evasion in cancer is a critical process that involves the expression of immunological checkpoints, including PD-1, PD-L1, and CTLA-4. Inhibiting these checkpoints is an effective approach for treating cancer. Multiple studies demonstrate that STAT3 is involved in directly or indirectly controlling these immunological checkpoint molecules [115–118]. Notably, HDAC6 emerges as a significant regulator of the STAT3 pathway [119–121]. Lienlaf et al. provided evidence that HDAC6 plays a role in the body's defence against tumours in melanoma by affecting the STAT3-PD-L1 pathway [121], this discovery was further supported by Keremu et al. in their study on osteosarcomas [120]. Elevated production of HDAC6 leads to the phosphorylation of STAT3 and its translocation into the nucleus, without causing any changes in acetylation of its co-protein PP2A. Phosphorylated STAT3 and HDAC6 coexist in the nucleus and target the PD-L1 promoter, resulting in the activation of transcription and the enhancement of PD-L1 gene expression [19, 121, 122] (Fig. 6). Notably, preclinical studies indicate that a combination of HDAC6 inhibitor and PD-L1 antibody enhances γδ T cell antitumor functions [123]. This underscores the potential of targeting the HDAC6 inhibition-PD-1/PD-L1 pathway as a novel approach to augment cancer immunotherapy. The concurrent use of pan-HDACis and cytokine-induced killer (CIK) cell treatment [124], which has demonstrated efficacy in preclinical multiple myeloma models [125, 126], provides additional validation for this idea. The presence of specific HDAC6 inhibitors such as ACY-1215, tubastatin A, and ricolinostat presents a potential opportunity for their use, either alone or in conjunction with CIK cell therapy, in medical environments. This offers a hopeful pathway for the treatment of cancer.

The combined use of HDACis and immunotherapy holds promise for enhancing cancer treatment outcomes, but it also presents several challenges. (1) Limited understanding of mechanisms: The mechanisms through which HDACis interact with the immune system

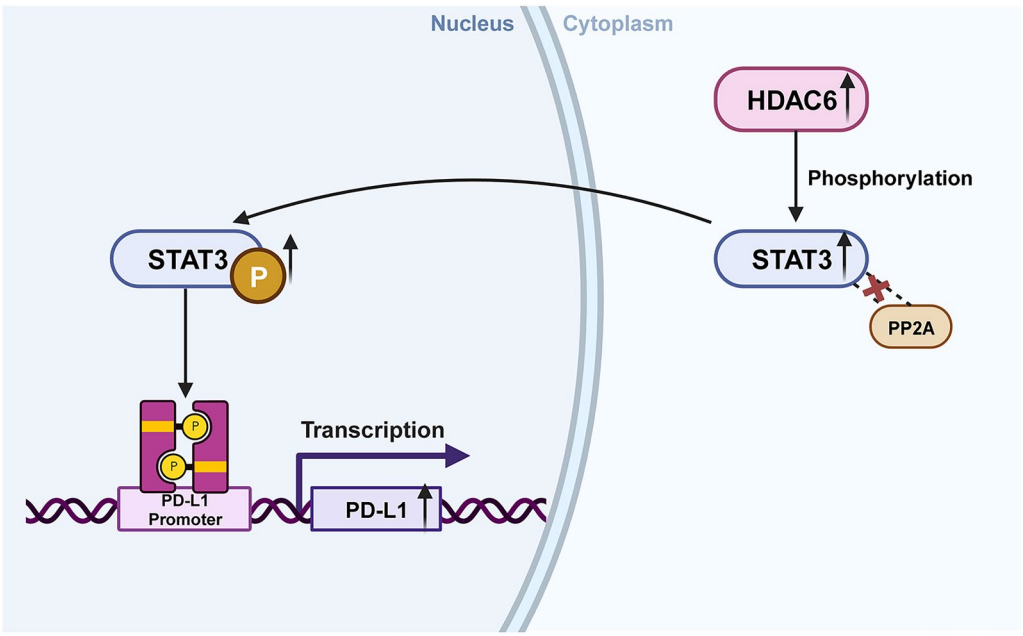

Fig. 6 Mechanistic illustration of HDAC6 in STAT3-PD-L1 pathway: When HDAC6 levels are high, STAT3 accumulates in a phosphorylated form, reducing the interaction between STAT3 and PP2A. After entering the nucleus, pSTAT3 and HDAC6 bind to the PD-L1 promoter, promoting PD-L1 expression. Figure created with BioRender.com

and modulate responses to immunotherapy are not fully understood. Better insights into these mechanisms are crucial for optimizing combination therapies. (2) Dose-dependent effects: The effects of HDACis can be dose-dependent, and finding the right balance is critical. High doses of HDACis may have immunosuppressive effects, counteracting the desired immune activation promoted by immunotherapy. (3) Off-target effects: HDACis can affect various cellular processes beyond histone acetylation, potentially leading to off-target effects [127]. Understanding and minimizing these off-target effects is important to avoid unintended consequences on immune cells and overall treatment efficacy. (4) Patient heterogeneity: Patient responses to HDACis and immunotherapy can vary significantly. Identifying biomarkers to predict which patients are more likely to benefit from the combination is a challenge. Personalized medicine approaches may be essential for optimizing treatment strategies. (5) Toxicity and side effects: HDACis can be associated with toxicities and side effects, including hematological toxicity and fatigue. Combining these agents with immunotherapy may exacerbate these issues, and managing the overall toxicity profile is crucial for patient safety and adherence. (6) Resistance development: Tumor cells can develop resistance to HDACis and immunotherapy. Understanding the mechanisms of resistance and developing strategies to overcome or prevent resistance is essential for long-term treatment success. (7) Optimal sequence and timing: Determining the optimal sequence and timing of HDACis and immunotherapy is challenging. The order in which these treatments are administered can impact their effectiveness, and finding the right schedule is critical for maximizing therapeutic benefits. (8) Synergistic vs. antagonistic effects: Achieving synergistic effects between HDACis and immunotherapy is the goal, but there is a risk of antagonistic interactions. Careful preclinical and clinical studies are needed to assess the compatibility of these treatments and avoid potential counteractive effects. (9) Clinical trial design: Designing clinical trials to effectively evaluate the safety and efficacy of combined HDACis and immunotherapy is challenging. Robust study designs, appropriate patient selection, and relevant endpoints are necessary to draw meaningful conclusions. (10) Regulatory hurdles: Regulatory approval for combination therapies can be complex. Coordinating the approval process for two or more agents may require additional evidence of safety and efficacy, and navigating regulatory pathways is a significant challenge. Addressing these challenges will require collaborative efforts from researchers, clinicians, and regulatory authorities to advance the understanding and implementation of combined HDACis and immunotherapy for cancer treatment. Ongoing research and clinical trials are essential to further elucidate the complexities and refine treatment strategies.

