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HDACi – going through the mechanisms

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1. ABSTRACT

Histone deacetylases inhibitors (HDACi) have recently emerged as potent antitumor treatment modality. They are currently tested in many phase I, II and III clinical trials as single agents as wells as in combination schemes. They have demonstrated promising antitumor activity and favorable clinical outcome. Histone deacetylases (HDACs) are involved in the process of epigenetic regulation of gene expression. Epigenetic changes are believed to be crucial for the onset and progression of cancer and have recently gained remarkable attention. Since epigenetic regulation of gene expression is a reversible process, targeting histone deacetylases provides a good rationale for anticancer therapy. The acetylation status of histones regulates the organization of chromatin and the access of transcription factors. Moreover, functions of many non-histone proteins are controlled by acetylation. The broad and complicated influences of HDACi on various molecular processes may account for the observed pleiotropic effects. In this review we summarize recent advances in the understanding of biology of HDACs and mechanism of action of their inhibitors.

2. INTRODUCTION

Epigenetic regulation of gene expression is a tightly controlled reversible process that does not affect the nuclear DNA sequence. Epigenetic changes such as histone modifications and DNA methylation are believed to be crucial for the onset and progression of cancer and have recently gained remarkable attention (1). In mammalian cells DNA methylation has been almost exclusively observed at the cytosine residues within CpG–enriched regions, widely referred to as CpG islands. The localization of CpG islands is frequently limited to promoter regions. In normal cells methylation or lack of methylation of promoter CpG islands highly correlates with, respectively, transcriptional silencing or activation of the associated genes. CpG dinucleotides in coding regions are in most instances hypermethylated (2-3), a phenomenon which ensures monoallelic expression (genomic imprinting), X-chromosome inactivation and chromosomal stability. Neoplastic cells have been reported to express two patterns of aberrant methylation - global hypomethylation linked to chromosomal instability and loss of imprinting and selective hypermethylation of CpG islands in promoter regions including tumor suppressor genes (4-5). Another important mechanism regulating the gene expression relies on "histone code" generated by various post-translational histone modifications (6).

Eukaryotic chromatin exists in two different forms – open, easily accessible for transcriptional machinery, lightly packed euchromatin and tightly compacted heterochromatin, which generally harbors transcriptionally inactive genes. Remodeling of chromatin (changes in chromatin organization) derives from modifications of structure of nucleosomes, basic units of DNA organization consisting of a segment of DNA wound around an octamer of histone proteins. Histones undergo various N-terminal tails modifications, such as acetylation, deacetylation, methylation, phosphorylation, ubiquitination, poly-ADP ribosylation, sumoylation, glycosylation, deimination (modification of arginine into citrulline) and carbonylation. One of the most widely studied modifications of histones is their acetylation. In the simplest view, this highly reversible process of addition of an acetyl moiety to the epsilon-amino group of lysine residues on histone proteins, results in neutralization of their positive charge, impairs their interaction with negatively charged DNA and therefore increases the accessibility of regulatory proteins to DNA (7). However, it became apparent that acetylated lysine residues are also recognized by specific protein domains, such as bromodomain, involved in protein-protein interactions and assembly of multi-component complexes essential for transcriptional activation. Bromodomains are generally found in over one hundred chromatin associated proteins including histone acetyltransferases.

3. HISTONE ACETYLTRANSFERASES

The process of histone acetylation is regulated by opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HDACs catalyze the removal of acetyl groups that leads to the compacting of the chromatin and transcriptional repression of the affected genes (7-8). The first HATs and HDACs have been identified in the mid-1990s (9). Since then several enzymes have been reported to have HAT activity. Four major families of HATs have been studied extensively, namely: cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB)-binding protein (CBP)/p300 family, general control of amino acid synthesis protein 5 (Gcn5)-related N-acetyl transferases (GNAT) family, MYST family and Rtt109 family (10). The role of highly homologous p300 and CBP in cancer is still under debate. Some studies indicate that they behave as tumor suppressors, other suggest a role of tumor promoters (10). The validation of HATs as potential antitumor drug targets still remains a challenge, since their biological functions and interactions are yet to be fully investigated. Theoretically, the modulation of HATs activity may be useful and effective as anticancer treatment. Indeed, some small-molecule HATs inhibitors have been described (10). The majority of them has been isolated from natural sources, namely curcumin, garcinol, anacardic acid and several polyisoprenylated benzophenone derivatives (PBDs) containing garcinol-like structural features (11). One of the most promising HATs inhibitors is curcumin, already tested in many clinical trials. It has been reported to have anti-inflammatory effects and thus has been tested in several pathologies, namely rheumatoid arthritis, psoriasis, Alzheimer's disease, some mental disorders. Due to antitumor effects curcumin has been also tested as single-agent or in combination with chemotherapy in several clinical trials in patients with multiple myeloma, colorectal cancer, pancreatic cancer, osteosarcoma (12). The majority of currently described HATs inhibitors suffers from scarce potency, poor cell-permeability, low specificity and unsatisfactory pharmacokinetics. So far they have been mainly used to unravel the functions of HATs in several assay systems. Drug delivery strategies and modifications will hopefully help in the future to create druglike HATs inhibitors.

4. HISTONE DEACETYLASES

In humans eighteen different HDACs have been identified so far and subdivided into four classes based on their structural homologies to yeast HDACs, activity, cofactor dependency and subcellular localization (8) (Figure 1). Class I, II and IV are referred to as "classical" HDACs and are reported to require Zn^{2+} for deacetylase activity and to be inhibited by zinc-chelating compounds (13). Class III HDACs belong to a silent information regulator 2 (Sir2)-related protein family (sirtuins). They are reported to be NAD^{+}-dependent and their enzymatic activity can be inhibited by nicotinamide (14).

Class I (HDAC1-3, 8) are generally localized in the nucleus (apart from HDAC3, which is found both in the nucleus and cytoplasm) and associate with multiprotein complexes including corepressors and coactivators (15).

