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Histone deacetylase inhibitors and cell death

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Abstract

Histone deacetylases (HDACs) are a vast family of enzymes involved in chromatin remodeling and have crucial roles in numerous biological processes, largely through their repressive influence on transcription. In addition to modifying histones, HDACs also target many other non-histone protein substrates to regulate gene expression. Recently, HDACs have gained growing attention as HDAC-inhibiting compounds are being developed as promising cancer therapeutics. Histone deacetylase inhibitors (HDACi) have been shown to induce differentiation, cell cycle arrest, apoptosis, autophagy and necrosis in a variety of transformed cell lines. In this review, we mainly discuss how HDACi may elicit a therapeutic response to human cancers through different cell death pathways, in particular, apoptosis and autophagy.

Keywords

Histone deacetylases (HDACs); Histone deacetylase inhibitor (HDACi); cell death; apoptosis; autophagy

1. Introduction

Epigenetic alteration, which refers to the regulation of gene expression *via* post-translational modification of the chromatin structure without changes in the underlying DNA sequence, plays crucial roles in diverse physiological and pathological cellular processes [1]. In particular, acetylation, one of the most common modifications in epigenetics, serves as a key regulatory mechanism for chromatin structure and gene expression [2]. Acetylation is tightly governed by opposing actions of two large families of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs): Hyperacetylation of the N terminus of histone tails induced by HATs results in an open chromatin that frequently correlates with gene activation, whereas deacetylation by HDACs has been shown to mediate a closed chromatin confirmation and transcriptional suppression [3, 4]. The balance between these two antagonistic actions governs numerous developmental processes and can result in disease if dysregulated.

It has been widely recognized in recent years that HDACs are promising targets for therapeutic interventions intended to reverse aberrant acetylation states. Therefore, there has been considerable effort to develop HDAC inhibitors (HDACi) [5]. In various transformed cells, HDACi can induce different phenotypes, including, but not limited to, growth arrest, differentiation, and apoptosis [6]. Although the effect of HDACi on histones is well understood, recent evidence suggests that the anti-proliferative action of HDACi might not be exclusively due to the modulation of gene expression through histone remodeling. A steadily growing number of non-histone proteins modulating a wide variety of cellular events and biological processes have now been identified as substrates for HDACs [7].

2. HDAC superfamily

According to functional and phylogenetic criteria, HDAC family proteins have been divided into four classes: class I, II, III, and IV, which differ in structure, enzymatic function, subcellular localization, and expression patterns [3, 8]. *Class I HDACs* include HDAC1, 2, 3, and 8, which are most closely to the yeast Rpd3 [9, 10]. Class I HDACs are found to be ubiquitously expressed, located almost exclusively in the nucleus, and show strongest enzymatic activity among the HDAC classes. Of note, HDAC1 and HDAC2 share a substantial functional redundancy and a high sequence similarity with 82% amino acid identity for the human isoforms [11-13]. They always co-exist in multi-protein repressor complexes such as Sin3A, NcoR/SMRT, Co-REST, Mi2/NuRD and EST1B [3]. However, other studies also show distinct functions for HDAC1 and HDAC2 [14]. *Class II HDACs* consist of two subclasses with similarity to yeast Hda1: class IIa (HDAC4, 5, 7 and 9) and class IIb (HDAC 6 and 10). Compared to class I HDACs, their expression pattern is more restricted and their function is more tissue specific. Class IIa HDACs can shuttle between the nucleus and the cytosol in response to different stimuli, whereas HDAC6 and HDAC10 mainly localize in the cytoplasm [15, 16]. HDAC11 is the only known member of *class IV HDAC*, but very little information is available about its expression and function [17]. Class I, II, and IV HDACs make up the “classical HDACs”, which require zinc as a co-factor.

Class III HDACs refers to sirtuins, homologues of yeast Sir2, which is independent of zinc and dependent on NAD⁺ [18]. Each of the seven mammalian sirtuin proteins (called Sirt1–Sirt7) has a distinct subcellular localization: Sirt1, Sirt6 and Sirt7 are localized in the nucleus, while Sirt2 is predominantly cytosolic, and Sirt3, Sirt4, and Sirt5 appear to be found exclusively in the mitochondria. Whereas much is known about Sirt1, comparatively little is known about other Sirt family proteins [19]. However, there is now a growing interest in understanding the function of these related family members, especially as increasing evidence has demonstrated that they are critical transcriptional regulators [20].

Although histones are the most extensively studied substrates of HDACs, accumulating evidence suggest that many, if not all, HDACs can deacetylate non-histone proteins, at least *in vitro*, and an increasing number of proteins are being identified as substrates of HDACs. The tumor suppressor p53 is one of the non-histone targets of acetylation/deacetylation: it can be deacetylated by HDAC1 and the class *III* HDAC Sirt1, resulting in inhibition of p53-induced transcription [21, 22]. More recently, HDAC1 and HDAC2 have been found to suppress p53 hyperacetylation in the embryonic epidermis [23]. In addition to transcription

factors, other classes of nonhistone proteins are regulated by dynamic acetylation and deacetylation, including, but not limited to, signal transduction mediators, cytoskeletal proteins, molecular chaperones, nuclear import factors, and viral proteins [7]. These HDAC substrates are directly or indirectly involved in numerous important cell pathways including control of gene expression, and regulation of cell proliferation, differentiation, migration, and death.