Conclusions and future directions

In the past decade, the landscape of MM treatment has undergone significant transformation, largely due to advancements in HDACis, immunomodulatory drugs, and other novel therapies. The incorporation of HDACis into the therapeutic arsenal has expanded the spectrum of effective treatment options, leading to increased patient longevity and improved quality of life. Presently, a wide array of potent therapy regimens that leverage HDACis as a backbone is available, indicating a pivotal shift in MM management strategies. Moreover, ongoing research is exploring innovative approaches, such as the integration of HDACis with monoclonal antibodies, targeted medicines, and cellular immunotherapy, aiming to further enhance treatment efficacy and patient outcomes.

A notable area of progress involves the synergistic combination of HDACis with anti-CD38 monoclonal antibodies, such as daratumumab, which received FDA approval in November 2015. For example, the combination of daratumumab with bortezomib and dexamethasone improved progression-free survival in patients with RRMM compared to just bortezomib and dexamethasone [128]. Panobinostat, MS-275, and ACY1215 enhance CD38 expression, thereby increasing daratumumab's anti-myeloma effectiveness [129, 130]. In general, it has shown promising results in vitro, underscoring the potential for HDACis to improve the efficacy of established therapies in both initial and relapse settings. Despite these advancements, the specific molecular mechanisms underlying the enhanced anti-tumor activity of these combination therapies remain to be fully elucidated. Furthermore, the development of isoform and/or class-selective HDACis presents a promising avenue to mitigate the adverse effects commonly associated with non-selective HDACis, while maintaining robust anti-tumor efficacy.

The ongoing challenge of addressing toxicity, resistance mechanisms, and the absence of reliable biomarkers for predicting HDACis response underscores the need for continued research. Efforts to identify predictive markers, understand the molecular basis of HDACis action, and explore novel therapeutic combinations are essential for optimizing MM treatment. As research progresses, it is anticipated that the targeted application of HDACis, either as monotherapies or in combination with other agents, will significantly advance the treatment paradigm for MM, offering patients more personalized and effective treatment options [131, 132]. This integration of novel HDACis-based therapies into MM treatment regimens not only reflects the current

progress but also sets the stage for future advancements that promise to further improve patient survival and quality of life.

Abbreviations

HDACs	Histone deacetylases
HDACis	Histone deacetylase inhibitors
MM	Multiple myeloma
IMiDs	Immunomodulatory drugs
ASCT	Autologous stem cell transplantation
mAbs	Monoclonal antibodies
ADC	Antibody–drug conjugates
BiTE	Bispecific T-cell engagers
CAR-T	Chimeric antigen-T-cell therapy
FDA	The United States Food and Drug Administration
RRMM	Relapsed/refractory multiple myeloma
CTCL	Cutaneous T-cell lymphoma
CMML	Chronic myelomonocytic leukemia
HIV	Human immunodeficiency virus
AIDS	Acquired immunodeficiency syndrome
DLBCL	Diffuse large B-cell lymphoma
PTCL	Peripheral T-cell lymphoma
NSCC	Non-small cell lung carcinoma
HCC	Hepatocellular carcinoma
ATLL	Adult T-cell leukemia-lymphoma
hERG	The human ether-a-go-go
ECG	Electrocardiogram
tdP	Torsades de pointes
CIK	Cytokine-induced killer

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JJP, TL, XZW, AS, IGHS-W, JH and LPJ contributed to conceive, design and revision of the manuscript sections. JJP wrote the manuscript. JJP and TL designed figures and created Tables. JJP, AS, IGHS-W, JH and LPJ supervised the manuscript by providing critical feedbacks and revisions. The authors read and approved the final manuscript.

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3.3 Publication 3: Histone deacetylase 6: at the interface of cancer and neurodegeneration

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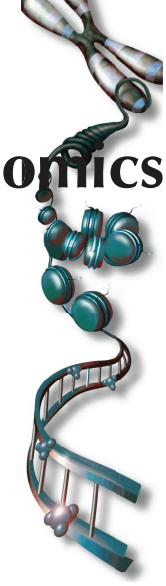

Histone deacetylase 6: at the interface of cancer and neurodegeneration

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With the recognition in the early 1960s that histones can be post-translationally modified, the list of different post-translational modifications of histones and their biological consequences has continued to expand. In addition, the idea of the 'histone code' hypothesis, later introduced by David Allis and colleagues, further broaden the horizon of chromatin biology. Currently, there is a wealth of knowledge about the transition between the active and the repressive state of chromatin, and modifications of histones remains at the center of chromatin biology. Histone deacetylases (HDACs) in particular are of great importance for the therapeutic success of cancer treatment. Focusing primarily on *HDAC6*, herein we have briefly highlighted its unique involvement in cancer and also apparently in neurodegeneration.

Plain language summary: Cancer (uncontrolled cell proliferation) and neurodegenerative diseases (loss of neurons/protein aggregation) are two distinct pathological conditions that share/overlap certain molecular determinants. Histone deacetylase 6 appears to be one such determinant for which researchers have made significant progress by accumulating sufficient evidence for its clinical translation in these aforementioned disease conditions.

Tweetable abstract: Be it cancer or neurodegeneration, understanding the dynamics of histone modifications continues to be of great interest, and histone deacetylase 6 (HDAC6) now apparently is at the forefront.

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In the early 1960s, the pioneering work of Vincent Allfrey and others [1] and further insights by David Allis and colleagues [2] contributed to a better understanding of the functional dynamics of chromatin-associated proteins such as histones. Of interest, it has now been recognized that histones can be modified in a number of ways to affect gene expression, with certain histone modifications such as acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, glycosylation and ADP-ribosylation being most widely investigated. In addition, the relative contribution of dysregulated epigenomic entities such as histone modifications alongside DNA methylation, which drive disease progression, particularly the cancer phenotype, continues to be a focus of research [3]. Disruption of these modifications has been shown to affect the function of the genome by altering chromatin structure, which in turn affects its accessibility and compactness. The interplay of histone acetylation and deacetylation remains an important mechanism for regulating gene transcription, implemented by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. To date, 18 enzymes have been identified that belong to the HDAC superfamily and are classified into four classes, including class I (HDAC1, HDAC2, HDAC3, HDAC8), class IIa (HDAC4, HDAC5, HDAC7, HDAC9), class IIb (HDAC 6, HDAC10), class III (SIRT1-SIRT7) and class IV (HDAC11) (Figure 1A). HDACs predominantly localize in the nucleus, but some have also been observed in the cytoplasm, for example HDAC6. Being a structurally and functionally unique cytoplasmic deacetylase, HDAC6 targets several nonhistone substrates, including HSP90, cortactin, peroxiredoxin, α-tubulin and HSF1 [4]. Several reports have been made on the involvement of *HDAC6* in various signaling pathways associated with early- and