Class IIa (4, 5, 7, 9) can shuttle between the nucleus and the cytoplasm due to interaction with 14-3-3 proteins in response to various stimuli (13), while class IIb (6, 10) are

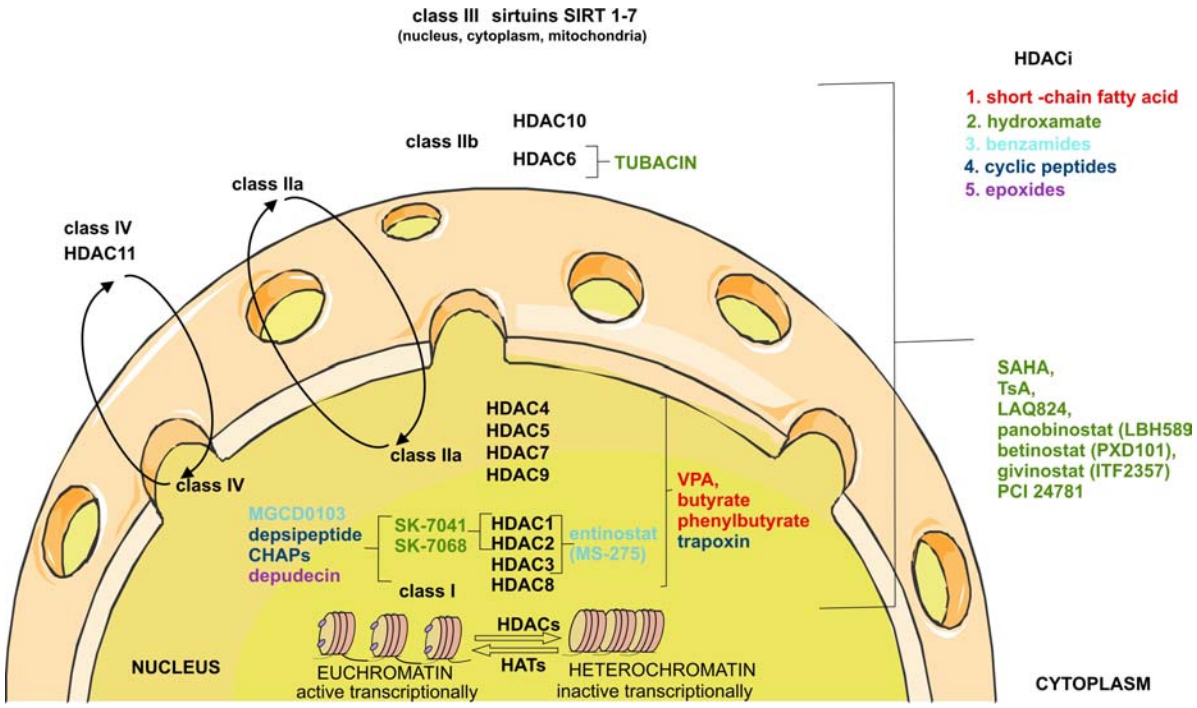

Figure 1. Histone deacetylases – targets for histone deacetylases inhibitors.

cytoplasmic proteins. Binding of class IIa HDACs with 14-3-3 proteins stimulates their nuclear export, accumulation in cytoplasm and subsequent derepression of target genes (16). Interestingly, class IIa HDACs have been shown to repress numerous transcription regulators due to the presence of specialized domain which binds to an array of transcription factors which in a consequence dictate gene targeting specificity (reviewed by Martin et al. (17)). Class IIa HDACs have been reported to interact among others with myocyte enhancer factor-2 (MEF2) (transcription factor involved in the regulation of cellular differentiation and stress response) and have been demonstrated to act as potent inhibitors of MEF2-dependent transcription (18). Interestingly, HDAC6 localizes actively in cytoplasm owing to the presence of a strong nuclear export signal (NES) that prevents the accumulation of the protein in the nucleus (19). HDAC6 is a unique enzyme that is proposed to play a critical role in mediating and coordinating various cellular events (reviewed by Boyault et al. (20)). HDAC6 contains two deacetylase domains which have been reported to deacetylase alpha-tubulin and heat shock protein 90 (HSP-90) (for the consequences of this process see below). Moreover, HDAC6 has one domain which binds specifically to mono- and poly-ubiquitin chains. It seems though that HDAC6 with its partner proteins play a critical role in determining the fate of ubiquitinated proteins in cells (21). It has been hypothesized that high-affinity binding of ubiquitinated proteins by HDAC6 delays their recognition and processing by proteasome that finally leads to accumulation of ubiquitinated, mainly misfolded proteins and formation of aggresomes (20). HDAC6 has been shown to be essential for transport of ubiquitinated proteins along microtubules and their accumulation in aggresomes, a process which ultimately induces lysosomal degradation and autophagic clearance (22).

Class IV is represented by only one enzyme - HDAC11, which shares some structural similarity with both class I and II HDACs, however the binding target substrates for this HDAC have not been identified so far.

Class III HDACs (sirtuins, SIRT1-7) regulate gene expression in response to changes in cellular redox balance and are reffered to as critical regulators of energy-dependent transcription (23). The role of sirtuins is to prevent the accumulation of mutations, promote replicative senescence under stressful conditions and to ensure that DNA damage is not propagated (24). Thus, sirtuins are described to be cancer-protective, although some studies also point out their role in tumor progression. The best described sirtuin is sirtuin 1 (SIRT1), although 7 sirtuins have already been discovered (25). This group has recently emerged as a potential new target for anticancer therapy, however still there is limited clinical experience with sirtuins modulators (reviewed by Liu et al. (26) and Saunders et al. (24)). It seems that sirtuins activators, such as resveratrol are promising anticancer therapeutics (24). Resveratrol is undergoing several phase I/II clinical trials as single agent in patients with colorectal cancer. It is also tested in combination with bortezomib in phase II trial in patients with multiple myeloma (MM).

5. HISTONE DEACETYLASES AND CANCER

The disrupted balance between histone acetylation and deacetylation has been frequently associated with tumorigenesis. Aberrant recruitment of HDACs has been

observed in both hematological malignancies and solid tumors (27). Altered expression of individual HDACs in neoplastic cells has been shown to depend on tumor type and tissue of its origin (28). Class I HDACs have been demonstrated to be overexpressed in primary colon, breast, cervix, prostate, gastric, and lung carcinomas (29-32). Some studies demonstrate that high expression of individual HDACs correlates with advanced stage of disease, increased rate of proliferation, poor prognosis, nodal metastases and diminished patient survival. However, some reports suggest that overexpression of HDAC1 in hormone-sensitive breast cancer is associated with improved patient survival (31). Relatively little is known about the expression of class II HDACs in tumors. In some cancer types overexpression of class II HDACs and correlation with advanced stage of the disease have been observed, while in others decreased expression seemed to correspond with advanced disease stage and inferior survival rate (33). In many human prostate cancer cell lines levels of SIRT1 have been shown to be significantly increased. The SIRT1 inhibition by nicotinamide as well as by RNA interference resulted in decreased proliferation of prostate cancer cells.