3. HDAC inhibitors

The dysfunction of transcriptional repression mediated by HDACs may lead to carcinogenesis. Indeed, altered expression of individual HDACs in tumor samples has been reported [24]. For example, overexpression of HDAC1 has been reported in prostate, gastric and breast cancer cells [25-27] and increased expression of HDAC2 and HDAC3 has been observed in colorectal cancer [28, 29]. However, increased levels of HDAC6 have been reported to correlate with improvement in recovery and overall survival in patients with hormone-sensitive breast cancer [30]. And reduced expression of class II HDAC enzymes HDAC5 and HDAC10 has been associated with poor prognosis in lung cancer patients [31]. Accumulation of HDAC2 in transformed mouse embryonic fibroblasts (MEFs) lacking a HECT domain ubiquitin ligase Mule (Mcl-1 ubiquitin ligase E3) [32] confers cellular resistance to HDACi-induced apoptosis. More importantly, the resistance can be fully rescued by lowering the elevated HDAC2 in Mule-null cells to the normal levels as in wild-type cells [33], suggesting that altered expression of HDACs could have an active role in cellular response to HDACi, and highlighting HDACs as attractive targets for therapeutic intervention. At the same time, the involvement of histone acetylation and deacetylation in numerous physiological and pathological cellular processes suggests that pharmacologic inhibition of HDACs could result in nonspecific effects as a consequence of global regulation of gene expression. It has been reported that 8-20% of genes are regulated by HDACi at the transcriptional level *via* their inhibition of HDAC function on histone tails [34-36]. Importantly, HDACi could also target gene transcription through indirect mechanisms by inhibiting HDAC interactions with non-histone proteins. To this point, HDACi have recently emerged as a novel therapeutic group of drugs with potent anti-cancer function [24].

On the basis of their chemical structure, HDACi can be classified into four different groups, including hydroxamates, cyclic peptides, aliphatic acids and benzamides [37] (Table 2). Furthermore, since HDACi do not inhibit all HDACs to the same extent, they may be grouped into pan- or class I specific inhibitors [38]. For example, Trichostatin A (TSA) and its structural analog suberoyl anilide hydroxamic acid (SAHA) are both hydroxamate derivatives and are pan-HDACi targeting class I, II, and IV HDACs. Aliphatic acids are another well-known group of HDACi including valproic acid (VPA) and sodium butyrate. These compounds are class I-specific inhibitors, although they are less efficient in their HDAC-inhibiting capacity with IC₅₀ in the milli-molar range compared to the nanomolar range for the pan-HDAC inhibitor TSA.

As mentioned above, Class III HDACs are structurally and enzymatically distinct from classes I, II and IV, thus classical HDACi's cannot inhibit class III HDACs. Much less is

known about the biological consequences of sirtuin inhibition. To date, only a small number of sirtuin inhibitors have been described [39] and majority of studies have focused on the inhibition of human Sirt1 and/or Sirt2. Class III HDACs require the binding of an NAD⁺ molecule in their active site to enable deacetylation of target proteins [40]. Nicotinamide competitively binds to the NAD⁺ binding site of sirtuins, preventing NAD⁺ from binding and thus inhibiting deacetylation of acetylated substrates such as histone proteins [40]. Therefore, it is an unspecific sirtuin inhibitor of all seven sirtuins. Another more specific inhibitor of class III HDACs is Suramin, a symmetric polyanionic naphthylurea, which has been shown to inhibit both Sirt1 and Sirt2 isoforms.

TSA, the most potent HDAC inhibitor so far, is also the first natural product and was found to possess HDAC inhibitor activity in 1990 [41, 42]. However, since the production of TSA is expensive and it showed toxicity in clinical trials, it is now mainly used as a reference compound for newly discovered HDACi. SAHA, the structural analog of TSA, is the first FDA-approved HDAC inhibitor for the clinical treatment of cutaneous T-cell lymphoma (CTCL) patients [43-46]. In a phase II trial enrolling 74 patients with stage IB and higher CTCL who failed two systemic therapies, 61 patients (82%) had stage IIB or higher grade CTCL and 30 patients (41%) had Sézary syndrome. The objective response rate was 30%, the estimated median response duration was 168 days, and the median time to tumor progression was 202 days [46]. Other inhibitors, for example, the class I-selective FK228 (Depsipeptide, romidepsin) [47-49] and MGCD0103 (mocetinostat) [50], the class I- and IIa-selective valproic acid [51], and the pan-inhibitor PXD101 (belinostat) [52, 53] and LBH589 [54, 55] have also been demonstrated therapeutic potential in CTCL and other malignancies. Currently at least 80 clinical trials are underway, testing more than 15 different HDAC inhibitory agents for both hematological and solid malignancies [56] (Table 3).

There are also numerous preclinical and clinical studies that support the synergistic effect of HDACi in combination with diverse chemical and biological therapeutic agents. For example, many of the earliest combination studies of HDACi with retinoic acid were conducted in patients with acute myeloid leukemia (AML), in which HDACs are aberrantly recruited to the fusion proteins PML-RARα or PLZF-RARα [57, 58]. In cancers that poorly respond to chemotherapy, treatment with HDACi can increase the sensitivity of the cancer cells to other drugs and treatments [59]. They have therefore been tested in solid tumors in combination with conventional chemotherapeutic agents including taxanes, gemcitabine, fluorouracil, imatinib, bortezomib, and ionizing radiation [5].

4. Signaling pathways involved in HDACi-induced apoptosis

Treatment of tumor cells with HDACi can lead to histone acetylation, and derepression of gene expression, thus increasing the susceptibility of tumor cells to apoptosis. Induction of apoptosis seems to be the predominant route of HDACi-induced cell death [60], and this is of considerable therapeutic value for the treatment of cancer [5]. In most cases, HDACi induce death of tumor cells by apoptosis through either the extrinsic (death receptor) or intrinsic (mitochondria) pathways.

4.1 Extrinsic (death receptor) pathway

In the extrinsic pathway, tumor necrosis factor (TNF) superfamily death receptors such as Fas, tumor necrosis factor receptor (TNFR), TNF-related apoptosis-inducing ligand-receptor 1 (TRAIL-R1) and TRAIL-R2 bind to their cognate TNF superfamily receptor ligand. This leads to the assembly of a death inducing signal complex (DISC) on death domains found in the cytoplasmic regions of the receptors that are required for death signal transmission [61, 62]. The DISC includes the adaptor protein FAS-associated death domain (FADD) together with procaspase-8 and procaspase-10, which induce the apoptosis signal by direct cleavage of downstream effector caspases such as caspase-3 [63-65].