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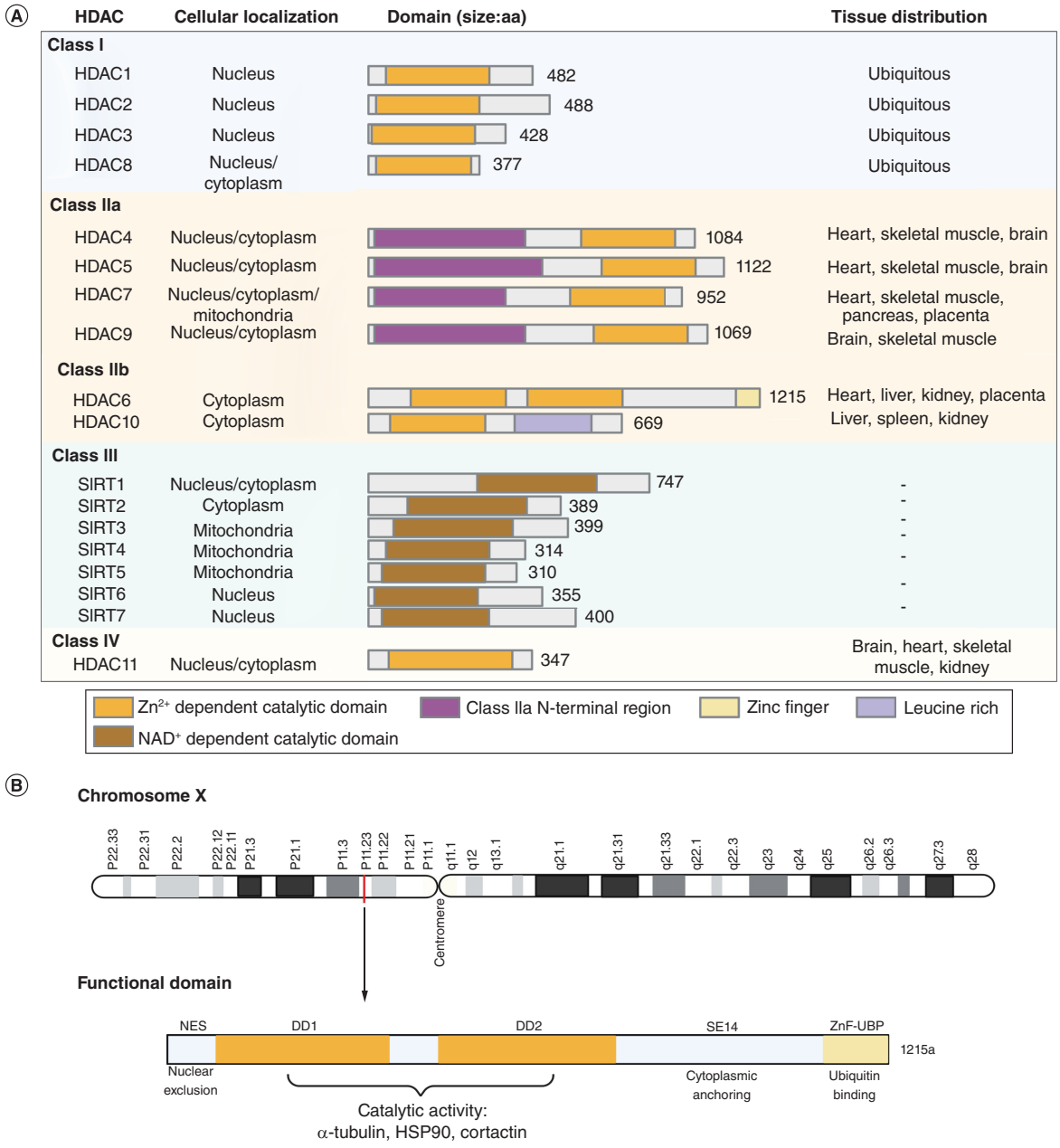

Figure 1. Classification of histone deacetylase family members and uniqueness of HDAC6. (A) Based on homology to yeast histone deacetylases (HDACs), subcellular localization and noncellular enzymatic activity, the 18 HDAC isoforms in humans are divided into four groups: class I, class IIa, class IIb, class III and class IV (HDAC11). Class I, II and IV HDACs have a Zn^{2+}-dependent deacetylase domain, while class III HDACs have a NAD^{+}-dependent domain. The SIRT1-7 family belongs to class III; however, they are not functionally linked to HDAC; their deacetylase activity is based on NAD^{+} rather than Zn^{2+}-dependent enzymes. **(B)** The gene encoding *HDAC6* is located on chromosome Xp11.23. The HDAC6 protein has two functional catalytic domains (DD1 and DD2) that catalyze α-tubulin, HSP90 and cortactin deacetylation. The nuclear export signal supports the protein's cytoplasmic localization and the Ser-Glu-containing tetrapeptide (SE14) region assures the enzyme's stable cytoplasmic anchoring. In its C-terminal region, the ubiquitin-binding zinc finger domain interacts with ubiquitinated proteins and regulates ubiquitination-mediated degradation.

advanced-stage cancer as well as neurological diseases [5–9]. Therefore, targeting *HDAC6* has attracted particular interest in recent years. Herein, we provide a brief insight into the involvement of *HDAC6* in two distant entities, namely cancer and neurodegeneration.