Although the results from correlative studies seem contradictory, generally the inhibition or knockdown of individual HDACs in various tumor cells lines inhibits tumor cell growth and impairs tumor cell survival (34-35).

6. HISTONE DEACETYLASES INHIBITORS

Since epigenetic regulation of gene expression is a reversible process, targeting histone deacetylases provides a good rationale for anticancer therapy. Inhibition of the HDAC activity results in the transcriptional activation of the corresponding genes including tumor suppressor genes, often silenced in cancer. However, as assessed by gene expression microarrays, HDAC inhibition results in transcriptional repression of approximately the same or even greater number of genes (36-40). Even if some mechanisms are still to be elucidated, it seems that inhibition of histone deacetylases activity plays an important role in blocking tumor progression. Recently, a large number of various HDACi has been developed, many of which have entered phase I, II and III clinical trials. Vorinostat (SAHA – suberoylanilide hydroxamic acid) is undergoing phase III clinical trials as an single-agent treatment in patients with advanced malignant pleural mesothelioma lung cancer and phase III/IV in patients with non-small cell lung cancer (NSCLC). Moreover, it is tested in combination with proteasome inhibitor – bortezomib in patients with multiple myeloma. Tacedinaline is undergoing phase III clinical trials in combination with gemcitabine as a second-line treatment in patients with advanced non-small cell lung cancer.

Encouraging results from clinical trials using newly developed epigenetic treatment in hematological malignancies, such as myelodysplastic syndrome (MDS), chronic myeloid leukemia (CML), acute myeloid leukemia (AML) (41-44) have led to investigation of epigenetic therapy in solid tumors. So far, only vorinostat has been approved in 2006 by the U.S. Food and Drug Administration (FDA) for treatment of advanced or refractory primary cutaneous T-cell lymphoma (CTCL). Vorinostat has been shown to act synergistically with various chemotherapeutics in phase I, II and III clinical trials (45).

Several classes of HDACi have been characterized based on distinct structure, origin and chemical properties. HDACi interfere with catalytic domain of HDACs with varying efficiency and among these agents both pan-(promiscuous) and selective inhibitors are described. The chemical structure of HDACi typically consists of three parts: a zinc-binding-group (ZBG), a hydrophobic group (CAP) for protein recognition and interaction and a linker connecting both of them (46). ZBG is analogous to the acetyl residue of acetylated histone lysine.

The group of short chain fatty acid include butyric acid (BuA), sodium butyrate (NaBu), phenylbutyrate, valproic acid (VPA) and a prodrug AN-9. Apart from AN-9 they inhibit HDAC activity at relatively high concentrations (mmol dose ranges).

The largest group, hydroxamate-based HDACi, inhibit zinc-dependent class I and II HDACs. Trichostatin A (TsA), vorinostat, panobinostat (LBH589), belinostat (PXD101) and droxinostat (4-(4-chloro-2-methylphenoxy)-N-hydroxy-butanamide) all mimic the lysine substrate and chelate essential zinc ion in the enzymatic centre of class I and II HDACs. In this group SK-7041 and SK-7068 have been shown to selectively inhibit only HDAC1 and 2, whereas tubacin is a specific inhibitor of HDAC6 (47). Tubacin specifically inhibits deacetylation of alpha-tubulin and subsequently impairs its function. Tubacin has also been reported to induce marked accumulation of ubiquitinated proteins and apoptosis of multiple myeloma cells (48). *In vitro* studies have shown that TsA induces strong acetylation of histones in nanomolar concentrations (49-50), but it has never entered clinical trials due to severe side effects and high toxicity. Hydroxamate-based HDACi have limited ability to inhibit HDACs specifically, so non-hydroxamate, isoform-selective inhibitors are currently under development (51).

The third class, benzamides, has been reported to inhibit HDAC activity by chelating zinc ion at micromolar concentrations. Entinostat (MS-275, SNDX-275), tacedinaline (CI-994) and mocetinostat (MGCD-0103), all are in clinical trials. Mocetinostat is an isoform-specific inhibitor which targets class I HDACs (52).

The fourth group, cyclic peptides, include depsipetide (romidepsin, FK228, FR901228), trapoxin A, CHAPs, apicidin. Depsipetide is activated in cells due to reduction of the disulfide bond to thiol that enters the active site of HDAC and chelates zinc ion (53). They are all potent irreversible HDAC inhibitors active in nanomolar concentrations.

The fifth group, epoxides includes depudecin which irreversibly inhibits HDACs at micromolar concentrations.

Depudecin has been also shown to inhibit growth of vascular endothelial cells and process of angiogenesis.

7. ACETYLATION OF NON-HISTONE PROTEINS

Post-translational acetylation of lysine residues of histones was first discovered in the early 1960s. Originally it was regarded as a powerful tool of gene expression regulation. 20 years later acetylysine residues were also found in non-histone proteins leading to the notion that N-acetylation process controls many basic cellular processes. Nowadays it is clear that functions of many non-histone proteins are controlled by acetylation (Table 1, reviewed by Glozak et al. (54)).The process of acetylation has been even proposed to rival that of phosphorylation. There is a growing body of evidence that acetylation status of non-histone proteins regulates their functions and influences their stability (54). The mechanism of this phenomenon has been suggested to depend on lysine residues that are targets for both acetylation and ubiquitination. Acetylation of proteins may protect them from ubiquitination and subsequent degradation, while deacetylation in many cases precedes ubiquitination.

