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REVIEW ARTICLE

Histone deacetylase inhibitors as a new anticancer option: How far can we go with expectations?

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Summary

Histone modification that occurs through the process of acetylation plays a key role in the epigenetic regulation of gene expression. The balance between histone deacetylases (HDACs) and histone acetyltransferases controls this process. Histone deacetylase inhibitors (HDACIs) can induce cancer cell cycle arrest, differentiation and cell death, reduce angiogenesis and modulate immune response. Therefore, HDAIs represent a group of enzymes that can be used for the development of pharmaceutical agents against a variety of malignant diseases. The mechanisms of their anticancer effect depend on many factors. HDACIs vorinostat, romidepsin and belinostat have been approved for some T-cell lymphomas and panobinostat for multiple myeloma. Other HDACIs are tested in clinical trials for the treatment of hematological and solid malignancies. The results of such studies are promising but further larger studies are needed.

Key words: *cancer, deacetylase, epigenetics, Histone, targeted, inhibitor*

Introduction

Mutation or inappropriate expression of various HDACs has often been observed in human diseases, particularly in cancer, making HDACs an important therapeutic target for anticancer therapies. The whole pattern of histone acetylation is deregulated in cancer. A research group reported

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[1] that cancer cells undergo loss of acetylation of histone H4 in place of lysine 16, suggesting that HDAC activity is crucial in the formation of the cancer phenotype [2]. In pathological conditions where classical HDACs are overexpressed. HDACIs have emerged as promising anticancer therapeutic agents. To date, four HDACIs have been approved by the US Food and Drug Administration (FDA) for anticancer therapy: vorinostat (SAHA, Zolinza) and romidepsin (FK228, Istodax) are used for cutaneous T-cell lymphoma, belinostat (Beleodaq) for peripheral T-cell lymphoma and panobinstat (Farydak) for multiple melanoma (Table 1), while several HDACIs are still on clinical trials [3]. However, most HDACIs have the disadvantage of lacking enzyme specificity and can cause a wide range of unwanted effects. In addition, it is worth mentioning that the contribution of HDACs to cancer may be through mechanisms other than overexpression, which may be related to truncating and inactivating mutations. Also, HDACs may be inappropriately mobilized to target genes by interacting with fusion proteins, as is the case in some leukemias. In this case, it will be necessary to investigate the use of alternative therapeutic agents. The previously known role of HDACs in neoplastic diseases is presented in two respects: the one concerning their expression in neoplastic patients and the one concerning their mechanism of action in cancer cell lines.

Histone acetylation and deacetylation

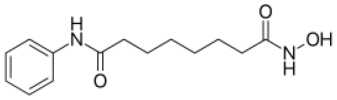
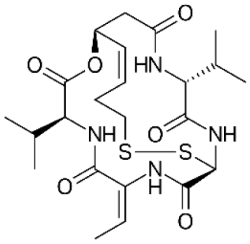
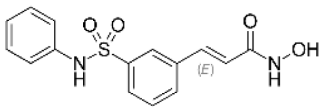
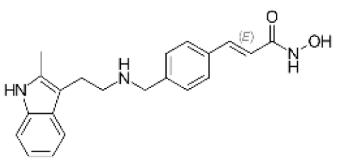
Acetylation is mediated by histone acetyltransferases (HATs), it occurs in lysine residues of protein tails and promotes transcriptional activation by neutralizing the positive lysine load resulting in a reduction in the degree of interaction with the negatively charged DNA molecule. This reduced interaction leads to less condensed / coiled chromatin formation, which facilitates access of transcription factors to DNA and thus facilitates transcription. This is reinforced by the mobilization of ATP-dependent chromatin rearrangement complexes, such as the SWI/SNF complex, which facilitate the binding of transcription factors by DNA de-scintillation [4].

Histone acetylation is a reversible process, where HDACs remove acetyl groups from protein tails. Deacetylation results in the restoration of the positive charge to the lysine residues, resulting in the return of electrostatic interaction with the DNA molecule, the transition of chromatin to a more concentrated conformation and suppression of transcription [5] (Figure 1).

Histone deacetylases (HDAC)

HDACs are classified in four classes I, II, III, IV depending on sequence homology to the yeast

Table 1. Histone deacetylase inhibitors (HDACIs) approved by the US Food and Drug Administration (FDA)

Drug	*Chemical name*	*Year of FDA approval*	*Classification*	*Chemical type*	*Anticancer treatment*
Zolinza	Vorinostat (SAHA)	2006	Hydroxamic acid		Cutaneous T-cell lymphoma
Istodax	Romidepsin (FK228)	2009	Cyclic peptide		Cutaneous T-cell lymphoma
Beleodaq	Belinostat	2014	Hydroxamic acid		Peripheral T-cell lymphoma
Farydak	Panobinstat	2015	Hydroxamic acid		Multiple melanoma

enzymes Rpd3, Hdal and Sir2 [5,6]. Class I, II and IV are considered as "classical" HDACs, whereas class III enzymes are known as sirtuins. Classical HDACs and sirtuins differ in their catalytic mechanism. Classical HDACs carry a zinc ion catalytic pocket on their base and can be inhibited by zinc-binding chelating agents. In contrast, sirtuins have a different catalytic mechanism, which requires the presence of NAD as a co-factor. Typically, the term "HDAC inhibitors" refers mainly to substances that act against "classical" HDACs.