HDAC6, a unique HDAC family member as a cancer target

As aforementioned, histone acetylation and deacetylation constitute important mechanisms of gene regulation, which are modulated by HATs and HDACs, respectively. While HATs induce chromosomal depolymerisation and activation of transcription, HDACs act to repress the transcription. Contrary to other members of the HDAC family, zinc-dependent HDAC6 is mainly located in the cytoplasm as it contains a nuclear export sequence and the SE14 motif, which is essential for cytoplasmic retention (Figure 1B) [10,11]. The *HDAC6* gene is located on chromosome Xp11.23 and encodes a protein of 1215 amino acids, the largest protein of the HDAC family [12]. Two functional catalytic domains that are homologous and functionally independent of the overall activity of HDAC6 make it unique [13]. In addition, the C-terminus of HDAC6 has a ubiquitin-binding zinc finger domain (ZnF-UBP domain, also known as DAUP, PAZ or PAZ domain) that is involved in the control of ubiquitin-mediated degradation [14,15]. In addition, HDAC6 regulates the acetylation of various nonhistone substrates, including α-tubulin, cortactin and HSP90 [9,15]. The first nonhistone substrate of HDAC6 was identified as α-tubulin, and reversible deacetylation of α-tubulin by HDAC6 has the potential to affect microtubule stability and function. Acetylation of α-tubulin has been shown to affect cell cycle progression by impairing intracellular trafficking and interfering with mitotic processes via the protein produced by the cylindromatosis gene [16,17]. Likewise, HDAC6 influences actin-dependent cell motility via another nonhistone substrate, cortactin [9]. Cortactin, an F-actin-binding protein, promotes aggregation and branching and is generally present at dynamic actin assembly sites. It is deacetylated by binding to the deacetylase domains of HDAC6 [18]. HSP90 is a further nonhistone substrate of HDAC6 whose main function is to support protein maturation and structure maintenance [19,20]. The presence of the ZnF-UBP domain distinguishes HDAC6 as a regulator of the ubiquitin and proteasome system, which controls physiological responses to protein misfolding [20]. It also stimulates autophagy by recruiting and deacetylating corticin, a protein required for autophagosomes and lysosomes. In addition, HDAC6 forms a complex with HSP90 and HSF1, contributes to the activation of HSF1, stimulates the production of HSP25 and HSP70, controls protein folding, and contributes to the repair and degradation of misfolded proteins [11]. Recently, an intriguing interaction between *HDAC6*-induced lncRNA (LINC00152) and its potential sponge miRNA (hsa-miR-499a-5p) has been discussed from the perspective of an avenue to study the mutual interactions between the noncoding genome and the epigenetic machinery that may exert biological functions in the dysregulated genome [10]. Overall, these findings collectively demonstrate the integral role of HDAC6 in a diverse array of cellular processes that hold significant relevance for the pathogenesis of cancer [11]. For instance, Zheng *et al.* recently pointed out that several cancer types, including bladder urothelial carcinoma, cervical squamous cell carcinoma, colon adenocarcinoma, esophageal carcinoma, glioblastoma, lung squamous cell carcinoma, prostate adenocarcinoma, rectum adenocarcinoma and uterine corpus endometrial carcinoma, had higher levels of *HDAC6* compared with normal tissue, thus suggesting that inhibition of HDAC6 may be particularly effective in these types. Nevertheless, moderate expression of *HDAC6* was also found to be inversely associated with overall patient survival in some cancers, such as head and neck squamous cell carcinoma and pancreatic adenocarcinoma, suggesting that targeting HDAC6 may be pivotal in these solid cancers [5]. In general, there have been numerous studies (as described later) showing altered expression of *HDAC6* in human cancer types ranging from hematological malignancies to solid cancers (Figure 2A & Table 1).

Patterns of HDAC6 expression in hematological malignancies

In cutaneous T-cell lymphoma, elevated *HDAC6* was the unique parameter that significantly affected survival in Cox analysis ($p = 0.04$). *HDAC6* expression was ultimately associated with the good outcome regardless of cancer subtype [12]. In chronic lymphocytic leukemia, correlation with survival time showed that high *HDAC6* levels were significantly associated with longer survival [13]. Likewise, in diffuse large B-cell lymphoma (DLBL), multivariate analysis revealed that elevated *HDAC6* expression emerged as an independent prognostic factor for individuals afflicted by DLBL [14]. Overall, overexpression of *HDAC6* appears to be associated with a better prognosis in cutaneous T-cell lymphoma, chronic lymphocytic leukemia and DLBL, thus suggesting that HDAC6 may function as a potential biomarker in hematological malignancies.

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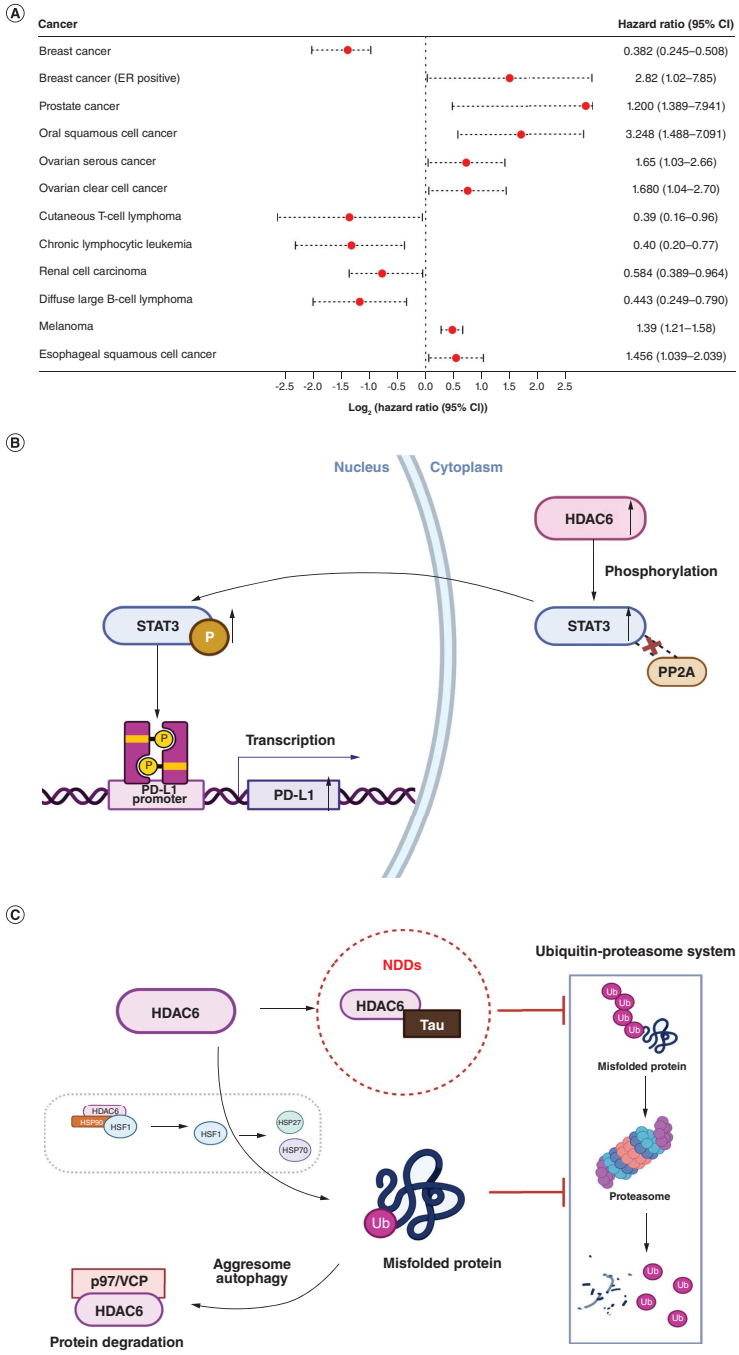