HDACi have been shown to augment signaling through the extrinsic apoptosis pathway *via* diverse mechanisms including upregulation of cell surface death receptors and/or ligand expression, reductions in the level of cytoplasmic FLICE-like inhibitory protein (c-FLIP) and enhanced recruitment of DISC formation. Importantly, the effects of HDACi on the extrinsic apoptosis pathway can enhance the sensitivity of many tumor cell types to activators of this pathway, such as TRAIL. For example, many investigators have observed that HDACi upregulates death receptor TRAIL-R2 expression and causes a decrease of c-Flip expression in various human malignant tumor cells [66-68], which provokes the rapid DISC production in the presence of TRAIL and the activation of caspase-8. Consequently, HDACi synergistically sensitizes cells to apoptosis induced by recombinant human TRAIL in human hepatocellular carcinoma cells and human leukemia cells. HDACi can induce the expression of TRAIL by directly activating the promoter of TNFS10 gene that encodes TRAIL, thereby triggering death signaling in acute myeloid leukemia (AML) cells [69].

Additionally, HDACi are reported to facilitate the recruitment of FADD and caspase-8 to form the active DISC complex. In chronic lymphocytic leukemia (CLL) which is inherently resistant to TRAIL-induced apoptosis, HDACi such as FK228 at low concentrations which is unable to induce apoptosis markedly sensitize CLL cells to TRAIL-induced apoptosis by facilitating the formation of an active DISC, leading to the rapid activation of caspase-8 [70]. While depsipeptide does not enhance TRAIL binding to TRAIL-R1, TRAIL-R1 aggregation, or internalization of TRAIL-R1, it does enhance FADD recruitment to TRAIL-R1 in the DISC [71].

The functional importance of the extrinsic pathway in HDACi-induced cell death has been investigated by RNA interference or knockout of the death receptors/ligands. In AML cells, RNA interference against TRAIL blocked downstream procaspase-8 activation and inhibited HDACi MS275-mediated apoptosis, suggesting that TRAIL mediates the anticancer action of HDACi [69]. However, studies by other groups using the E μ -myc lymphoma model show that death receptor pathway blockade by knockout of TRAIL has no effect on the therapeutic activities of LAQ824 and LBH589 [72]; Therefore, the importance of the extrinsic pathway in HDACi-induced apoptosis is possibly cell-type dependent.

4.2 Intrinsic (mitochondria) pathway

The mitochondrial pathway is initiated by the release of cytochrome *c*, from the mitochondrial intermembrane space to the cytoplasm, where it binds to Apaf-1 [73, 74].

Cytochrome *c* binding to Apaf-1 triggers the formation of the apoptosome, which activates procaspase-9 [75]. Activated caspase-9 cleaves and activates caspase-3 and caspase-7, which subsequently cleave many intracellular substrates, leading to the characteristic morphological changes associated with apoptosis [76]. In addition to cytochrome *c*, several other apoptogenic proteins are also released from the mitochondrial intermembrane space, including Smac/Diablo [77, 78], which antagonize the caspase-inhibitory IAP proteins [79-81].

The intrinsic cell death pathway involves the interplay of pro- and anti-apoptotic Bcl-2 superfamily proteins [82]. The pro-apoptotic Bcl-2 members promote cytochrome *c* release [83], while anti-apoptotic Bcl-2 proteins such as Bcl-2, Bcl-xL, and Mcl-1 protect mitochondrial integrity [32, 84-87]. The pro-apoptotic Bcl-2-family proteins consist of two groups: 1) multidomain proteins Bax and Bak that may initiate mitochondrial membrane permeability [88], and 2) BH3-only proteins Bad, Bik, Bid, Bim, Bmf, Puma, and Noxa which act as sensors of cellular stress for activation of the intrinsic apoptotic pathway [89]. Among the anti-apoptotic members of the Bcl-2 family, Mcl-1 is unique in that it is an early-response gene that can be rapidly induced and turned over, which enables it to function at an early step in a signaling cascade consisting of Bcl-2 family proteins and provides an acute protective function against apoptotic stimuli [32, 84].

In addition to acting through the extrinsic death receptor pathway, HDACi also induce apoptosis in a series of cancer cells through activation of the intrinsic mitochondria pathway *via* decreased expression of anti-apoptotic proteins Bcl2, Bcl-xL and enhanced expression of proapoptotic proteins Bax and Bak [90-92]. For example, TSA treatment repressed the transcription and downregulated the expression of the anti-apoptotic Bcl-2 proteins Bcl-2 and Bcl-xL in TRAIL-resistant myeloma cells [93]. A significant decrease in Bcl-xL protein expression was noted after sodium butyrate exposure in mesothelioma, and this was corroborated at the transcriptional level with selectively decreased Bcl-xL mRNA production [94]. These studies collectively suggest that decreased expression of anti-apoptotic Bcl-2 family genes might be a trigger for HDACi-induced apoptosis. In accordance with this, inhibition of anti-apoptotic Bcl-2 family proteins sensitizes cells to the cytotoxic activity of HDACi. Conversely, over-expression of anti-apoptotic Bcl-2 family proteins can antagonize HDACi-induced apoptosis in a series of cancer cell lines such as human malignant B cells and melanoma cells [95-98]. Moreover, an *in vivo* model of Eμ-myc-driven lymphoma further demonstrated that overexpression of Bcl-2 or Bcl-xL protected lymphoma cells from HDACi-induced killing [72, 99].

It has been reported by several groups that HDACi can also up-regulate pro-apoptotic Bcl-2 family proteins, such as Bim, Bmf, Bad, Bid, Noxa, Puma, Bax, and Bak in different tumor cell types at both transcriptional and (or) posttranscriptional levels [100-102]. Transcriptional activations of Bim, Bmf, Noxa, and Puma upon HDACi treatment have been found to be due to H3 and/or H4 hyperacetylation in their promoter regions [103, 104]. The functional importance of the pro-apoptotic Bcl-2 family proteins in HDACi-mediated apoptosis has been widely studied. For example, silencing of Bim by siRNA effectively abolishes E2F1-mediated cell death sensitization to HDACi [101]. Similarly, a key role for both Bim and Noxa has been proposed in HDACi-mediated apoptosis based on the finding

that RNAi against Bim and Noxa largely prevents the HDACi-induced losses in mitochondrial membrane potential and caspase processing [105, 106]. In support of these data, *in vivo* studies also suggest that Bim plays a pivotal role in mediating the apoptotic effects of SAHA [99]. SAHA specifically induces Bim mRNA in mice bearing E μ -myc lymphomas, whereas knockout of Bim in these lymphomas leads to inhibition of vorinostat-mediated apoptosis *in vitro* and *in vivo* and suppression of the therapeutic effects of vorinostat.