It is apparent from their name, that the enzymatic activity of HDAC is the deacetylation of histone proteins. Through this activity, HDACs control the interaction of positively charged histones with negatively charged DNA, thereby altering chromatin modulation, access to transcription enzymes therein and, consequently, transcriptional activity. The high activity of HDACs is associated with concentrated, inactive chromatin. Apart from this epigenetic function of HDACs, it is now recognized that certain HDACs also exhibit significant cytoplasmic function by controlling the acetylation state and functionality of various cytoplasmic proteins and transcription factors. As a result, the term "lysine deacetylases" would have probably been more precise to indicate that their substrates are not limited to histones [7].

In general, more and more substrates for HDACs are identified, such as p53, E2F, GATA1, Bcl-6, Stat3, HMG, HSP90, NF-kb, tubulin, ibortine, nuclear hormone receptors and β-vacuine [8]. HDACs regulate the activity of various agents of central importance for the cell, which are involved in the regulation of transcription, intracellular signaling, cell cycle and apoptosis, among others. This clearly shows that HDACs regulate important cellular functions regardless of their epigenetic role in controlling the chromatin structure in the cell nucleus [9,10].

In summary, HDACs have emerged as crucial co-repressors of transcription in a variety of physiological and pathophysiological systems. To date, 18 human HDACs have been identified and categorized as described in Table 2.

Table 2. Classification of histone deacetylases (HDACs)

Classification		*Agent*	*Dependence*
Class I	Classical	HDAC-1, -2, -3, -8	Zn^{2+}
Class II		IIa: HDAC-4, -5, -7, -9 IIb: HDAC-6, -10	
Class III	Sirtuins	SIRT-1, -2, -3, -4, -5, -6, -7	NAD
Class IV	Classical	HDAC-11	Zn^{2+}

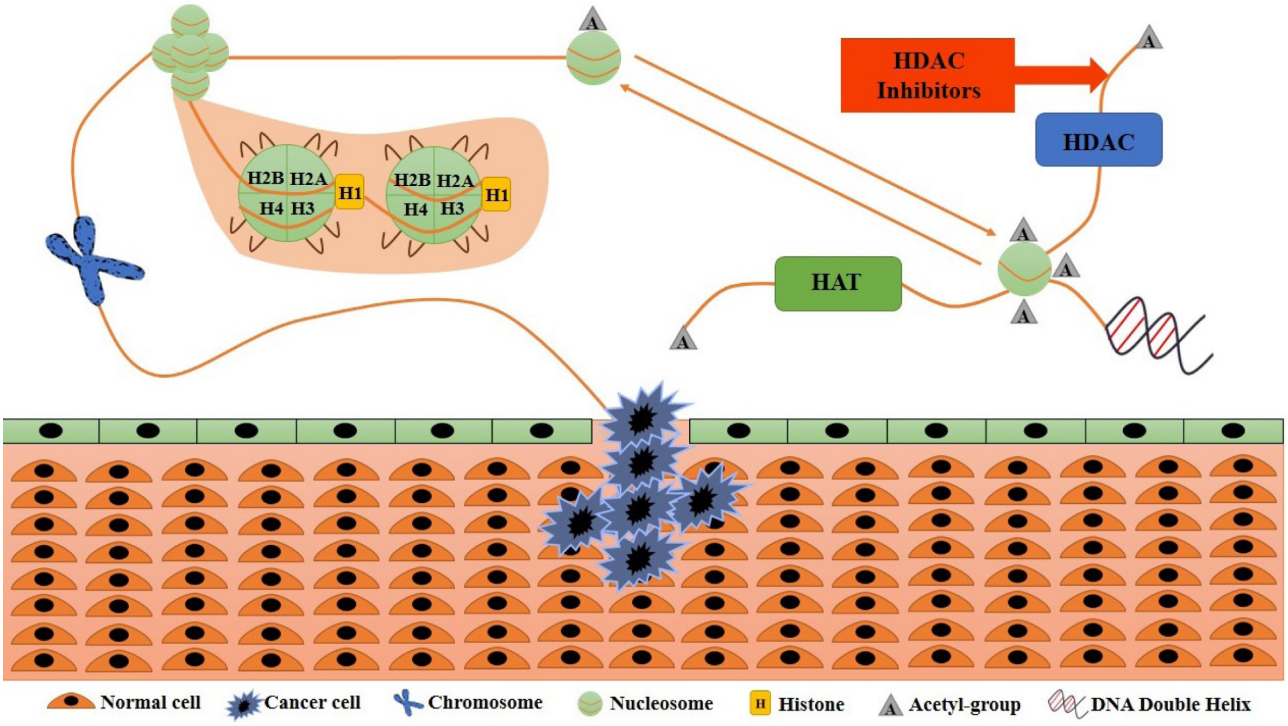


Figure 1. Therapeutic anticancer strategy using histone deacetylase inhibitors. HAT: Histone acetyltransferases; HDAC: Histone deacetylases.

HDAC Class I

Class I HDACs are expressed in all tissues and consist of subunits of polyprotein nuclear complexes that play a key role in the transcriptional repression and epigenetic landscaping. HDAC-1 and -2 are components of the co-rest complex that inactivates the expression of neuronal genes in non-neuronal tissues [11], while other complexes containing HDAC-1 and -2 are NURD and SIN3 suppressive complexes [12]. HDAC-3 is found in the N-COR and SMRT complexes [13]. HDAC-8 has so far not been found to be part of any repressive complexity, but that attaches particular importance to it.