Figure 2. Molecular interplay of HDAC6 in cancer and neurodegenerative diseases. (A) *HDAC6* expression and patient prognosis (adapted from Table 1). Hazard ratios and 95% CIs for the expression of *HDAC6* in a variety of human cancer types. **(B)** Mechanistic illustration of HDAC6 in the STAT3-PD-L1 pathway: when HDAC6 levels are high, STAT3 accumulates in a phosphorylated form, reducing the interaction between STAT3 and PP2A. After entering the nucleus, pSTAT3 and HDAC6 bind to the PD-L1 promoter, promoting PD-L1 expression [21–23]. **(C)** HDAC6 in neurodegenerative diseases (interaction of HDAC6 with ubiquitin and tau protein). The ubiquitin–proteasome system is known to be disrupted in neurodegenerative diseases, leading to an accumulation of highly ubiquitinated misfolded proteins that tend to aggregate. HDAC6 attaches to ubiquitinated protein aggregates, forms an aggresome, and is subsequently removed by autophagy. To induce protein degradation, p97/VCP may disrupt the complexes formed between HDAC6 and polyubiquitinated proteins. The binding of HDAC6 to polyubiquitinated proteins can also cause the HDAC6/HSP90/HSF1 complex to dissociate, leading to the activation of HSF1 and consequently promoting the production of HSP70 and HSP27 genes. HDAC6 is also able to modulating Tau aggregation and stability in Alzheimer's disease neurons [24,25].

Table 1. HDAC6 expression and prognostic impact in different tumor types.

Cancer	HR	95% CI	p-value	End point	Material	Ref.
Breast cancer (n = 228)	0.382	0.245–0.508	**<0.01**	OS	Protein	[15]
Breast cancer (ER positive, n = 48)	2.82	1.02–7.85	**0.047**	OS	Protein	[16]
Prostate cancer (n = 16)	7.282	1.389–7.941	**0.007**	OS	Protein	[20]
Oral squamous cell cancer (n = 133)	3.248	1.488–7.091	**0.003**	OS	Protein	[26]
Ovarian serous cancer (n = 88)	1.65	1.03–2.66	**0.039**	PFS	Protein	[18]
Ovarian clear cell cancer (n = 106)	1.680	1.04–2.70	**0.034**	OS	Protein	[19]
Cutaneous T-cell lymphoma (n = 59)	0.39	0.16–0.96	**0.04**	OS	Protein	[12]
Chronic lymphocytic leukemia (n = 200)	0.40	0.20–0.77	**0.006**	TFS	RNA	[13]
Renal cell carcinoma (n = 132)	0.584	0.389–0.964	**0.038**	OS	Protein	[27]
Diffuse large B-cell lymphoma (n = 132)	0.443	0.249–0.790	**0.006**	OS	Protein	[14]
Melanoma (n = 80)	1.39	1.21–1.58	**0.0001**	OS	Protein	[28]
Esophageal squamous cell cancer (n = 209)	1.456	1.039–2.039	**0.029**	OS	Protein	[29]

The cohorts indicated in the references were used to obtain data. Only articles from which a multivariate survival analysis (including hazard ratios and 95% CIs) could be obtained were included. $p < 0.05$ were deemed significant and reported in bold.
OS: Overall survival; PFS: Progression-free survival; TFS: Treatment-free survival.

HDAC6 expression in solid cancers

As with hematological malignancies, the involvement of HDAC6 in solid cancers is also considerable. For instance, higher expression of androgen receptor and *HDAC6* and their co-expression have been linked to a poorer clinical outcome in breast cancer [15]. In estrogen receptor-α-positive breast cancer, multivariate analysis showed that *HDAC6* expression was an independent prognostic factor (odds ratio: 2.82; p = 0.047). These findings highlight the biological importance of HDAC6 regulation via estrogen signaling [16]. In ovarian cancer, *HDAC6* expression was found to be high compared with normal tissue. HDAC6 appears to affect pathways associated with ovarian cancer by modulating various cellular processes, including stress response, oncogenesis, cellular motility and multiple signaling networks pertinent to cancer [17]. In particular, in high-grade serous ovarian cancer, an independent prognostic factor for progression-free survival (PFS) was identified in the form of heightened expression of *HDAC6* [18]. In ovarian clear-cell cancer and nuclear expression of *HDAC6* was also found to be an independent prognostic factor for poor prognosis [19]. In prostate cancer, a study showed that the dysregulation of cortactin and HDAC6 may be implicated in the invasiveness and migration of prostate cancer cells [20]. Of interest, high expression of *HDAC6* was shown to be related to poor prognosis in both oral squamous cell cancer and esophageal squamous cell cancer [26,29]. Zhang *et al.* showed that high expression of *HDAC6* is an independent adverse prognostic factor in renal cell cancer patients and can be used as a biomarker for prognosis [27]. In melanoma, Hu *et al.* showed that the high expression of *HDAC6* was associated with melanoma metastasis and shortened survival time of patients [28]. Contrary to hematological malignancies, increased *HDAC6* expression has a poor prognosis and shortened survival time in most solid cancers.

HDAC6 in cancer immunity & immunotherapy

The expression of immune checkpoints (PD-1, PD-L1 and CTLA-4, and so on) remains an important mechanism for evading the immune system and their inhibition is one of the most effective cancer treatments. There is ample evidence that STAT3 is able to directly or indirectly regulate these immune checkpoint molecules [30–33]. Intriguingly, HDAC6 appears to be an important regulator of the STAT3 pathway [21–23]. In this context, Lienlaf *et al.* showed that HDAC6 contributes to antitumor immunity via the STAT3-PD-L1 pathway in melanoma [23]. A similar finding was also reported in osteosarcomas by Keremu *et al.* [22]. Mechanistically, high *HDAC6* expression induces phosphorylation and ectopic entry of STAT3 into the nucleus, but no acetylation changes in its co-protein PP2A. Overall, pSTAT3 and HDAC6 are recruited to the *PD-L1* promoter after entry into the nucleus to activate transcription and enhance *PD-L1* gene expression (Figure 2B) [6,23]. Furthermore, a preclinical study has shown that PD-L1 antibody and HDAC6 inhibitor enhance the antitumor functions of γδ T cells [34]. Thus, a new avenue of the HDAC6 inhibition-PD1/PDL1 pathway to improve cancer immunotherapy can be imagined. Here it is worth mentioning that the combined use of pan-HDAC inhibitors and cytokine-induced killer (CIK) cell therapy, which has recently completed 30 years in clinics, has provided very effective results in preclinical multiple myeloma models [35,36]. Now that selective HDAC6 inhibitors (e.g., ACY-1215, tubastatin A, ricolinostat, and so

on) are available, their implementation (alone or in combination with compatible CIK cell therapy) in the clinics can be expected to yield positive results in the treatment of cancer.