8. MECHANISMS OF ACTION OF HDACi

8.1. Cytotoxicity

8.1.1. Apoptosis

8.1.1.1. HDACi activate both intrinsic and extrinsic apoptotic pathway

In vitro studies have demonstrated that HDACi induce tumor cell death that biochemically and morphologically resembles apoptosis. This process can be mediated by regulating both histone function and subsequently gene transcription and/or hyperacetylation of non-histone targets of HDACs. Apoptosis, depending on the stimulus, can proceed via two pathways which are molecularly linked and converge at the level of the effector proteolytic enzymes called caspases. It has been established that exposure of cancer cells to HDACi leads to the activation of both intrinsic and extrinsic programmed cell death pathways (68-70). The extrinsic pathway is initiated by ligand-dependent activation of the transmembrane death receptors from TNFR family. Activation of death receptor signaling, via recruitment of adaptor proteins, leads to activation of caspase 8 or caspase 10 and initiation of cell death cascade. HDACi have been demonstrated to induce expression of genes encoding both death receptors (TRAILR1 (DR4), TRAILR2 (DR5), FAS) and their cognate ligands (TRAIL - tumor necrosis factor-related apoptosis-inducing ligand), FASL (71). HDACi have been reported to stimulate extrinsic apoptotic pathway both *in vitro* and *in vivo*. In *in vitro* studies inhibition of death receptors and/or their ligands with siRNA and neutralizing antibodies have been shown to protect tumor cells from HDACi-mediated apoptosis (72-75). siRNA targeting TRAIL and FAS has been reported to protect leukemic cells from valproic acid-induced apoptoisis in a murine APL (acute promyelocytic leukemia) model (73). Interestingly, in this study HDACi have been shown to induce apoptosis selectively in leukemic cells, but not in normal hematopoietic progenitors. Moreover, HDACi also downregulate various inhibitors of death receptor pathway including c-FLIP (a protease-deficient caspase homolog widely reported as inhibitor of a procaspase 8 activation) (71, 76). A recent study identified a small-molecule - droxinostat, already known to sensitize cancer cells to death receptor stimuli by decreasing the expression of FLIP, to be a selective inhibitor of HDAC3, HDAC6, and HDAC8. Inhibition of these HDACs was functionally important for the ability of droxinostat to sensitize cells to death ligands (77).

The intrinsic apoptotic pathway, dependent on mitochondria, is usually activated in response to stress stimuli such as DNA damage, oxidative stress, chemotherapeutic drugs, disruption of growth factor signaling. Disruption of mitochondrial transmembrane potential leads to release of cytochrome c, subsequent activation of caspase 9, formation of apoptosome and activation of downstream effector caspases 3, 6 and 7. Proapoptotic and antiapoptotic members of the Bcl-2 family play a crucial role in modulation of apoptotic process. Even if some mechanisms of proapoptotic activity of HDACi are still to be elucidated, there is a body of evidence that HDACi alter the balance between the pro- and antiapoptotic proteins and thus activate the intrinsic apoptosis pathway. HDACi upregulate proapoptotic proteins of Bcl-2 family, including Bak, Apaf-1 and BH3-only proteins (Bid, Bim, Bmf, Noxa) at transcriptional and/or translational level (76, 78-79). Moreover, inhibitors of apoptosis of IAP family (survivin, IAP, XIAP) and anti-apoptotic proteins of Bcl-2 family, such as Bcl-2, Bcl-xL, Mcl-1 have been shown to be downregulated upon inhibition of HDAC enzymes with a number of various HDACi (78, 80-81). It has been also established that vorinostat is involved in post-translational modification and cleavage of pro-apoptotic proteins, such as Bid and subsequent initiation of intrinsic apoptotic pathway in acute T-cell leukemia cell line (82). Depsipeptide has been reported to induce apoptosis by activating caspase-3 and decrease in the protein level of surviving (83); panobinostat by degradation of Aurora A and B kinases (84-86). Entinostat induces apoptosis in erbB2-overexpressing breast cells by reduction of the levels of growth factor receptors erbB2 and erbB3, as well as significant decrease in downstream signaling (87). Overexpression of prosurvival Bcl-2 family proteins protects tumor cells form HDACi-induced apoptosis both *in vitro* and *in vivo* (88-89). Further studies have shown that knockdown of Bad, Bim, Noxa and Bmf with siRNA substantially impairs HDACi-induced apoptosis (79, 90-91).

8.1.1.2. HDACi regulate the activity of tumor suppressors p53 and p73, important inducers of apoptosis

Recently, it has been shown that depending on the trigger p53 undergoes site-specific acetylation and subsequent activation (92-93). Different acetylation patterns influence DNA-binding ability of p53, spectrum of its targets and thus cellular fate. For instance, acetylation of p53 at K320 activates promoters of genes with high-affinity p53 binding sites (e.g. *p21*), while acetylation at K373