They are primarily located in the cell nucleus and exert a strong catalytic effect on histone lysine residues. HDAC-1 and -2 show great similarity and they are involved in various cellular processes, such as proliferation, cell cycle and apoptosis [14]. HDAC-3 plays a role in cell cycle processes and in response to DNA damage [15]. Finally, HDAC-8 is predominantly found in the cytoplasm and is expressed in smooth muscle differentiation cells [16]. The protein structure of HDAC class I presents an active deacetylase catalytic center flanked by short N-terminal and C-terminal groups [17]. HDAC-1 and HDAC-2 are catalytic subunits of the Sin3, Mi-2/NurD and CoREST complexes while HDAC-3 is mainly employed by the N-CoR/SMRT complex. In contrast, as has been said, HDAC-8 has not been described so far as part of any protein complex [7].

HDAC Class II

Class II of HDACs is further divided in Class IIA and IIB. Class IIA members have a large, functionally important N-terminal portion that regulates the transition between nucleus and cytoplasm and specific DNA binding. The intracellular traffic of these HDACs is regulated by endogenous nuclear insertion and extraction signals, as well as binding sites for 14-3-3 proteins. HDAC-4, -5, -7 and -9 contain three such positions. Binding of 14-3-3 proteins results in maintenance in the cytoplasm or extraction from the core of HDAC class IIA, in a phosphorylation-dependent manner, which in turn regulates the activity of transcription factors such as MEF2 [7,18,19]. Various signaling pathways regulate the phosphorylation of these 14-3-3 binding sites. These pathways include Ca^{2+}/ calmodulin-dependent kinases (CaMKs) [7], protein kinase D [20], kinases that regulate the affinity for microtubules [21], salt-inducible kinases [22] and kinase 1 of cell cycle checkpoints (CHK1) [23].

HDAC Class IIB includes HDAC-6 and HDAC-10. The HDAC-6 contains two active deacetylase catalytic centers in series and a carboxy-terminally located zinc finger. HDAC-6 has now been shown to regulate cell mobility, adhesion and chaperone. Its cellular functions are not affected by its deacetylation activity. By ubiquitin binding via the zinc finger of its catalytic center, it regulates intracellular aggregates (aggresome), autophagy and function of heat shock factor 1 (HSF-1) and platelet-derived growth factor (PDGF) [24,25]. HDAC-10 is structurally related to HDAC-6 apart from a catalytically inactive protein domain. Its function is largely unknown.

Class IIA HDACs are expressed in specific tissues and are involved in cell differentiation and growth. They exert their suppressive effect on transcription in the striated and smooth muscles and myocardium, vasculature, bone, immune and central nervous system, among others. They also have a long regulatory N-terminal segment, through which their interactions are mediated with tissue-specific transcription factors and co-transcriptors [7,10]. The catalytic activity of class IIA HDACs remains unclear, but it has been found to be part of the SMRT / N-CoR suppressor complex [26,27].

The HDAC IIb family consists of HDAC-6 and HDAC-10. The first is predominantly found in the cytoplasm, where its main molecular target is a-tubulin, containing two active deacetylase centers and a C-terminal zinc finger. The latter is found in both the nucleus and the cytoplasm and also contains a second active deacetylase center [7, 28]. Its specific substrate remains unclear at present.

HDAC Class III

Sirtuins are widely expressed and have a very wide range of biological functions, such as regulating oxidative stress, DNA repair, metabolic regulation and cell aging [29, 30]. Sirtuins are located in different cell compartments: SIRT-1, SIRT-6, and SIR-T7 are located in the nucleus, SIRT-2 is found in the cytoplasm, and SIRT-3, SIRT-4 and SIRT-5 are predominantly found in mitochondria.

HDAC Class IV

HDAC-11 is currently the only class IV HDAC member. It is structurally related to both Class I and Class II HDACs. HDAC-11 contains amino acid residues in the regions of catalytic active sites, which share both class I and class II HDAC [31]. Its expression is higher in the liver, brain, testicles, heart and skeletal muscles, but its function has not been adequately studied. It has been associated with the development of oligodendrocytes and the immune response [32,33].

Histone deacetylase inhibitors (HDACIs)

As mentioned, four classes of HDACs are distinguished. Class I, II and IV deacetylases rely their effect on zinc-dependent catalysis. Under these enzymatic conditions, a hydrophobic pocket leads to the active catalytic central position of the zinc and most inhibitors come into contact with this center due to their ability to enter the hydrophobic pocket and thus block access of the enzyme substrates. Class deacetylases III are called SIRT-1 to -7 and use NAD + as a co-factor for their action [34-36].

Hydroxamic acids

These substances have chelating activity against metallic atoms and can bind the zinc ions necessary for the catalytic activity of HDACs. It has been shown that, with the exception of some of them, they are generally well tolerated by the body. They can bind to the active center of the deacetylases, directly inhibiting the catalytic action [37]. The deacetylation inhibitors, which fall into the hydroxamic acid class and have been more studied in various malignancies, are trichostatin A, vorinostat, panobinostat and belinostat [38,39]. Trichostatin A (TSA) is a natural inhibitor of deacetylases which inhibits class I and II deacetylases [40]. The action of trichostatin has been studied *in vitro* in many cancer cell lines and also *in vivo* using allografts in nude mice. However, due to its toxicity, its clinical use has never been favored, and instead synthetic analogues such as SAHA are preferred [41-44]. Vorinostat is a synthetic substance that belongs, like TSA, to class I / II deacetylase inhibitors [45] and has been approved by the FDA for the treatment of recurrent or reversible T-cell lymphoma (CTCL) [46]. In 2007, Arnold et al. were the first to describe the effect of vorinostat in pancreatic cancer cell lines, indicating that vorinostat induces cell cycle arrest in the G1 phase by increasing p21 in BxPC-3 and COLO-357 cells, but not to gemcitabine-resistant PANC-1 cells. However, the inhibitor exhibited synergistic effect with gemcitabine on BxPC-3 and COLO-357 cells and sensitized PANC-1 cells to gemcitabine [47]. In contrast, Kumagai et al. showed in 2007 that vorinostat therapy leads to inhibition of PANC-1 cell growth, induces p21 in these cells and causes arrest in the G2/M transition instead of the G1 phase of the cell cycle [48]. Other recent studies have investigated the effect of vorinostat in combined therapies in gastric cancer, specific types of lymphoma and non-small cell lung cancer [49-52].