Death receptors can also activate the intrinsic pathway in addition to the extrinsic death pathway. For example, simultaneous exposure of leukemia cells to TRAIL and SAHA results in a dramatic increase in mitochondria damage, caspase activation and apoptosis [107]. This increase is significantly diminished with dominant-negative caspase-8, dominant-negative FADD, CrmA (receptor pathway), Bcl-2 or Bcl-xL (mitochondrial pathway), indicating that the extrinsic and intrinsic pathways are simultaneously activated. In this way, the intrinsic pathway becomes activated by caspase-8-mediated cleavage of Bid, which serves as a molecular link between the extrinsic and intrinsic pathways and can amplify the death receptor-stimulated apoptotic effect.

Besides the classical caspase-dependent pathways, several studies have indicated that other mitochondrial proteins such as AIF could be released from the mitochondria to execute the cell death process [108]. Fandy et al demonstrated that cytosolic release of AIF and shuttling of AIF to the nucleus was induced by SAHA or TSA in multiple myeloma [109]. The activation of AIF may be a common theme because its cytosolic release has been observed in human leukemia cells following treatment with HDACi MS-275 and LAQ824 [110, 111].

4.3 Alternative mechanisms of HDACi-activated cell survival and death

The mechanism by which HDACi induce cell death is still not completely understood. Although many actions are shared by the majority of HDACi, the detailed mechanisms of individual HDACi appear to vary with each agent. Those molecular mechanisms involving regulation of apoptosis are discussed here.

4.3.1 Non-histone substrates—Acetylation of non-histone proteins has been demonstrated to modulate protein function by altering their stability, cellular localization, and protein-nucleotide/protein-protein interactions. Interestingly, many non-histone proteins targeted by acetylation are important for tumorigenesis, cancer cell proliferation, cell death, and immune functions. To name a few of them:

4.3.1.1 NF- κ B: NF- κ B is now firmly established as a key transcriptional regulator of gene expression in the inflammatory response with anti-apoptotic effect [112]. Mammalian NF- κ B functions as homodimers or heterodimers generated from combinations of five Rel family proteins (RelA, c-Rel, RelB, p50 and p52) which share many conserved structural and functional features. RelA and p50 constitute the so-called canonical NF- κ B pathway that is thought to play a dominant role in the control of inflammation. Under resting conditions these dimers are associated with the inhibitory protein I κ B α , which prevents NF- κ B transport to the nucleus and interferes with DNA binding [113]. In response to inflammatory stimuli, I κ B α is phosphorylated, ubiquitinated and degraded. Degradation of

I κ B α allows for NF- κ B release and translocation to the nucleus, leading to increased gene transcription.

Several studies have found that HDAC activity is required for the efficient initiation and/or elongation of pro-inflammatory gene transcription mediated by NF- κ B. Inhibition of NF- κ B activation contributes, at least partially, to the antitumor activity of HDACi in multiple cancer cell lines [114-117]. However, other reports demonstrate that acetylated forms of RelA are subject to deacetylation by HDAC3 that promotes I κ B α binding and terminates the NF- κ B response. For example, Chen *et al* reported that RelA subunit of NF- κ B is subject to inducible acetylation and that acetylated forms of RelA interact weakly, if at all, with I κ B α . Acetylated RelA is deacetylated through a specific interaction with HDAC3. This deacetylation reaction promotes effective binding to I κ B α and leads to I κ B α -dependent nuclear export of the complex. Therefore, deacetylation of RelA by HDAC3 acts as an intranuclear molecular switch that controls the duration of the NF- κ B transcriptional response [118, 119]. Based on these data, in the presence of TSA, HDAC3 inhibition leads to a prolonged activation of NF- κ B and a subsequent increase in the pro-inflammatory response. In line with this, HDAC inhibitors block HDAC3-mediated deacetylation of the p65/RelA NF- κ B subunit, leading to impairment of I κ B α binding and increased NF- κ B nuclear translocation [118, 120, 121]. According to these studies, activation of NF- κ B following HDAC inhibition may well be cytoprotective (pro-survival) and thus an important mediator of HDACi-resistance. Taken together, these data suggest that the regulation of NF- κ B in HDACi treatment can play a dual role in cancer therapy -cytoprotective or cytotoxic, which is dependent on the nature of HDACi, concentration and/or time of exposure, and the cell type.

4.3.1.2 p53: p53 is often inactivated or mutated in cancers. In response to stress such as DNA damage, p53 is modified and accumulates in the nucleus following its dissociation from its E3 ubiquitin ligase Mdm2. In the nucleus, p53 activates a series of target genes, including the cyclin-dependent kinase inhibitor p21 resulting in cell cycle arrest, and pro-apoptotic genes (e.g. Bax, Puma and Noxa) to induce apoptosis [122-127]. These stress-induced modifications of p53 mainly involve phosphorylation mediated by kinases (ATM, CHK1, CHK2), and acetylation controlled by acetyltransferases such as CBP/ p300 and deacetylases including the HDAC1/mSin3 complex and Sirt1 [21, 22, 128].

The importance of p53 acetylation in HDACi-mediated apoptosis is controversial. In most reports, apoptosis and p21 induction upon HDAC inhibition can be induced in a p53-independent manner [129-131]. However, following HDAC inhibition, acetylation of p53 has been reported in several tumor systems, which has been related to p53 stabilization, cell cycle arrest, and apoptosis [132-134]. More recently, studies have shown that HDAC inhibitor TSA prompts PUMA expression to induce apoptosis in gastric cancer cells *via* two independent ways: p53 stabilization and p53 interaction with the PUMA promoter through the inhibition of HDAC3 [135].