Table 1. Non-histone substrates for acetylation activity

Function	Protein	Role	Acetylation-induced functional changes
Transcriptional factors			
	p53	Tumor suppressor protein Induces apoptosis and growth arrest by holding the cell cycle at the G_1/S regulation point on DNA damage recognition Activates DNA repair	Acetylation by p300/CBP protects the protein from ubiquitination and proteasome-dependent degradation (55), it guarantees protein stability and transcriptional activation of its target genes (56) (e.g. *p21* – cell cycle inhibitor)
	c-Myc	Proto-oncogene upregulated in many cancer types Stimulates cell proliferation, differentiation and self-renewal, blocks apoptosis	Acetylation leads to increased stability but not necessarily activity (ubiquitination of c-Myc is also required for transcriptional activation of its target genes (57))
	GATA family	Transcriptional factors that play important role in hematopoiesis, erythroid development and differentiation and T-cells development	Acetylation activates protein and increases its DNA binding capability (58-59)
	E2F family	Regulators of cell cycle and apoptosis, family consists of three activators and six suppressors, tumor suppressor rb binds to the E2F1 and prevents activation of its target genes involved in DNA replication, cell proliferation, chromosomal replication	Acetylation increases DNA binding affinity, stabilizes E2F1, increases its half-life by preventing ubiquitination and degradation (60) Acetylation of E2F1 also leads to its recruitment to the promoter of proapoptotic *p73* (61)
	Runx 3[1]	Tumor suppressor often functionally inactive in tumor cells, in a subset of tumors it has been reported to have an oncogenic function	Hyperacetylation inhibits ubiquitin-mediated degradation of Runx3 (62)
	YY-1[2]	Ubiquitously distributed transcription factor involved in repressing and activating gene promoters It has been hypothesized that YY-1 has an ability to direct HDACs[3] and HATs[4] to a promoter in order to activate or repress the promoter (54)	YY-1 is regulated by acetylation and deacetylation It contains two domains – repression domain (acetylated and deacetylated) and DNA-binding domain (acetylated which greatly decreases its DNA-binding activity)
	FOXO[5] family	Regulators of cell-cycle, DNA repair and apoptosis genes in response to DNA damage and oxidative stress, claimed to be tumor suppressor	Acetylation exerts inhibitory effects on transactivation activity (63) SIRT1[6] deacetylates and deactivates FOXO
	NF-kappaB[7]	Regulator of genes responsible for cell proliferation and survival constitutevely active in many cancer cells	Acetylation and deacetylation dynamically regulate NF-kappaB in a complicated and controversial manner that has to be elucidated (14)
	STAT-3	Member of STAT[8] family Downstream effector of cytokine signal transduction pathways When phosphorylated dimerizes and activates target genes	Acetylation enhances its DNA binding and transactivation (64) Deacetylation inhibits transcription of STAT3 target genes such as *cyclin D* and antiapoptotic gene *bcl-xL*
	MEF2 family[9]	Regulators of cell differentiation Induce stress response in some tissues	Deacetylation induces its functional inhibition (18)
DNA repair enzymes			
	Ku70	DNA repairing enzyme Inhibits apoptosis by binding directly to Bax	Acetylation disrupts Ku70-Bax interaction and allow Bax to translocate to mitochondria and induce apoptosis (65)
Structural protein			
	alpha-tubulin	Cytoskeletal protein Influences tumor cells motility	Hyperacetylation causes its functional disruption (47) HDAC6 and SIRT2 deacetylate tubulin and increase cell motility
Chaperones			
	HSP90[10]	Chaperone protein protecting client proteins from proteasome-dependent degradation Critical for maturation of various proteins	Acetylation decrease its binding to VEGFR[11]-1 and 2 and enhance their degradation in the proteasome Acetylation induces degradation of HSP90 client proteins: HER2/neu[12], ERBB1[13], c-Raf, BCR-ABL (66) and FLT3[14]
Cell adhesion/transcription regulator			
	beta-catenin	Component of Wnt signaling pathway When activated translocates to the nucleus Might function as an oncogene	Deacetylation promotes beta-catenin nuclear accumulation Inactivation of HDAC6 inhibits beta-catenin nuclear accumulation and subsequent *c-myc* induction (67)

Abbreviations: [1]Runx 3 – Runt-related transcription factor 3, YY-1 - Yin Yang-1, [3]HDACs - histone deacetylases, [4]HATs - histone acetyltransferases, [5]FOXO - Forkhead box proteins, [6]SIRT1 – sirtuin 1, [7]NF-kappaB - nuclear factor kappa-light-chain-enhancer of activated B cells, [8]STAT - signal transducers and activator of transcription, [9]MEF2 - myocyte enhancer factor-2, [10]Hsp90 - heat shock protein 90, [11]VEGFR - vascular endothelial growth factor, [12]HER2/neu - human epidermal growth factor receptor 2, [13]ERBB1 - epidermal growth factor receptor1, [14]FLT3 - FMS-related tyrosine kinase 3

activates promoters of genes with low-affinity p53 binding sites (e.g. *Bax*) (94). Acetylation increases also stability of p53 and protects it from ubiquitination and subsequent degradation by proteasomes *in vitro*. However, some studies of knock-in mice with lysines replaced by arginines indicate that acetylation of p53 may not be required for its stability *in vivo*, although it influences p53 activation upon DNA damage (95). Interestingly, it has been reported that HDACi specifically induce degradation of mutant p53, a process preceded by induction of p53-dependent transcription (96). E2F and p73 have been also implicated in HDACi-mediated apoptosis. Doxorubicin-triggered acetylation of E2F1 has been demonstrated to trans-activate its target gene – *p73* – a p53-related protein known to induce apoptosis (61). However, the exact role of p73 remains to be determined as many studies suggest that p73 may not be a tumor suppressor but rather an oncoprotein.

8.1.1.3. HDACi target DNA-repairing enzymes involved in induction of apoptosis

Both classical and class III HDACs have been reported to deacetylate and increase DNA binding of Ku70. Ku is a multi-functional heterodimer of two proteins – Ku70 and Ku80. It binds to DNA double-strand breaks (DSBS) and is required for the non-homologous end-joining (NHEJ) DNA repair pathway. Upon DNA damage Ku70 levels have been reported to increase (54). In an unclear mechanism HDACi have been reported to decrease Ku70 and Ku80 in melanoma cells and thus increase their sensitivity to radiation-induced DNA damage. Ku70 also plays an important role in apoptosis regulation. It binds and sequesters in the cytoplasm the proapoptotic protein Bax. HDACi-mediated acetylation of Ku70 releases Bax, allowing it to translocate to the mitochondrial outer membrane and initiate apoptosis, a phenomenon described in several cell lines (65).

8.1.1.4. HDACi modulate the activity of chaperone protein HSP90

The activity of HSP90, a molecular chaperone, is regulated by acetylation status (54). HSP90 binds a diverse array of proteins, enables their maturation, proper folding and prevents their ubiquitination and subsequent proteasomal degradation. Client proteins of HSP90 include key oncogenic (Her2/neu, ERBB1, ERBB2, c-Raf, BCR-ABL, FLT3) and antiapoptotic proteins. Acetylation of HSP90 impairs its ability to form complexes with client proteins, ultimately leading to their proteasomal degradation. HDAC6 has been demonstrated to mediate HSP90 deacetylation and HDAC6-inhibitors have been reported to induce HSP90 acetylation and subsequent degradation of its client proteins (97-99).