It has been found *in vitro* that the combination of gemcitabine, the proteasome inhibitor bortezomib and vorinostat exhibits the greatest inhibitory effect on cell growth. This finding, however, has not been confirmed *in vivo*, as experiments on nude mice did not show a significant benefit of the triple combination vs gemcitabine with bortezomib [53]. A further study by Millward et al., which is a phase I clinical study, demonstrated in 2012 a significant synergism of the proteasome inhibitor marizomib and vorinostat in cancer cell lines *in vitro* with cells derived from non-small cell lung cancer, melanoma and pancreatic cancer. However, the study on its initial phase did not detect tumor response to treatment [54]. Although there have been few encouraging results in preclinical and clinical studies, the anticancer activity of vorinostat observed *in vitro* and other types of cancer leads researchers to design new studies with vorinostat [55]. In an ongoing phase I/II clinical study, the combination of vorinostat with radiotherapy and 5-FU is considered in patients with locally advanced adenocarcinoma of the pancreas [56]. Another ongoing study attempts to evaluate the efficacy of vorinostat, capecitabine (a 5-FU prodrug) and radiotherapy combination in patients with non-metastatic pancreatic carcinoma [57].

Panobinostat inhibits all classes of deacetylases of zinc-dependent histones and it is therefore called a universal inhibitor (pan-HDAC inhibitor) [58]. It was tested for the first time against pancreatic cancer in 2008 by Haefner et al., who showed that panobinostat caused interruption in the cell cycle G2/M transition, up-regulation of p21 and *in vitro* apoptosis. In *in vivo* conditions, the substance significantly reduced the tumor mass in nude mice and regulated the efficacy of gemcitabine, but apoptosis increased only slightly and no significant reduction in cell proliferation was observed [59]. Panobinostat is tested in multiple phase II clinical studies in combination with bortezomib against different malignancies [60-63]. However, a recent study with panobinostat in combination with PI3K and mTOR BEZ235 inhibitor demonstrated inhibition of growth both *in vitro* and *in vivo* using allografts in nude mice [64].

Belinostat is a relatively new universal inhibitor (pan-HDAC inhibitor) of HDACs [58]. In 2010, it was tested in a phase I clinical study in combination with carboplatin and/or paclitaxel in patients with solid tumors, experiencing a partial response to the belinostat combined with carboplatin [65]. Like panobinostat, belinostat has also been tested in a preclinical study in combination with bortezomib, and the results demonstrated synergistic action against cell proliferation and in favor of apoptosis for both drugs in pancreatic cancer and multiple myeloma [66,67]. Two more recent stud-

ies have shown that belinostat induces cell growth inhibition both in vitro and *in vivo* in immunocompromised mice, either alone or in combination with gemcitabine [68,69].

Short chain fatty acids (SCFAs)

These substances are less potent inhibitors of deacetylases than hydroxamic acids, probably because they have no access to the zinc ion found in the pocket of the active center of HDACs [70]. However, the fact that they are considered to be a bacterial fermentation product of fiber foods by the intestinal flora and potentially may protect the intestine from developing tumors, makes this category of HDACI extremely important for anticancer research. When it comes to pancreatic cancer, the best studied and most promising SCFAs are valproic acid and butyrate. Valproic acid is a class I/IIa HDACI that was invented as an antiepileptic drug and later its inhibitory effects on HDACs became evident [71]. In a phase I clinical trial of valproic acid and epirubicin in solid tumors, one patient showed a partial response to this drug combination [72]. Also, *in vitro*, valproic acid has been shown to strongly downregulate cell proliferation and adhesion of cancer cells [73].

In 2011, two distinct studies by the Iwahashi et al. group on pancreatic cancer cell lines showed that valproic acid on its own was incapable of inducing a significant degree of inhibition of growth but potentiated the inhibition of growth by 5 -FU and the combination of gemcitabine and pegylated interferon α2β [74,75]. More recently, a phase II study was conducted in order to evaluate the toxicity and efficacy of valproic acid in combination with gemcitabine and radiotherapy [76]. The long-term use of valproic acid as an antiepileptic drug - in addition to the advantage of well-documented knowledge of its clinical pharmacology - offers the advantage of a previous knowledge of its effect on certain solid malignancies, mainly driven by elements of the nervous system [77].

Valproic acid seems to have a potential role in the treatment of medullary thyroid cancer, as it induces metabolic stress, activates AMP-activated protein kinase and increases autophagic flux in the thyroid cell lines [78].

Butyrate is a class I/II HDACI which has been shown to induce apoptosis and prevent penetrance/infiltration in cancer cell lines [79]. It is estimated to exert a significant influence on chemotherapy activity [80,81]. It is reported, however, that it is pharmacologically deficient, as far as its half-life and its clearance in its first hepatic passage are concerned [82]. Butyrate prodrugs with better pharmacological features could offer an alternative therapeutic option as it has been shown by the inhibition of cellular growth that tributyrin prodrug induces in pancreatic cancer cells [83]. Preclinical and clinical studies are required to evaluate the therapeutic value of butyrate-related substances.