4.3.1.3 STAT1: STAT1 has been known to repress NF- κ B-mediated cell signaling [136]. Similar to NF- κ B, STAT1 can interact with HATs and HDACs [137]. In human melanoma cell lines undergoing apoptosis in response to HDACi and interferon α , there is an increase

expression and acetylation of STAT1. The acetylation of Stat1 functions as a molecular switch, which permits binding of Stat1 to NF- κ B, interferes with NF- κ B function and ultimately apoptosis by preventing its DNA-binding ability, nuclear localization and expression of anti-apoptotic genes [138].

4.3.1.4 Tubulin: Stable microtubules contain high levels of acetylated α -tubulin, while dynamic microtubules are largely hypo-acetylated. HDAC6 and Sirt2 are able to deacetylate α -tubulin and control the level of tubulin acetylation and assembly of the microtubule network [139, 140]. In the combined treatment of TSA and paclitaxel, a significant increase in acetylated tubulin and microtubule stabilization was observed, leading to the dramatic activation of the apoptotic cascade in endometrial cancer cell. The *in vivo* evidence in the mice model also suggests α -tubulin acetylation is one possible mechanism by which HDACi reduces cancer growth [141].

4.3.2 Reactive oxygen species (ROS)—Accumulation of ROS occurs in transformed cells cultured with HDACi, such as vorinostat, TSA, MS275, or LAQ824 [142-145]. Exposure of human leukemia cells to a higher concentration of LAQ824 resulted in the early generation of ROS before disruption of mitochondria, which has been implicated in increased oxidative stress following treatment with HDACi [144]. Pre-treatment with the ROS scavenger N-acetyl cysteine (NAC) reduces apoptosis induced by HDACi, suggesting that oxidative stress may play an important role in tumor cell death induced by HDACi [144, 146, 147]. Other studies show that enhanced ROS generation is associated with the synergistic induction of apoptosis by co-treatment with HDAC inhibitor and other drugs [146, 147]. For example, Rosato *et al.* demonstrated a synergistic effect on apoptosis in human leukemia cells induced by the combined treatment of LAQ824 and the chemotherapeutic drug fludarabine [148]. An early ROS peak was observed dependent on HDACi treatment, and ROS scavengers NAC diminished LAQ824/ fludarabine lethality, suggesting HDACi-mediated ROS induction is a cause rather than a consequence of the synergistic cell death effect of fludarabine and HDACis.

Cellular redox homeostasis is maintained through a complex set of reactions. Members of the thioredoxin (Trx) family which function as hydrogen donors and potent scavengers of ROS, participate in this process. HDACi treatment results in increased expression of Trx binding protein 2 (TBP2), which binds Trx and inhibits its activity [102, 149], and can cause downregulation of Trx in transformed but not normal cells. Additionally, RNH1, a ribonuclease inhibitor, has been found to dampen HDACi-induced ROS in gastric cancer cells. Genetic knockdown and overexpression assays show that RNH1 is both necessary and sufficient to induce HDACi resistance. Therefore, RNH1 has been identified as a regulator of HDACi resistance in gastric cancer, highlighting a functional role for ROS induction in the cellular effects of HDACi [150].

Oxidative stress can promote apoptosis due to the upregulation of the pro-apoptotic proteins and (or) activation of the extrinsic apoptotic pathway. It has been shown that ROS production is central to SAHA-induced cell death resulting from the cleavage of the BH3-only pro-apoptotic Bcl-2 family member Bid [144]. The synergistically increased apoptosis following combined treatment with a HDAC inhibitor and a PI3K inhibitor LY294002 has

been shown to be dependent on the accumulation of intracellular ROS and Bim induction. In human AML cells, the synergistic effect of HDACi and resveratrol is associated with sustained ROS production, and treatment with a free radical scavenger Mn(III)tetrakis (4-benzoic acid) porphyrin chloride blocked ROS generation, DR5 upregulation, caspase-8 activation, DNA damage, and apoptosis, indicating a primary role for oxidative injury in death of HDACi/resveratrol-treated AML cells. This process requires multiple ROS-dependent actions including death receptor up-regulation, extrinsic apoptotic pathway activation, and DNA damage induction [151].

4.3.3 Cell cycle regulator (p21)—HDACi can concurrently induce both cell cycle arrest and apoptosis. It has been well documented that increased expression of p21 is a common molecular response to HDACi. Interestingly, increased p21 expression has been correlated with both p53-dependent and independent pathways [131, 152]. It has been proposed that p21 can influence the decision of tumor cells to undergo apoptosis and/or cell cycle arrest following HDACi treatment [153, 154]. For example, overexpression of p21 leads to the cellular resistance to HDACi-induced apoptosis [155]. However, the effects of HDACi on tumor cell apoptosis in the absence of p21 are inconsistent. One study in breast cancer cells showed that HDACi-mediated p21 upregulation is required for the synergistic induction of apoptosis by the combined treatment of HDAC inhibitor and TNF α / TRAIL/anti-Fas agonist antibody, and that blocking it results in a protection in cell death [156]. In contrast, another *in vivo* study using E μ -myc/p21^{-/-} tumors demonstrated that induction of p21 does not regulate HDACi-mediated tumor cell apoptosis and refuted the notion that p21 is an obligate mediator of HDACi-induced cell cycle arrest [157]. Thus, the role of p21 as a determinant of HDAC inhibitor response is not totally understood and needs further investigation.

5 HDAC inhibitor-induced autophagy

Autophagy, a ubiquitous process of ‘self-digestion’ of cellular compartments in response to physiological changes such as starvation, growth factor signaling or cellular stress, is essential for cellular homeostasis [158-160]. In several cancer models, it has been reported that HDAC inhibitor treatment can elicit caspase-independent autophagic cell death. The autophagic cell death is characterized by the conversion of unconjugated microtubule-associated protein light chain 3 (LC3-I) to conjugated light chain 3 (LC3-II), the localization of LC3 to autophagosomes, and the accumulation of acidic vesicular organelles and/or increased expression of autophagy-related proteins such as autophagy-related gene (Atg) 5 [161-163]. The importance of acetylation in autophagy control has emerged since the last few years [164].