HDAC6 as a therapeutic intervention in CNS malignancies & neurodegenerative diseases

Among CNS malignancies, glioblastoma (GBM) is the most common and aggressive malignant primary brain tumor in adults. Recently, a study demonstrated high levels of HDAC6 in GBM tissues and patient-derived GBM stem cells (GSCs), and further characterized a novel HDAC6i (JOC1) that inhibits GBM cell growth and GSC activity [37]. Certainly, the blood–brain barrier is a major obstacle to the treatment of malignant CNS diseases, as it impedes the penetration of drugs and other therapeutic inventions into the brain. However, the relative success of randomized clinical trials using CIK cell therapy in GBM patients [38,39] has now opened up the possibility of further defining the therapeutic paradigm for GBM patients. Because CIK cell therapy is compatible with HDAC inhibitors, one can envision a potential clinical treatment with favorable outcomes for GBM patients or CNS-related malignancies, in particular using HDAC6.

Neurodegenerative diseases (NDDs) are as incurable as GBM (or other CNS malignancies). Undoubtedly these two entities (cancer and NDDs) are phenotypically distinct, yet the involvement of certain common genes or shared pathways have been recently discussed [40,41]. Importantly, studies have shown that HDAC inhibitors can also be used for NDDs [42], thus providing a rationale to discuss any possible involvement of HDAC6 in NDDs [24]. First to be mentioned in this context are the α-synucleinopathies (Parkinson's disease [PD] and dementia with Lewy bodies), in which the involvement of HDAC6 in the accumulation of α-syn oligomers and the formation of protein aggregates has been extensively discussed [43]. One of the first indications that HDAC6 may be a component of Lewy bodies comes from a study reporting that HDAC6 had high expression in α-synuclein- and ubiquitin-positive Lewy bodies in brain sections of patients with PD [44]. Currently, there has been sufficient evidence (mostly from cultured cells or animal models) that the inhibition of HDAC6 can have neuroprotective effects in PD [45]. In the case of tauopathies, one study demonstrated that a HDAC6-dependent surveillance mechanism suppresses toxic tau accumulation, which may protect against the progression of Alzheimer's disease (AD) and related tauopathies [25]. Among the tauopathies the role of HDAC6 has been best studied in, independent studies have reported significantly elevated HDAC6 levels in various brain regions of AD patients compared with controls [46–48]. Despite repeated debate about the protective and/or detrimental effects of HDAC6 in AD over the years, its potentially valuable clinical benefits cannot be ignored. Recently, Li *et al.* provided a detailed description of HDAC6 inhibitors reported over the last decade and discussed the key rationale that may contribute to their pharmacological success in AD [48]. Interestingly, a novel HDAC6 inhibitor (CKD-504) has recently been shown to be effective in treating preclinical models of Huntington's disease [49]. CKD-504 is also currently being investigated in phase I clinical trials for the treatment of Huntington's disease, as it is believed to penetrate the blood–brain barrier (NCT03713892). Broadly speaking, cytoplasmic HDAC6 regulates acetylation of a variety of nonhistone proteins related to intracellular transport, neurotransmitter release and aggregate formation in healthy neurons, whereas it is abundant in the nucleus under pathological conditions and affects transcriptional regulation and synapses [7]. In addition, the pivotal role of HDAC6 in neuronal dysfunction (e.g., the interaction of HDAC6 with ubiquitin and the tau protein; Figure 2C) renders it as a key determinant of NDD progression. In the future, it will certainly be of interest to see if selective inhibition of HDAC6 can provide some clinical benefit in NDDs.

Conclusion

Understanding histone modifications and their interplay in key biological processes has certainly helped to delineate the human genome and various disease states. Undeniably, HDAC6 (among HDAC family members) holds a unique position when addressing pathological conditions such as cancer and NDDs. Deeper insights into the molecular regulation of HDAC6 in these entities (cancer and NDDs) including a favorable outcome in the clinical landscape is expected.

Future perspective

HDAC6 is involved in several fundamental and common molecular processes that are shared across diseases ranging from cancer to neurodegeneration, thus making it ideal for clinical implementation (as a single-target approach) in the treatment of various diseases. However, it cannot be ruled out that the disease-specific mutations and other underlying factors may dictate the role of HDAC6 as a cause or consequence of a given disease state. Moreover, HDAC6 targets multiple substrates, including HSP90, and it is known that disruption of either of these

counterparts leads to unexpected negative outcomes in cancer models. Given that HSP90 and its co-chaperones are equally well known to be involved in various NDDs [50,51], it cannot be excluded that selective inhibition of HDAC6 (in NDDs) may interfere with the functional process of HDAC6–HSP90 interplay, leading to a similar fate. Notwithstanding, researchers have made significant progress by accumulating sufficient evidence for the clinical translation of epigenetic players such as HDAC6 across diseases having distinct characteristics (accelerated cell proliferation in cancer and loss of neurons/protein aggregation in NDD). Here, controversy about the efficacy of selective HDAC6 inhibitors (e.g., Ricolinostat) should also be mentioned, as their use at high concentrations may lead to low selectivity, potential off-target effects and certain discrepancies regarding their activity [52–54]. Therefore, stringent criteria to identify selective HDAC6 inhibitors with targeted anticancer activity (not attributable to the off-target effect of other members of the HDAC family) could help to support their clinical utility. Considering that the cellular mechanisms eliciting HDAC6 in disease states might extend beyond its deacetylase activity or ubiquitin-binding properties, a careful re-evaluation is needed that could benefit clinical strategies.

Executive summary

The function of HDAC6 in cancer

- HDAC6, a zinc-dependent HDAC, is primarily located in the cytoplasm due to specific motifs and sequences. It encodes a large protein with unique catalytic domains and a ubiquitin–binding domain.
- HDAC6 also deacetylates nonhistone substrates, including α-tubulin, cortactin and HSP90, affecting processes such as microtubule stability, cell motility and protein folding.
- HDAC6 plays a role in the regulation of the ubiquitin and proteasome system and is involved in autophagy and the repair of misfolded proteins.
- HDAC6 may serve as a potential biomarker for cancer.
- The potential of HDAC6 inhibition in enhancing antitumor immunity through the STAT3-PD-L1 pathway suggests that combining HDAC6 inhibitors with cytokine-induced killer cell therapy could be a promising approach in cancer treatment.