Cyclic peptides

Romidepsin is a pentapeptide that interacts with the zinc ion, which is found at the active site of HDACs. It is classified as class I/II HDACI. In 2009, romidepsin was approved by the FDA for the treatment of CTCL patients [84]. Its function is to induce G1 or G2/M phase disruption of the cell cycle and subsequent apoptosis in treatment-resistant pancreatic carcinoma [85]. In addition, romidepsin is reported to cause *in vivo* growth inhibition in allografts of pancreatic cancer [86]. Recently, a phase I study by Jones et al. tested ro-

Table 3. Classification of most important histone deacetylase inhibitors (HDACIs) with anticancer effects

Classification	*Agent*	*Specify to HDACs*
Hydroxamic acids	Trichostatin A (TSA)	Classes I, II
	Vorinostat	Pan inhibitor
	Panobinostat	Classes I, II
	Belinostat	Pan inhibitor
Short chain fatty acids (SCFAs)	Valproic acid	Class I, IIa
	Butyrate	Class I / IIa
Cyclic peptides	Romidepsin	Class I
Benzamides	Entinostat	Class I
	Mocetinostat	Class I

midepsin in combination with gemcitabine in solid tumors. Although cumulative haematological toxicity of the drug combination was observed, the disease was stabilized in 14 patients and a partial response was seen in 2 patients. That fact requires further investigation with appropriately designed studies [87,88].

Benzamides

Entinostat is a characteristic synthetic benzamide derivative that inhibits class I HDACs [58]. Its anticancer activity was firstly studied in 1999 by Saito et al. This group reported strong anticancer activity against human cancers in nude mice [89]. In 2008 a phase I study of entinostat included a patient with metastatic pancreatic cancer who developed disease progression [90]. A recent phase I study of entinostat in combination with 13-cis retinoic acid in solid tumors included once more a patient with pancreatic cancer, which however resulted in prolonged disease stabilization [91]. Entinostat seems to be also effective against advanced breast cancer and colon cancer [92,93]. Mocetinostat belongs to the same category and it has also been reported to inhibit colon cancer cell proliferation by a different mechanism of upregulating the WNT ligand DKK-1 expression [93]. Since then, it has been withdrawn from clinical applications due to the adverse effects recorded during its use.

Table 3 summarizes the most important HDACIs with anticancer effects.

HDACIs-mechanism of action

HDACIs can induce cell death via various molecular pathways, depending partially on the degree of cell exposure to them and on the specific molecular features of each cell. Normal cells, however, exhibit relative resistance to HDACI-induced cell death [94,95].

Damage and repair of DNA

It should be noted that there are no indications so far that HDACIs cause directly mutations. HDACI-induced histone acetylation induces structural changes in chromatin, which may expose DNA to harmful agents with mutagenic power, such as ultraviolet radiation, cytotoxic drugs and oxygen radicals. Those lead to breaks of the double helix [9,36,96]. HDACIs can induce accumulation of reactive oxygen species (ROS) resulting in DNA damage [94,95,97]. They also induce accumulation of the phosphorylated form of H2AX, a double-helix disintegration marker [98]. HDACIs can still lead to downregulation of proteins related to DNA repair in homologous recombination (such as RAD51, BRACA1, BRAC2) and to non-homologous repair of double helix disruption (such as Ku70, Ku86 and DNA- PKcs) [99-104]. Accumulation of DNA damages causes changes in gene expression and leads to apoptotic cell death. Affected cells may have many defects in the double helix repair pathways and unlike normal cells, they do not have the ability to repair DNA damage. The synergy of HDACIs and DNA-damaging therapeutic substances, such as cytotoxic drugs and radiotherapy, arises not only from the effect of HDACI on inhibiting DNA repair procedures but from activating endogenous and exogenous pathways of apoptotic cell death as well, as mentioned below in particular [105].

Alteration in gene expression

HDACIs modulate gene transcription by induction of histone acetylation as well as transcription factors and other proteins that regulate gene expression [36,106]. Early experiments with cultures of TSA-treated lymphatic cell lines showed that the percentage of genes undergoing change in expression was only about 2% of the expressed genes as compared to untreated cells. The change was defined either as an increase or as a decrease in expression [9,107]. More recent studies using cDNA sequences have demonstrated that 10 to 20% of the expressed genes exhibited a change in their expression in leukemia, multiple myeloma cell lines as well as colon, kidney, prostate and breast cancer cells, which were treated with HDACIs [108-111]. The number of genes with change in expression increased according to the duration of the culture and the concentration of HDACIs. Some changes in gene expression are estimated to be a direct result of HDACIs, while others may be manifestations of subsequent derivatives and crucial points of the biochemical circuits that are affected. The pattern of changes noted in gene expression is similar among different HDACIs, although there are differences in induced changes -special to certain factors- in relation to the molecular status of the reference cells [108-110].

Cyclin-dependent kinase inhibitor (CDKI) p21WAF1/Cip1 is one of the most frequently HDACI-induced genes [112]. HDACI-induced expression of p21 is independent of p53. In ARP-1 cells, vorinostat has been reported to cause specific modifications to the acetylation and methylation pattern of lysines on H3 and H4 histones, which are associated with the proximal promoter region of the p21 gene [113]. Acetylation or methylation of histones in the promoter region of the expressed p27 (KIP1) or silent e globin in HDACI-treated ARP-1

cells was not reported to be altered. Similarly, the expression of these genes was neither reported. Vorinostat caused a significant decrease in HDAC-1 and Myc as well as recruitment of RNA polymerase II to the protein complex which is associated with the proximal promoter region of the p21 gene. The detectable changes in HDAC-2, Brg1, GCN5, P300 and Sp1 proteins in the complex were few. These findings suggest that selective alteration of the transcription of a gene by HDACIs may be determined by the composition of proteins involved in the transcriptional complex, including HDACs. HDACIs can inhibit STAT5-mediated gene expression [114]. HDACIs can also suppress the transcription of androgen receptor (AR) gene as well as inhibit the transcriptional activation of other genes mediated by AR [115]. HDACIs as well as SAHA can also alter the expression of miRNA in cancer cells. These miRNAs have gene targets related to angiogenesis, apoptosis, chromatin modifications, cell proliferation and cell differentiation [116]. HDACIs can activate a Sp1/Sp3-mediated induction of multiple response genes to cellular stress (such as fos, Juh, egr1, egr3, a3, arc, mr4a1, mdrg4, Mt1B, MtiE, Mt1f, ME1H), which are associated with cellular apoptosis [117].