5.1 Acetylation/deacetylation and autophagy

So far, many ATG proteins have been demonstrated to be acetylated and their acetylations are regulated by specific HAT-HDAC pairs. When cells are deprived of growth factors, glycogen synthase kinase-3 (GSK3) can activate acetyltransferase TIP60. The activated TIP60 directly acetylates and stimulates the autophagy-initiation kinase ULK1, which is essential for starvation-induced autophagy [165]. In line with this, studies in yeast have also demonstrated that starvation-induced Atg3 acetylation is controlled by HAT Esa1 and

deacetylase Rpd3. Specifically, Atg3 acetylation at K19 and K48 regulates autophagy by controlling Atg3 and Atg8 (LC3) interaction and lipidation of Atg8. Therefore, increased Atg3 acetylation after deletion of the deacetylase Rpd3 caused increased autophagy [166, 167]. Autophagy can also be regulated through the acetylation of transcriptional factors such as FOXO [168].

In particular, HDACs can influence autophagic activity. The most extensively studied in this context is HDAC6. Expression of HDAC6, a microtubule-associated deacetylase that interacts with polyubiquitinated proteins, is sufficient to rescue degeneration associated with ubiquitin-proteasome system (UPS) dysfunction *in vivo* in an autophagy-dependent manner [169-171]. Thus, HDAC6 provides an essential mechanistic link in the compensatory interaction of induced autophagy when the UPS is impaired. Phenylephrine (PE)-triggered autophagy activation, which is required for cardiomyocyte hypertrophy, is mediated by HDAC1 and HDAC2. Knockdown of HDAC2 is more effective at inhibiting autophagy, indicating HDAC2 plays a dominant role in mediating PE-induced autophagy in cardiomyocytes [172]. In contrast, both inhibition and genetic knock-down of HDAC1 in HeLa cells significantly induce autophagic vacuole formation and lysosome function and disruption of HDAC1 leads to the conversion of LC3-I to LC3-II [173]. Although poorly studied, HDAC10 has been reported to promote autophagy-mediated cell survival in neuroblastoma. Inhibition of HDAC10 sensitized tumor cells for cytotoxic drug treatment that suggests that HDAC10 could serve as a potential biomarker for treatment response of high-risk tumors likely to bypass drug resistance [174].

The Class III HDAC Sirt1 forms a molecular complex with several essential components of the autophagy machinery, including autophagy genes Atg5, Atg7, and Atg8 and was found to stimulate basal rates of autophagy through its deacetylase activity [175]. Recently, the regulatory role for Sirt6 in autophagy activation during cigarette smoke-induced cellular senescence was examined, and it was found that Sirt6 overexpression induced autophagy *via* attenuation of IGF1-Akt-mTOR signaling. Autophagy inhibition by knockdown of ATG5 and LC3B attenuated the anti-senescent effect of Sirt6 overexpression, suggesting that Sirt6 is involved in cigarette smoke extract-induced human bronchial epithelial cells senescence *via* autophagy regulation [176].

5.2 The role of autophagy in HDACi-induced cell death

The matter of whether autophagy is protective or cytotoxic for HDACi treatment in cancer cells is an area of active investigation. Varied results have been obtained depending on the model system used in each study [169, 177]. However, in most cases, autophagy functions as a prosurvival mechanism to enable cells to recoup ATP and other critical biosynthetic molecules. For example, disruption of the autophagy pathway by chloroquine markedly augments the anti-neoplastic effects of SAHA in chronic myelogenous leukemia (CML) cell lines and primary CML cells expressing wild type and imatinib-resistant mutant forms of Bcr-Abl. These results provide a promising new strategy to treat imatinib-refractory patients who fail conventional therapy [178, 179]. Similarly, inhibition of autophagy by chloroquine significantly enhances cell death effect of a HDAC inhibitor in t(8;21) AML cells, suggesting that combined treatment of chloroquine and HDACi may serve as an attractive

treatment option for AML1-ETO-positive leukemia [180]. Knock-down of ATG7 by RNA interference in glioblastoma cells also results in an increase in SAHA-induced apoptosis [181]. Similar effects from autophagy inhibition by both genetic and chemical methods are also described in a series of cancer models including oral squamous cell carcinoma cells [182], breast cancer cells [183, 184], colon cancer cells [185], Jurkat T-leukemia cells [186], malignant rhabdoid tumors [187], and malignant peripheral nerve sheath tumors [188]. Moreover, it has been observed that when apoptosis is pharmacologically blocked, HDAC inhibitor-induced nonapoptotic cell death can also be potentiated by autophagy inhibition [181]. Therefore, targeting autophagy and its role in HDACi-induced cancer cell death may leads to potential combination therapy for cancer treatment.

On the other hand, extensive autophagic degradation of intracellular content in response to cellular response may serve as a potent death signal and lead to the cytotoxic effect of autophagy. For example, in hepatocellular carcinoma, SAHA-induced cytotoxicity is compromised when autophagy is inhibited by 3-methyladenine (3-MA) or Atg5 knockout, suggesting a pro-death function of autophagy [189]. In addition, in some cases, autophagic rather than apoptotic processes are responsible for SAHA-induced cell death in endometrial stromal sarcoma cells [190].

It should be noted that there are still doubts regarding the importance of autophagy in regulating the anti-tumor responses of HDACi. One *in vivo* study utilized genetically tractable mouse model bearing Eμ-myc/*Apaf-1*^{-/-} lymphomas and demonstrated that depletion of two essential autophagy proteins, Atg5 and Atg7, resulted in survival similar to that of the control group, indicating that inhibition of autophagy neither protected Eμ-myc/*Apaf-1*^{-/-} cells from HDACi-mediated cell death nor potentiated its anticancer effect [157]. The discrepancy might be due to different model systems and different cancers in these studies.