HDAC6 as a therapeutic option for CNS malignancies & neurodegenerative diseases

- Glioblastoma is a common and aggressive brain tumor, and recent research highlights the potential of HDAC6 inhibition as a treatment option.
- The blood–brain barrier presents a challenge, but clinical trials with cytokine-induced killer cell therapy show great promise.
- There is a connection between HDAC6 and neurodegenerative diseases, particularly in alpha-synucleinopathies and tauopathies. HDAC inhibitors may have therapeutic potential in these conditions. In addition, a novel HDAC6 inhibitor is being studied for Huntington's disease treatment.
- HDAC6 plays a crucial role in both cancer and neurodegenerative diseases, and selective inhibition of HDAC6 may offer clinical benefits in the future.

Author contributions

Writing, original draft: J Pu, A Sharma, J Hou & IGH Schmidt-Wolf. All authors read and approved the final manuscript.

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Writing disclosure

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3.4 Publication 4: Balancing the interplay of histone deacetylases and non-coding genomes: a step closer to understand the landscape of cancer treatment

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COMMENT Open Access

Balancing the interplay of histone deacetylases and non-coding genomes: a step closer to understand the landscape of cancer treatment

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Abstract
Histone deacetylase (HDAC) inhibitors have enormous therapeutic potential as effective epigenetic regulators, and now with the focus on the selective HDAC6 inhibitor in ongoing clinical trials, more advantages over other non-selective pan-HDAC inhibitors are foreseeable. As it is of paramount importance to understand the complex regulatory web of mutual interactions involving epigenetic machinery and non-coding genome in regulating gene expression, herein, we investigated the intriguing interactions between HDAC6-induced lncRNA (LINC00152) and its possible sponge miRNA (hsa-miR-499a-5p) in multiple myeloma.

Keywords Histone deacetylases, Non-coding genome, Epigenetics, Cancer

Main text

The intriguing interactions between the non-coding genome and the epigenetic machinery that regulate gene expression have preoccupied researchers over the past decades. Certainly, the question of how organisms/cells have adapted to these multiple regulatory mechanisms continues to resurface. Despite the fact that the functional existence of microRNAs relies exclusively on miRNA-mRNA interactions, another obvious concern is why the crosstalk of lncRNAs and microRNAs is so pivotal and what is the rationale behind the involvement of epigenetic enzymes in this complexity. With recent advances, it is undeniable that a deregulated noncoding genome poses a critical factor in diseases [1], particularly cancer, and epigenome mapping has highlighted that certain patient cell populations can be sensitive to drugs and therapies [2]. Thus, the mutual interactions between the noncoding genome and the epigenetic machinery exert their biological functions in the dysregulated genome.

Since it is of utmost importance to understand the complex regulatory web of these mutual interactions, Wu and colleagues presented some interesting results on this concept using glioblastoma model [3]. The authors successfully confirmed the altered expression of lncRNA (LINC00461) after inhibition of histone deacetylase 6 (HDAC6) and also identified the interaction of HDAC6 and RNA-binding proteins in regulating its stability. A further section to be appreciated was the methodology for predicting lncRNA-miRNA mRNA networks, which prompted us to try a similar approach in multiple myeloma (MM). We directly used reliable publicly available MM datasets and identified an HDAC6-induced

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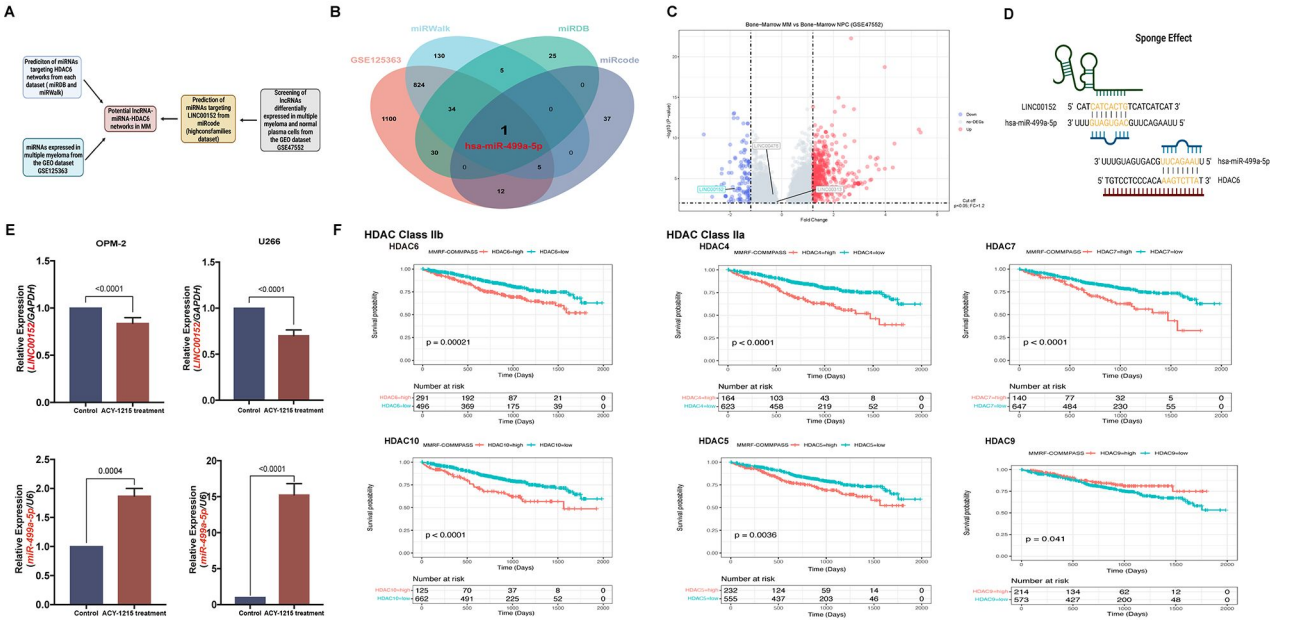