8.1.1.5. HDACi influence redox state of the cancer cells and activate ROS-mediated apoptosis

Accumulation of reactive oxygen species (ROS) during oxidative stress is implicated in cellular damage and mediation of programmed cell death. ROS have been widely reported to oxidatively damage nucleic acids, proteins and lipids. ROS have been reported to activate and induce transcription of Bim, a proapoptotic protein of BH3-only Bcl-2 family. Accumulation of ROS in HDACi-treated transformed cells has been reported in various studies (78, 100). The generation of ROS is believed to be an important and early event in HDACi-induced cell death that precedes the loss of mitochondrial outer membrane potential (82, 101). LAQ-824 has been shown to trigger ROS generation as early as 30 minutes after drug treatment in human leukemia cells (102). Treatment of leukemia cells with entinostat or vorinostat enhances ROS generation and downregulates expression of Mn-superoxide dismutase (103). Pretreatment of cells with ROS scavengers such as N-acetylcysteine protects cells form HDACi-triggered apoptosis Moreover, HDACi-triggered ROS accumulation is believed to appear selectively in transformed but not in normal cells. The mechanism of this phenomenon remains unclear, however it has been hypothesized that selective induction on the transcriptional level of the thioredoxin, an endogenous ROS scavenger, helps normal cells to cope with HDACi-trigerred oxidative stress (100). On the contrary, vorinostat-treated transformed cells have been shown to upregulate the transcription of negative regulator of thioredoxin – TBP2 (thioredoxin binding protein 2) (78, 104). It has been also demonstrated that vorinostat downregulates thioredoxin transcription in human bladder carcinoma (104), while depsipeptide in human lung cancer cells downregulates thioredoxin reductase (TrxR), an enzyme known to reduce thioredoxin (105). Interestingly, thioredoxin has been identified to be an acetylated protein in HeLa cells and mouse liver mitochondria, however it has not been established how the acetylation influences Trx functions and whether it is hyperacethylated in response to HDACi treatment (106). Interestingly, hyperacetylation of peroxiredoxins (Prx) I and II increases their ability to reduce H_2O_2, the phenomenon that probably contributes to resistance of HDACi-treated cells to ROS-mediated damage (107).

8.1.2. Autophagy

HDACi induce autophagy-associated cell death. Autophagy is a catabolic, tightly regulated process involving sequestration of cellular proteins and organelles in autophagosomes followed by fusions with lysosomes and subsequent degradation via acidic lysosomal hydrolases. A variety of ATG (autophagy-related) proteins is implicated in autophagosome formation. Recent studies indicate that mTOR regulates autophagy by inhibiting activation of ATG5 and ATG7 among others. In a study by Hrzenjak et al. vorinostat decreased mTOR expression and induced cytotoxicity via autophagic mechanisms (108). It seems that HDAC1 as well as HDAC6 activity plays an important role in autophagy process. HDAC1 inhibition with a specific inhibitor or siRNA has been reported to induce autophagic death. Vorinostat and butyrate have been reported to induce autophagic cell death in HeLa cells deficient in Apaf-1 protein or with overexpression of Bcl-xL (109-110). The relative contribution of apoptosis and autophagy in HDACi-mediated cytotoxicity still has to be elucidated. Moreover, it has recently been argued that autophagy might actually be a cytoprotective mechanism that under metabolism stress promotes cell survival (111).

8.2. Cell cycle arrest

A cell-division cycle, vital and tightly regulated process, consists of four distinct phases that leads to cell

duplication. Cells with deregulated cell cycle components divide actively and may potentially lead to tumor formation. In various studies HDACi have been reported to induce cellular differentiation and cell cycle arrest at G1/S and G2/M checkpoints based on cell type, dose and type of HDACi . Hence, treatment with HDACi decreases the proportion of cells in S phase and increases the proportion of cells in the G(0)-G(1) and/or G(2)-M phases of the cell cycle (84-87, 112-113), and subsequently results in profound cell growth arrest (70, 83-84, 112, 114-116). G1/S arrest is mediated by complexes of cyclins and cyclin-dependent kinases (CDKs) that phosphorylate and inactivate tumor suppressor retinoblastoma protein (Rb). Once phosphorylated Rb releases transcription factors of E2F family which stimulates the progression along the cell cycle. Two families of cell cycle inhibitors at G1/S checkpoint have been described - the *cip/kip* family (p21, p27 and p57) and INK4a/ARF family (p16). Due to inhibition of cyclin/CDK complexes retinoblastoma protein (Rb) is kept in a state of low phosphorylation, tightly bound to the transcription factor E2F. It has been established that various HDACi induce p21 in a p53-independent manner on both transcriptional and translational level (117-118). It seems that G1 arrest may also result from HDACi-mediated downregulation of cyclin proteins, mainly cyclin D and A (119). The mechanism of a much rarer HDACi-mediated G2/M arrest is poorly understood. HDACi have been reported to downregulate cyclin B1, a protein expressed predominantly during G2/M phase of cell cycle (120).

8.3. Angiogenesis, metastasis and invasion

8.3.1. HDACi downregulate HIF-1alpha activity and target growth factors responsible for angiogenesis

Hypoxia-inducible factor 1 (HIF-1) is a heterodimer consisting of alpha and beta subunits. The post-translational modification of HIF-1alpha by prolyl hydroxylases (PHDs) in normoxia leads to a series of modifications, which result in its polyubiquitination and proteasomal degradation. The ubiquitination is a consequence of interaction between HIF-1alpha subunit and pVHL (von Hippel-Lindau protein), which binds to HIF-1alpha only when a conserved proline is hydroxylated (121). In hypoxia the process of hydroxylation of HIF-1alpha by PHDs is inhibited and the molecule is stabilized and activated. The stabilized form of HIF-1alpha is translocated to the nucleus, where it dimerizes with HIF-1beta, binds to p300/CBP and nuclear receptor coactivator 1 (NCOA1) and finally activates target gene transcription (e.g. *vegf*) (122). VEGF is described as one of the most important proangiogenic factors that inhibits endothelial cell apoptosis by increasing the expression of Bcl-2 (123). Four isoforms of this molecule bind to three VEGF receptors: VEGFR-1, VEGFR-2 and VEGFR-3 . The principal form of VEGF is VEGF-A which binds to VEGFR-2. Both overexpression of HIF-1alpha and high transcription rate of VEGF mediated by HIF-1alpha have been observed in various tumors (124-125), which makes HIF-1alpha an attractive target for antitumor therapies.