5.3 Molecular mechanism of HDACi-induced autophagy

Although it is unclear under which conditions autophagy induces cell survival or cell death, so far several signaling pathways have been reported to play a role in HDAC inhibitor-induced autophagy:

- 1) The nutrient-sensing kinase mammalian target of rapamycin (mTOR). mTOR is a major suppressive regulator of autophagy, is inactivated by SAHA treatment in several cancer models [181, 186, 189, 191]. mTOR suppresses autophagy by phosphorylating and inactivating the ULK1 complex, an upstream component of the autophagy pathway. mTOR inactivation by SAHA restores the function of the ULK1 complex and thereby induces autophagy.
- 2) NF-κB. In prostate cancer cells, the increased expression of several ATG genes upon SAHA treatment is achieved by the stimulation of NF-κB activity in a reversible manner *via* modulation of RelA/p65 signaling, leading to enhanced vesicular stomatitis virus oncolysis, whereas inhibition of autophagy by either 3-MA treatment or genetic ablation of Atg5 decreased virus replication and oncolysis [192].

3) ROS. In Jurkat T-leukemia cells, proteomic analysis reveals several groups of enzymes associated with energy metabolism, anti-oxidative stress and cellular redox control, which suggests an abnormal reactive oxygen species (ROS) production upon SAHA treatment. It is further confirmed by ROS chemiluminescence assay. Mechanistic studies revealed that SAHA-triggered autophagy is mediated by ROS production, which can be attenuated by ROS inhibitor NAC [186, 193].

Taken together, these studies suggest that the targeted induction of autophagic cell death by HDACi is a promising therapeutic strategy to treat cancer.

6 HDAC inhibitor-induced necrosis

Necrosis, which was initially considered as an accidental and uncontrollable process, can occur in a regulated manner [85]. For example, although death receptors usually activate apoptosis, necrotic cell death can be initiated by activation of death receptors in the presence of a caspase inhibitor to block apoptosis [194]. So far, the most extensively characterized pathway leading to necroptosis is initiated by ligation of TNFR1 [195], which requires the formation of the necrosome signaling complex containing receptor interacting protein kinase 1 (RIPK1) and RIPK3 [196-198]. There are few reports in the literature about the effect of HDACi on necrotic cell death. Transmission electron microscopy (TEM) analysis showed that HDAC inhibitor depsipeptide-treated malignant rhabdoid tumor cells exhibited necrotic morphology including cell swelling and rupture of the plasma membrane [187]. Cotreatment with HDACi SAHA and v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) inhibitors kill melanoma cells by induction of necrosis which was supported by caspase-independent release of high-mobility group protein B1 and TEM analysis [199]. Thus, it deserves further investigation to explore the necrotic effect of HDACi.

There are also some other molecular mechanisms that contribute to the execution of TNFR1-initiated necrosis. For example, the production of ROS is crucial for TNF-stimulated necrosis [200-203]. Accumulation of ROS occurs in transformed cells cultured with HDACi, such as vorinostat, TSA, butyrate or MS-275. Thus, it is reasonable to assume that ROS production is potentially essential for HDAC inhibitor-mediated necrotic cell death.

7 Conclusions and further directions

HDACi are a promising new group of anticancer agents. HDACi can induce diverse biological responses including suppression of cell proliferation, induction of differentiation, as well as cell death across a broad spectrum of different types of hematologic and solid neoplasms, which act cooperatively to mediate the potent anti-tumor activities of HDACi. The molecular processes underlying the effects of HDACi remain to be fully elucidated. Importantly, identifying the molecular target of individual HDACi will remarkably help us to understand the efficacy as well as side-effect of individual HDACi for its further improvement.

At a more fundamental level, the selectivity of HDACi in altering gene transcription is incompletely understood, as are the differences of the biological functions of different HDACs. Since most HDACi target either all or at least a wide range of HDACs, another

interesting perspective will be to differentiate the role of individual HDACs in different tissues and in different tumor models, and to develop isoform-specific HDACi. Because HDACs can affect various substrates, it will be meaningful to develop isoform-selective inhibitors to block the pathological actions of HDACs. Understanding this question will most likely broaden the therapeutic window of HDACi.

HDACi have histone and many non-histone protein substrates. Although the exact number and variety of proteins that are acetylated/ deacetylated in the cell is still unknown, it is clear that far more proteins are modified by this mechanism than initially expected. How to identify the non-histone substrates of HDACi? What kind of roles do non-histone substrates of HDACs have in the induction of cell death or other cellular effects caused by inhibiting the deacetylation? Answers to these questions will help us to further understanding of the process of oncogenic transformation and may lead to the development of more effective agents to treat cancers.

It's apparent that HDACi-mediated cell death is a complexity and is regulated by more than one pathway, such as apoptosis, autophagy and possibly necrosis. Although it's still under doubt whether autophagy contributes to HDACi-induced cell death, accumulating evidence suggest that acetylation can regulate autophagic process at multiple levels, including the core machinery of autophagy such as ATG proteins. Investigating the autophagic process by acetylation is emerging as a new and exciting field. It will also provide insights into the development of novel cancer therapeutic approaches that specifically target the autophagy machinery.

Moreover, HDACi have synergistic or additive antitumor effects with many other antitumor reagents, and such combinations represent a very attractive therapeutic strategy. Thus far, a large number of clinical trials have been completed and are ongoing in patients with both hematological and solid malignancies using a wide variety of HDACi. To this end, a synergistic screening will provide an unbiased approach to determine the synergistic effect of HDACi and other drugs and a more clearly mechanistic rationale for their therapeutic application with defined genetic lesions.