Fig. 1 HDAC6- LINC00152- hsa-miR-499a-5p network in multiple myeloma. (**A**) Workflow for the identification of potential regulatory LINC00152 miRNA-HDAC6 networks. (**B**) Overlapping miRNA was identified using relevant public databases (GSE125363, miRDB, miRWalk and prediction of miRNAs targeting LINC00152 from miRcode (highconsfamilies dataset)). (**C**) Volcano plot for screening the lncRNAs differentially expressed in multiple myelomas obtained from GEO dataset GSE47552. (**D**) LINC00152 acted as a sponge for hsa-miR-499a-5p in MM cells. Schematic representation of the binding sites between has-miR-499a-5p and HDAC6 3'UTR. (**E**) Relative mRNA expression levels of LINC00461 and hsa-miR-499a-5p in MM cell lines (OPM-2 and U266) treated with either DMSO or 2 μM HDAC6 inhibitor (ACY-1215). Results represent data from three separate experiments. Data are presented as mean ± standard deviation (SD). ($p < 0.05$, unpaired Student's t-test). (**F**) HDAC class IIb (HDAC6 and HDAC10) and HDAC class IIa (HDAC4, HDAC5, HDAC7 and HDAC9) showed significant prognostic ability in multiple myeloma retrieved from MMRF-COMMpass database

lncRNA (LINC00152) and its possible sponge miRNA (hsa-miR-499a-5p) (Fig. 1, supplementary file 1). Interestingly, we confirmed that the clinically applicable HDAC6 inhibitor (ACY-1215/Ricolinostat) was capable of inducing alterations in the expression of LINC00152 and hsa-miR-499a-5p in MM cell lines (OPM-2 and U266). To determine whether targeting HDAC6 and its non-coding network is vulnerable in the clinic, we further examined the expression pattern of the HDAC family in MM and found both members of HDAC class IIb (HDACs 6 and 10) as prognostically relevant. Except for some (HDAC11, SIRT2 and SIRT4), several other members of the HDAC family also showed prognostic significance in MM (Supplementary Fig. 1).

Undeniably, HDAC6 as a selective inhibitor has more advantages over other non-selective pan-HDAC inhibitors [4]. While the availability of newer HDAC6 selective inhibitors for the treatment of MM is exciting [5, 6], so is the synergistic compatibility of HDAC6 with non-oncology drugs (e.g., Meticrane) [7], raising the possibility of broader clinical application. Being a pioneer in cytokine-induced killer cell (CIK) immunotherapy, we have already demonstrated the beneficial effect of CIK cells with HDAC inhibitors against MM cells, and therefore HDAC6-specific clinical trials in this context can reasonably be anticipated in the future [8]. Independently, the role of the non-coding genome in MM, which has long been underappreciated, is increasingly being recognized [9, 10, 11]. Therefore, it will be of future interest to find out whether the integrated network of HDAC6, non-coding genome, and targeting mRNAs has any potential overlaps in cancer types (e.g. in MM) or diseases in general [12]. Nevertheless, the involvement of HDACs (especially HDAC6) in inhibiting or promoting cancer development and progression is becoming more apparent [12. Ongoing research to identify the underlying mechanisms is, of course, also receiving a boost [13]. Similar scenario is also quite apparent for non-coding genome [14, 15]. Now the significant work by Wu and colleagues has added an additional layer of information by offering insights into the HDACs- lncRNA-miRNA-mRNA axis. It is now foreseeable that more studies using the same axis in different cancer types may help to find a common module with anticancer potential.

Abbreviations

HDAC	Histone deacetylates
lncRNAs	Long noncoding RNAs
miRNAs	MicroRNAs

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Authors' contributions
Writing, original draft—J. P, T.L, A.S, IGHS-W. The authors read and approved the final manuscript.

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Declarations

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4. Discussion with references

The mechanisms underlying HDACis have been extensively explored through preclinical experiments and clinical trials, significantly expanding the scope of cancers treatable with these agents, particularly MM. Clinical trials have provided a wealth of knowledge on the efficacy of HDACis, whether used alone or in combination with other drugs. Panobinostat, an orally administered HDAC inhibitor, stands out as a particularly effective option for MM patients seeking additional treatment, especially in cases of relapsed or refractory MM (San-Miguel et al., 2014). However, several preclinical studies suggest that HDACis, including in combination therapies for MM, could be promising alternatives to conventional regimens (Ferro et al., 2023; Zhou et al., 2022). Similarly, CIK cell immunotherapy, which marks its 30th anniversary, has shown success in MM clinical trials (Sharma & Schmidt-Wolf, 2021). Surprisingly, there have been no clinical trials testing HDACis in combination with CIK cells, an area that may hold potential for future research. Our primary objective is to delve into the distinct contributions of HDACis and CIK cell therapy in the onset and progression of MM. Additionally, we aim to uncover the synergistic effects of these treatments when used in combination, particularly focusing on the molecular mechanisms that enhance their efficacy against MM. This investigation not only deepens our understanding of individual therapies but also sets the stage for innovative, more effective treatment strategies.

In the first publication, we tested the effects of HDACis, specifically panobinostat and romidepsin, in combination with CIK cells on different MM cell lines. We found that panobinostat combined with CIK cells enhanced cell destruction in most MM lines, while romidepsin was effective only in certain lines. Notably, both drugs increased early apoptosis and IFN-γ secretion, suggesting that HDACis may boost CIK cells' ability to kill tumor cells. We also explored how these drugs might interact with immune cells, finding that both increased the expression of certain molecules that activate NKT cells, enhancing their tumor-killing effects. This suggests a promising mechanism by which CIK cells can be more effective against myeloma when combined with HDACis. Given these positive results and the established use of CIK cell therapy in clinical settings, our findings support the

potential of combining CIK cells with HDACis as a promising treatment strategy for myeloma, warranting further investigation and application in clinical (Pu et al., 2024; Liu et al., 2024).

In the second publication, we provided a comprehensive analysis of the pivotal role that HDACis play in MM, as well as their clinical evaluation (Pu et al., 2024; Stephan et al., 2017). We focus on the various effects of inhibiting histone deacetylation in MM and explored the rationale for combining HDACis with other medications or immunotherapies that target different pathways to enhance their effectiveness. Additionally, we examine the mechanisms underlying resistance to histone deacetylation inhibition and discuss potential strategies to overcome this resistance through combination therapies. Ultimately, we offer an in-depth review of the clinical efficacy and safety data for HDACis-based treatments in various MM treatment scenarios, highlighting the importance of these drugs as a primary treatment option for MM.

Altogether, our study provides preliminary evidence that HDACis can potentiate the therapeutic effects of CIK cells in MM. This combinatory approach holds potential for improving patient outcomes in MM, warranting further investigation in clinical trials to assess its efficacy and safety in a more diverse patient population.

4.1 Implications for MM treatment

The potential for HDACis to modify the tumor microenvironment and improve the efficacy of immunotherapeutic strategies presents a promising avenue for the treatment of MM. By combining HDACis with CIK cell therapy, there may be an opportunity to overcome some of the limitations faced by current monotherapies, such as resistance and limited duration of response. Moreover, the safety profile of HDACis in our study was favorable, which supports the feasibility of this combinatory approach in clinical settings.

4.2 Limitations and future directions

While the results are promising, our study faces limitations, including the small sample size and the in vitro model system that may not fully recapitulate the complexity of the human immune system and tumor microenvironment. Future studies should aim to validate these findings in a clinical setting with a larger cohort of MM patients. Additionally, exploring the mechanistic pathways through

which HDAC inhibitors enhance CIK cell function could further optimize this therapeutic strategy.

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6. List of academic publications

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