Interruption of cell growth

HDACIs can induce cell growth disruption in both normal and malignant cells according to cell culture results. Vorinostat primarily causes a cell-cycle disruption in G1 phase at low concentration and in G1 and G2/M phases at higher concentrations [112]. In culture cells treated with HDACI, elevated levels of cyclin-dependent kinase inhibitors (CDKIs) and decreased levels of cyclins may be a cause of decreased activity of CDKs, causing dephosphorylation of Rb and inhibiting E2F activity in gene transcription for G1 phase progression and transition from G1 to S phase of the cell cycle [118,119]. HDACIs can affect both cell growth and non-proliferating transformed cells [95,120]. This is in contrast to the action of many other chemotherapeutic drugs, which are active only against the transformed cells being divided.

Induction of apoptosis

HDACIs can induce death of transformed cells by activating exogenous and/or endogenous apoptotic pathways [8,121-126]. Mechanisms in the course of downstream components, such as caspase 3 activation, are common steps between the exogenous and endogenous pathway [122]. The exogenous apoptotic pathway is activated by binding of the cell death receptor as well as Fas, TNF-1 receptors, TRAIL receptors (DR4 and DR5), DR3 (Apo3) and DR6, with their ligands, resulting in activation of caspases 8 and 10. HDACIs can upregulate not only cell death receptors but their ligands as well in vitro and *in vivo* in transformed cells but not in normal cells. Also, a vorinostat therapy followed by TRAIL has been shown to target multiple pathways as far as the progression of malignancies, angiogenesis and metastases are concerned. HDACIs can cause TNF-dependent apoptosis by inhibiting the ubiquitin-dependent pathway, which may be the basis for the effectiveness of combining HDACIs with the proteasome inhibitor in inducing death of malignant cells [126]. Combined treatment of HDACIs with agents that induce exogenous apoptotic pathway is likely to be critical in the development of effective therapeutic strategies.

The endogenous apoptotic pathway is mediated by disorders of mitochondrial function and release of mitochondrial transmembrane proteins, including AIF, Smac and cytochrome c, resulting in activation of caspases [8,9,36,127,128]. HDACIs induce the endogenous apoptotic pathway by deactivating or suppressing antiapoptotic proteins and activating apoptotic proteins. HDACIs can promote apoptosis-degrading Bid, which initiates the endogenous pathway and affects the mitochondria of cancer cells. High levels of Bcl-2 or Bcl-XL, which protect the mitochondria, have been found in some malignant cells that are resistant to HDACI-mediated cell death [123]. Inhibition of Bcl-2 by a chemical inhibitor may increase the sensitivity to HDACI-mediated cell death. The HDACIs cause upregulation in Bcl-2 family of apoptotic proteins, such as Bim, Bmf, Bax and Bik, while reducing the antiapoptotic proteins of the same family as Bcl-2, Bcl-XL, Bcl-w and Mcl-1, as well as the genetic inhibitor of XIAP apoptosis, that further causes the degradation of surviving cell death [123,129].

Disorder of mitosis

HDACIs can induce inappropriate accumulation of acetylated histones in heterochromatin and centromeric areas, resulting in the death of neoplastic cells. In transformed with TSA cell cultures, histones found in recently synthesized chromatin remain acetylated and disrupt the structure and the function of centromere and pericentric chromatin by loss of attachment to heterochromatin binding proteins (HBPs). Histone acetylation also inhibits histone phosphorylation, by disrupting the function of mitotic spindle and cell cycle checkpoint proteins, such as BubR1, hBUB1, CENP-F and CENP-E. As a result, the cell cycle is temporarily discontinued in the pre-metaphase so the accumu-

lation of chromosomal disorders in the process of mitosis leads to cell death [130-133].

Reactive oxygen species radicals reduction–oxidation changes

HDACIs cause accumulation of ROS in transformed cells, but not in normal cells. Increase in cellular ROS may occur within two hours of HDACI culture prior to mitochondrial disorder. Free radical scavengers, such as N-acetylocysteine, reduce the HDACI-mediated apoptosis, suggesting that ROS production is an important factor in the death of cancer cells. Thioredoxin is a hydrogen donor that is required to activate various proteins including ribonucleotide reductases -which are necessary for DNA synthesis- and transcriptional factors such as NF-κB. Reduced thioredoxin is a scavenger of ROS [134-136]. Vorinostat increases the expression of TBP-2 which binds and inhibits the activity of reduced thioredoxin, causing downregulation of thioredoxin to malignant but not to normal cells. Thioredoxin is a kinase 1 inhibitor, which regulates apoptosis (apoptosis signal-regulating kinase 1, ASK1). Inhibition of thioredoxin by binding to TBP2 activates ASK1, which in turn promotes apoptosis by induction of SET1-JNK and MKK3/MKK6/p38 signaling cataract, but also by enhancing the expression of Bim apoptotic protein [95,97,137,138].