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Figure 1. The molecular mechanism of HDACi-induced cell death

So far, induction of apoptosis is the predominant route of HDACi-induced cell death. HDACi-mediated apoptosis is initiated by the hyperacetylation of histone and non-histone proteins: Core histone hyperacetylation results in an open chromatin that frequently correlates with gene activation. Non-histone protein acetylation can promote the induction of apoptosis by modulating protein function through altering their stability, cellular localization, and protein nucleotide /protein-protein interactions. In particular, A series of transcription factors (including NF- κ B, p53, and signal transducers and activators of transcription – STATs) are directly acetylated by HDACi, which augments their capacity to bind DNA and selectively modulate the expression of apoptotic genes. Through the combined effects on histone and non-histone acetylation, HDACi activates either extrinsic pathway (such as up-regulation of death receptors and/or ligand expression) and (or) intrinsic pathway (such as decreased expression of anti-apoptotic proteins Bcl2, Bcl-xL and enhanced expression of pro-apoptotic proteins Bax, Bak) to induce apoptosis. HDACi can augment p21 expression in tumor cells, which can influence the decision of tumor cells to undergo apoptosis and/or cell cycle arrest following HDACi treatment. Moreover, HDACi-treatment causes the generation of reactive oxygen species (ROS) through Trx down-regulation to possibly initiate or enhance apoptotic signalling in tumor cells. More recently, HDACi have been shown to induce tumor cell death with morphological features of autophagy, although there are doubts regarding the importance of autophagy and the pro-survival/ pro-death function of autophagy in regulating the anti-tumor responses of HDACi.

Several signaling pathways including ROS regulate HDACi-mediated autophagy. Another possibility is that HDACi may induce necrosis in tumor cells.

Table Characteristic of HDAC family proteins

classes	HDACs	Localization	Size (AA)	non-histone targets
Class I	HDAC1	Nucleus	483	p53[21, 22], STAT3[204], YY1[205], androgen receptor[206], estrogen receptor[207], SHP[208], MyoD[209], E2F-1[210]
	HDAC2	Nucleus	488	STAT3[204], YY1[205]
	HDAC3	Nucleus	423	SRY[211], STAT3[204], YY1[212], SHP[208], GATA1[213], GATA2[214], RelA[119]
	HDAC8	Nucleus	377	
	HDAC4	Nucleus/cytoplasm	1084	GATA1[213]
Class IIa	HDAC5	Nucleus/cytoplasm	1122	GATA1[213]
	HDAC7	Nucleus/cytoplasm	855	
	HDAC9	Nucleus/cytoplasm	1011	
Class IIb	HDAC6	Mainly cytoplasm	1215	SHP[208], α -Tubulin[140], Hsp90[215]
	HDAC10	Mainly cytoplasm	669	
Class IV	HDAC11	Nucleus/cytoplasm	347	
	Sirt1	Nucleus	747	p53[216], NF- κ B[217], FOXO1[168], MyoD[218], Ku70[219], p300[220]
Class III	Sirt2	cytoplasm	389	α -Tubulin[139]
	Sirt3	Nucleus/mitochondria	257	acetyl-coA synthetase[221]
	Sirt4	mitochondria	314	Glutamate dehydrogenase[222]
	Sirt5	mitochondria	292	
	Sirt6	Nucleus	355	DNA polymerase[223]
	Sirt7	Nucleus	400	

Table 2

The classification of HDAC inhibitors

	Compounds	Selectivity	Potency	Clinical status
Hydroxymates	SAHA (vorinostat)	pan	μM	Approved, II /III
	PXD101 (belinostat)	pan	μM	Phase I/II
	4SC-201 (resminostat)	pan	μM	Phase I, II
	PCI24781 (CRA-024781)	pan	nM	Phase I
	LBH589 (panobinostat)	pan	nM	Phase II /III
	LAQ824	pan	nM	Phase I
Cyclic tetrapeptides	FK228 (romidepsin)	Clas I	nM	Approved, I/ II
Benzamides	MS275 (entinostat)	HDAC1, 2, 3	μM	Phase II
	MGCD0103 (mocetinostat)	Class I	μM	Phase II
Short-chain fatty acids	Valproic acid	Classes I and IIa	mM	Phase II
	Butyrate	Classes I and IIa	mM	Phase II

Table 3

Results of clinical trials using HDACi as a single agent in various types of cancer

HDACi	Phase	Type of cancer (No. of patients)	outcome				Ref
			Response Rate(RR)	median time to response	median duration of response	median time to tumor progression	
SAHA	II	Persistent, progressive, or refractory CTCL (n=74)	30%		168 days	202 days	[46]
	II b	refractory CTCL(n=74)	29.7%	56 days	NR	4.9 months	[45]
	II	refractory CTCL(n=33)	24.2%	11.9 weeks	15.1 weeks	30.2 weeks	[43]
PXD101	I	advanced hematological neoplasia(n=13)	5 in 13	MTD is determined to be 1000 mg/m (2)/d days 1-5 in a 21-d cycle.			[53]
	II	refractory advanced thymic epithelial tumors (n=41)	8%		NR	5.8 months	[52]
LBH589	II	relapsed/refractory WM(n=36)	25%	1.8 months	6.6 months		[55]
	II	patients with low or intermediate-1 risk MDS(n=13)	1 in 13 (8%)			3 months	[54]
LAQ824	I	patients with advanced solid tumors(n=9)	None	MTD ranges from 24 to 72 mg/m (2).			[224]
FK228	II	relapsed or refractory PTCL(n=131)	25%		17 months	13.4 months	[47]
	II	PTCL(n=47)	38%		8.9 months		[48]
	II	CTCL(n=96)	34%	2 months	15 months	6 months	[49]
MS275	I	refractory and relapsed acute leukemias(n=38)	MTD is 8 mg/m ² weekly for 4 weeks every 6 weeks.				[225]
	II	pretreated metastatic melanoma(n=28)	29% in arm A and 21% in arm B		8.8 months	55.5 vs 51.5 days in both arms	[226]
MGCD 0103	II	relapsed classical HL (n=51)	85 mg three times per week(RR=25%), has promising clinical activity with manageable toxicity in HL patients				[227]
	II	chronic lymphocytic leukaemia (n=21)	none	Limited activity is observed			[50]
Valproic acid	II	AML (n=75)	24%		4 months		[51]
Butyrate	II	sickle cell patients (n=25 for control, n=7 for treated group)	24% for control 78% for treatment		3 months		[228]

Abbreviations: CTCL: cutaneous T-cell lymphoma, NR: not reached, MTD: maximum tolerated dose, WM: Waldenstrom macroglobulinemia, MDS: myelodysplastic syndrome, PTCL: peripheral T-cell lymphoma, HL: Hodgkin's lymphoma, AML: acute myeloid leukemia.