Histone deacetylase inhibitors downregulate HIF-1alpha expression in a complex mechanism both directly and indirectly. It has been established that class I and II HDACs play crucial role in HIF-1alpha regulation. An increased level of HDAC1 has been shown to be commensurate with the reduction of expression of p53 and pVHL. As mentioned above pVHL participates in HIF-1alpha ubiquitination and proteasomal degradation, while p53 provokes HIF-1alpha probably by competing for shared coactivator p300 (126). Reduced expression of p53 an pVHL leads to suppression of factor inhibiting HIF-1alpha (FIH) (127), which can be fully inversed by TsA both *in vitro* and *in vivo* (128). Some reports suggest an exceptional role of class II HDACs in the process of angiogenesis. They have been noted to have a direct impact on HIF-1alpha stability. In a coimmunoprecipitation assay those isoenzymes were identified to associate with HIF-1alpha protein (129). What's more, cells with siRNA-silenced HDAC4 and HDAC6 show a reduced HIF-1alpha expression. HDAC7 has been shown to bind selectively to HIF-1alpha and under hypoxia cotranslocate to the nucleus, where it takes part in HIF-1alpha-p300 binding (130). It has been demonstrated that HDACi even in small doses have a potential to block the activity of HIF-1alpha CAD (C-terminal transactivation domain of HIF-1alpha), induce hyperacetylation of p300 and repress the formation HIF-1alpha-p300 complex *in vivo* and thus inhibit HIF-1alpha transcriptional reactivity (131). There is also a hypothesis that HDAC1 and HDAC3 have a potential to directly interact with ODDD (oxygen dependent degradation region) of HIF-1alpha (132), which promotes the molecule binding to pVHL ubiquitin E3 complex.

HDACi are also believed to be effective VEGF signaling inhibitors. In a study conducted by Deroanne et al. TsA and vorinostat have been shown to inhibit VEGF, VEGFR1 and VEGFR2, as well as upregulate the expression of VEGF competitor protein semaphorin III at both protein and mRNA level. Both TsA and vorinostat have a potential to prevent human umbilical cord endothelial cells (HUVEC) stimulated by VEGF from invading a type I collagen gel and forming tubular structures (133). Moreover, vorinostat and TsA inhibit the formation of capillary-like network in embryonic bodies with high specificity without affecting the growth and differentiation of other cells. Depsipeptide has been demonstrated to suppress more efficiently the expansion of large solid tumors, whose growth depends on the vasculature network development, than small solid tumors depending mainly on little capillaries. It has been though hypotesized that this effect is linked to the inhibition of angiogenesis. Further studies have shown that depsipeptide reduce VEGF mRNA expression without affecting *hif-1alpha* gene (134).The mechanism, in which this agent inhibits VEGF gene expression, is probably linked to histone acetylation of VEGF promoter regions. Panobinostat, which efficiently inhibit angiogenesis *in vitro* and *in vivo* in the presence of high concentrations of VEGF-A and bFGF (135), has been described to modulate

VEGF-A signaling pathway by preventing AKT phosphorylation in a dose-dependent manner.

8.3.2. HDACi downregulate matrix metalloproteinases MMP-2 and MMP-9

Matrix metalloproteinases are a group of zinc-binding endopeptidases secreted as zymogenes that desintegrate extracellular matrix components. They participate in the process of metastasis by promoting cell invasion. Multiple studies have revealed a special importance of MMP-2 (type IV collagenase, gelatinase B) and MMP-9 (type IV collagenase, gelatinase A) in this process (136). Antimetastatic effect of HDACi-based therapies is associated with inhibition of matrix metalloproteinases. In an *in vitro* study conducted by Kim et al. (137) apicidin has been reported to inhibit MMP-2 and MMP-9 (both zymogen and protein), to suppress the formation of new blood vessels in CAM (chicken chorioallontoic membrane) assay and significantly attenuate the vasculature of v-*ras*-transformed murine fibroblast NIH3T3 cells and human melanoma A2058. The function of MMP in tumor cells can be also inhibited by the activity of RECK - a membrane glycoprotein negatively regulating MMP activity, widely expressed in normal untransformed human cells and often undetectable in many tumor lines (138-139). A study performed by Liu et al. shows that TsA has a potential to upregulate RECK expression by its transcriptional activation in lung cancer cells CL-1 cells *in vitro,* which subsequently caused a decrease in MMP-2 level in gelatin zymography assay (140).

8.3.3. HDACi up-regulate the expression of angiostatic protein ADAMTS1

ADAMTS1 is an extracellular metalloproteinase found mostly in cartilage, which in contrast to other known metalloproteinases is reported to be a potent inhibitor of angiogenesis (141). The findings of Chou and Chen show that HDACi such as TsA, vorinostat and specific HDAC6 inhibitor - tubacin are able to upregulate the expression of ADAMTS1 in human lung carcinoma A549 cells (142). An increased level of ADAMTS triggers the release of angioinhibitory proteins - trombospondin 1 and 2.

8.3.4. HDACi downregulate the expression of chemokine (C-X-C motif) receptor 4 (CXCR4)

CXCR4, a CXC chemokine family receptor, plays an important role in the homing of bone marrow progenitor and circulating endothelial cells to active sites of angiogenesis (143) . During testing its angiostatic activity on HUVEC cells, panobinostat has been reported to down-regulate the expression of CXCR4 both at mRNA and protein level (135).

8.3.5. HDACi downregulate the expression of endothelial nitric oxide synthase (eNOS) affecting endothelial cells vasorelaxation

eNOS generates nitric oxide which plays a key role in the regulation of vascular tone and modulates angiogenesis in response to tissue ischemia (144). TsA, BuA and entinostat have been shown to downregulate eNOS protein and mRNA expression in a time and dose-depending manner in HUVEC cells (145). Surprisingly, the downregulation of eNOS mRNA by TsA correlated with pronounced activation of *eNOS* promoter region.