Activation of HDAC-6 and target proteins

HDAC-6 is unique among zinc-containing HDACs because it carries two catalytic domains: a ubiquitin binding site as well as a region associated with non-histone substrates such as HSP90 and a-tubulin [24,139-145]. Overexpression of HDAC-6 leads to deacetylation of a-tubulin and to increase in cellular mobility. HDAC-6 can bind both monoubiquitinated and poly-ubiquitinated proteins, while promoting ubiquitination of itself. Specific inhibition of HDAC-6 activity with tubacain or downregulation via siRNA causes accumulation of acetylated a-tubulin, HSP90, peroxiredoxin and other proteins that are related to its activity. Acetylation of HSP90 causes loss of its chaperone function and exposes the proteins that it affects - such as the survival and proliferation-related proteins Akt, Bcr-Abl, c-Raf and Erb-2- in multi-ubiquitination and degradation via the proteasome pathway [24,146,147]. The chaperone function of HSP90 is essential for the stability and function of various proteins such as steroid hormone receptors and protein kinases that are involved in cell signaling pathways and cell homeostasis. Recent studies have demonstrated both a direct interaction between HDAC-6 and HSP90 as well as HDAC-6 as a regulator of HSP90 activity through its deacetylation [147,148]. HDAC-6 can bind directly to protein phosphatase 1 (PP1) and cause simultaneous changes in phosphorylation and acetylation of cell proteins. As HSP90 affects a large number of proteins, numerous molecular changes may occur as a result of the inactivation of HSP90 via inhibition of HDAC-6 by HDACI. HDAC-6 is a component of the *aggresome*, a cellular structure that is the major breakpoint of defective protein aggregates with defective tertiary structure with respect to both ubiquitinated and non-ubiquitinated proteins characterized by defective tertiary structure. These proteins are susceptible to the formation of cytotoxic aggregates which may adversely affect normal cellular function. HDAC-6 acts as a bridge between the machines of dynein and the process of ubiquitination, leading the poly-ubiquitinated proteins to the aggresome. The BUZ region of HDAC-6 exhibits high affinity for the ubiquitin molecule and it is involved in the transport of multiple labeled proteins. Loss of function of HDAC-6 increases the sensitivity of transformed cells to stress associated with defective-forming proteins caused by proteasome inhibition [24,146]. Overall, these findings are important in the development of therapeutic strategies that combine the use of HDACI and proteasome inhibitors, as well as HSP90 inhibitors, possibly in the treatment of certain types of cancer.

Anti-angiogenesis

HDACIs can exert their antitumor activity by inhibiting angiogenesis that feeds tumors [149]. Solid tumors are often highly dependent on angiogenesis. Tumor angiogenesis may be mediated either by secondary hypoxia of cell growth or by increased tumorigenic signaling and consequently inducing the HIF-1A hypoxia factor and its transcriptional target, VEGF. HDACIs inhibit angiogenesis by suppressing HIF-1A and VEGF in animal models experiments. Under normal conditions, HIF-1A binds to the von Hippel-Lindau protein and it is inactivated by ubiquitination and then deconstruction to the proteasome. Hypoxia conditions can enhance the transcription of HDAC-1, HDAC-2 and HDAC-3 in cancer cells, resulting in decreased expression of the von Hippel-Lindau protein and hence increased expression of HIF-1A, which promotes angiogenesis. This sequence can be controlled by HDACIs. Also, HDACIs can induce the degradation of HIF-1α through a mechanism independent of von Hippel-Lindau protein. Class II HDACs are in direct association with HIF-1A and

their selective inhibition by siRNA induces HIF-1A degradation. The disturbance of HSP90 accompanying function via acetylation exposes HIF-1A to signaling and degradation to the proteasome [150-152]. These observations support the development of combined HDACIs therapies and drugs that restrict neovascularization.

Anti-metastatic effect

HDACIs cause increased regulatory expression in genes that suppress metastasis, such as Kangai (KAII), Ras homologs, RhoB, RECK protein and TIMP-1. In contrast, metastasis-promoting genes may have a regulatory restriction of their expression by HDACIs. In this group genes related to metalloproteinases (MMPs), integrin-α5 and forms of collagen are included [153]. These findings suggest that HDACIs may be effective in reducing the metastatic potential of some primary tumors, which is certainly worth exploring.

Glucose metabolism

HDACIs target against glucose transporter 1 (GLUT1) and exocinase I, inhibiting the use of glucose in transformed cells [154]. This effect of HDACIs may be important in the selective removal of nutrients from the transformed cells, which contributes to the inhibition of cell growth and to the death of these cells. The currently available data, which are primarily based on cell culture studies, demonstrate that HDACs have multiple targets and they are involved in almost every cellular biochemical pathway that affects cell survival as well as differentiation, proliferation, migration and death. In cell culture studies, HDACIs induce the death of transformed - but not normal cells - which probably reflects the ability of normal cells to recover after exposure to HDACIs (reversible) inhibition.

Figure 2 summarizes the mechanism of action of HDACIs as anticancer agents.

Conclusion

HDACIs constitute a promising group of anticancer agents with emerging applications in the treatment of both haematological and solid malignancies. The interest in this drug category has led to an increase of competition in the pharmaceutical research towards the composition of clinical as well as additional uses in non-neoplastic diseases, testing newer and more effective substances of the same category. Given the fact that HDACIs do not only affect histones but a broader category of proteins referred as lysine deacetylases, it is important to mention that the spectrum of cellular functions directly or indirectly affected by this class of enzymes is also wide and includes, among other functions, the regulation of gene expression, cell proliferation, cell migration and cell death. Body processes such as angiogenesis and immune response are also affected by the action of HDACIs. In case of induction of cell death in modified neoplastic cells, HDACIs are estimated to mobilize simultaneously multiple molecular pathways and

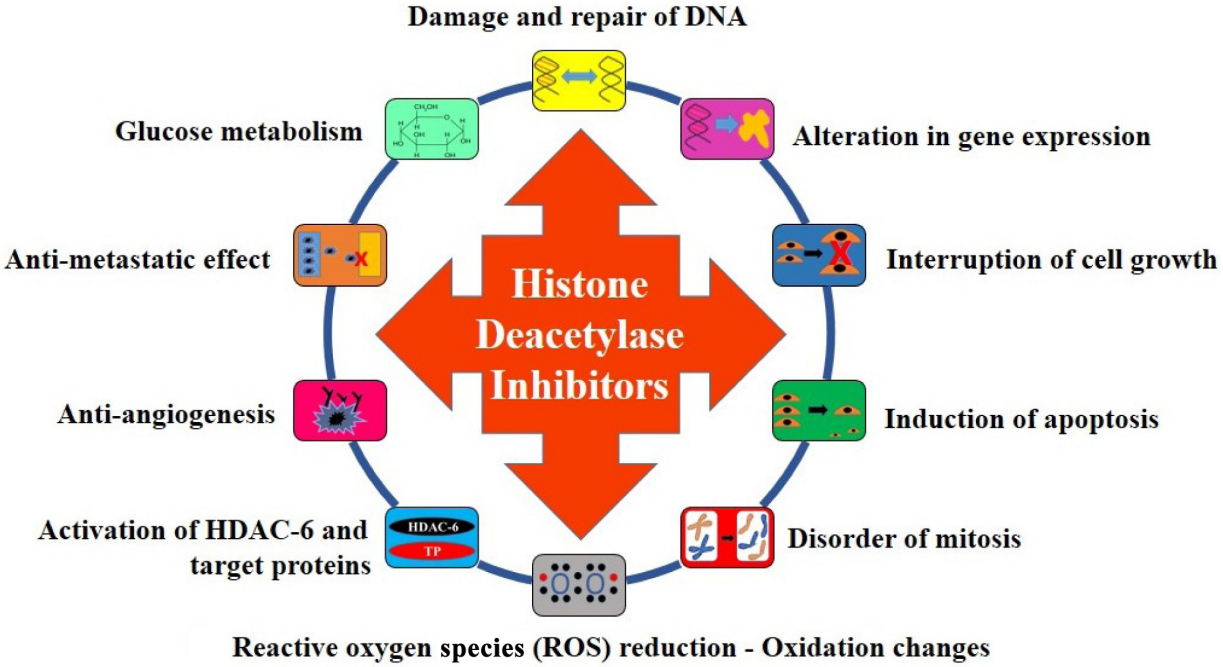


Figure 2. Mechanisms of action of histone deacetylase inhibitors (HDACIs) as anticancer agents.

their combined action is responsible for the final experimentally observed result.

Normal cells compared to malignant cells show resistance in the induction of cell death by HDACIs. It is believed that the genetic lesions that are accumulated in the neoplastic cells are to be blamed. Normal cells have a comparatively greater ability to overcome the obstacles that the HDACIs effects bring to their biochemical processes.

In the field of clinical therapeutics, this means that the exposure of cells to these substances can be adjusted in order to take advantage of the therapeutic window resulting from this difference between normal and malignant cells. In other words, the transient and intermittent mode of administration can ensure minimization of toxicity on healthy cells.

Clarification of the biochemical actions of HDACIs is also ongoing. A major question is whether the use of universal inhibitors of deacetylase (pan-inhibitors: inhibitors acting on both HDAC class I and HDACI class IIb) shows a comparative clinical advantage over the administration of selective inhibitors of individual enzymes. The development of inhibitors that act not only on specific proteins but also on their particular isoforms will probably help clarify this question. In the clinical studies done so far, the HDACIs used were not selective for a single enzyme. An additional point of interest in clinical research is the development of biomarkers that can predict the potential response of a patient to the administration of these drugs. This is highly important because, despite their documented action against solid organ and haematological malignancies, HDACIs do not appear to be effective in all patients for any given diagnosis but only in a percentage of them. Furthermore, interest in clinical pharmacology is based on optimizing the pharmacokinetic properties of HDACIs, both in terms of their water solubility and the choice of oral administration. Achieving these goals would provide a tremendous boost to the further development of HDACIs in the future as it would greatly enhance their clinical value. Finally, it is very important to seek a better understanding of the effect of HDACIs on protein interactions in addition to catalytic enzyme inhibition. These interactions are of dual importance because they help clarify the causes of HDACIs toxicity in normal cells, but also to understand better their pharmacodynamics on cancer cells, which they have been noted to have multiple molecular lesions and complicated pathophysiology.

Both clinical and preclinical studies show that HDACIs have the greatest therapeutic efficacy when they are administered in combination with other anticancer agents including all three major categories (other than of course surgical treatment), i.e., cytotoxic chemotherapeutic agents, targeted factors and radiotherapy [155]. As mentioned above, appropriate coordination is needed for the coadministration of HDACIs with the implementation program of these therapies, in order to achieve the best result. In all cases, combined therapies are expected to be more effective due to the various types of lesions that cancer cells carry. Therefore, there is a need for new clinical studies that examine the coadministration of HDACIs with other anticancer agents, aiming at upgrading our anticancer weaponry.

Conflict of interests

The authors declare no conflict of interests.

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