8.4. Immunomodulatory effects of HDACi

The antineoplastic effect of HDACi-based regimens has been reported to be linked with their immunomodulatory function. HDACi have been demonstrated to induce antitumor immunity either directly by changing malignant cells into more attractive targets or indirectly by modifying immune effector cells activity and cytokine production. The influence of HDACi on immune system has a complex character and engages many different pathways. HDACi have been demonstrated to up-regulate the expression of various proteins involved in major histocompatibility complex (MHC) class I antigen presentation. The antigen presentation in MHC class I context involves the translocation of proteasome-degraded peptides into the lumen of ER, a process accomplished by TAP proteins (TAP-1 and TAP-2). A decrease of MHC class I is a common strategy employed by malignant cells to escape the immune surveillance. Moreover, deficiency in TAP-1 resulting from mutations within *tap-1* gene, decrease in TAP-1 mRNA stability or in *tap-1* promoter activity are considered to correlate with tumor progression and metastasis (146-147). A study conducted by Setiadi et al. has shown that treatment of TAP-deficient metastatic carcinoma cells with TsA increases levels of TAP-1 mRNA and protein due to enhanced recruitment of RNA polymerase II to the *tap-1* promoter. Increased *tap-1* promoter activity correlated with higher (about 10 fold) MHC class I expression on tumor cells that made malignant cells more vulnerable to CTLs-dependent lysis (147). HDACi also have a potential to enhance MHC class II and costimulatory molecules (CD40 (148), CD80 and CD86 (149)) level on tumor cells . A study by Magner et al. shows that TsA treatment leads to enhanced transcription and significant increase in MHC class II protein expression on tumor cell surface. Furthermore, up-regulation of MHC class II molecules was observed also in some tumor lines unresponsive to INF-gamma. Some authors suggest that HDACi convert neoplastic cells into effective antigen presenting cells (APC), which together with crosspresentation of tumor antigens by host APC could establish antitumor immunity. HDACi-treated tumor cells have been shown to be an effective vaccine strategy both in prevention and treatment model (150). Vaccination with TsA-treated melanoma cells elicited mobilization of cytotoxic and IFN-gamma secreting splenocytes, CD4+ T cells, CD8+ T cells and NK cells, which were shown to play a key role in a vaccination effectiveness. Depletion of these cells prior to vaccination completely abrogated its antitumor effect. HDACi have also been noted for their impact on sensitizing malignant cells into NK-induced cytotoxity by enhancing the expression of MHC class I chain-related molecules MICA and MICB on the surface of tumor cells (151). Those two molecules are ligands for natural killer cell protein group 2D (NKG2D), the activating immunoreceptor expressed on the surface of NK cells, gamma delta and CD8+ T cells. In the study by Schmudde et al. HDACi have been shown to sensitize tumor cells to the cytotoxic effect of IL-2-activated peripheral blood mononuclear cells (PBMCs), mainly NK cells. In the study performed on HeLa and HepG2 tumor cell lines, NaBu upregulated the expression of the MICA and MICB molecules

at both mRNA and protein level that directly increased susceptibility of tumor cells to NK-dependent lysis. HDACi treatment promoted increased expression of heat shock protein 70 (HSP70), Sp1 transcription factor and subsequent elevated expression of MICA and MICB proteins, which directly leads to increased susceptibility of tumor cells to NK-dependent lysis (152). Although NKG2D ligands trigger the activation of cytolytic NK cells and act as co-stimulatory signals for CD8+ T lymphocytes, it has been demonstrated that prolonged exposure to NKG2D ligands may make effector cells functionally anergic (153). This effect can be reversed by various cytokines such as IL-15, IL-12, IL-2 and INF-alpha, which provides a rationale for combinated HDACi-cytokines regimens (154-155). The exact role of HDACi in immunomodulation requires further studies in preclinical and clinical settings.

9. HDACi IN COMBINATION SCHEMES

It seems that despite their efficacy as single agents HDACi will find most clinical use in combination with other chemotherapeutics and ionizing radiation (IR) (156). A rationale for combining HDACi with chemotherapeutics and IR is HDACi-mediated hyperacetylation of core histones and reversal of chromatin compaction which may increase accessibility of DNA damaging agents. What's more, HDAC1 has been shown to interact both *in vitro* and *in vivo* with ataxia teleangiectasia mutated kinase (ATM) recruited and activated by DNA doublestrand breaks (157). HDACi treatment has been reported to induce phosphorylation-dependent activation of ATM (158), which might subsequently phosphorylate its targets including p53. The effect of sensitization might be further enhanced by the ability of HDACi to induce ROS and block DNA repair pathways by down-regulating the activity of repairing proteins such as Ku70 and Ku80 (159). Finally, HDACi are known to modulate apoptosis threshold in cancer, so that they can potentiate the effects of chemo- and radiotherapy.

9.1. Combinations with chemotherapy

Many preclinical and clinical trials (reviewed by Batty el al. (160) and Ma et al. (27)) combining HDACi with chemotherapeutic agents have been performed. Some of them suggest additive or even synergistic antitumor effects of such combinations. TsA has been demonstrated to act synergistically with paclitaxel both *in vitro* and in xenograft murine model (161). Vorinostat has been shown to synergize *in vitro* with SN38 TopoI inhibitor (162), potentiate the cytotoxic effect of gemcitabine (163) and sensitize cancer cells to cisplatin (164). The efficacy of combinations of vorinostat with paclitaxel or with carboplatin has been assessed in phase I and II clinical trials (165-166). The sequence of drug administration in such schemes has been shown to be of an utmost importance. HDACi administration before chemotherapy sensitizes tumor cells to genotoxic agents, as it has been demonstrated in an *in vitro* assay by Kim et al. where TsA and vorinostat administered before VP-16, ellipticine, doxorubicin and cisplatin increased the sensitivity more than 10 fold, whereas the inverse order of drugs was without result(167). Combinations of HDACi and DNA methylation inhibitors such as 5-aza-2'deoxycytidine (168) and hydralazin (169) have been tested in phase I/II trials. Although response rates were not definitively superior, a relatively rapid response time was suggested to be a surrogate for synergy (160). Moreover, synergistic effects with ABL kinase inhibitor imatinib (170), activators of FAS and TRAIL pathways, remarkable sensitization to trastuzumab, taxol, gemcitabine, epothilone B (97) and additive effects in combination with retinoic acid (171) have been reported in in vitro studies. An interesting example of synergy is the cooperation between HDACi and a HSP90 inhibitor 17-allyloamino-demethoxy geldanamycin (17-AAG), which triggers client proteins degradation and proteasome inhibitor bortezomib (172-173). Independent in vitro studies by Yu et al. (172) and Pei et al. (174) have shown that preincubation of malignant cells with HDACi before treatment with bortezomib results in a mitochondrial dysfunction, caspase activation, and apoptosis, reflected by caspase 9, 8, and 3 activation and poly(adenosine diphosphate–ribose) polymerase (PARP) degradation. The mechanisms underlying this synergy remain to be fully elucidated, but they probably results from supression of NF-kappaB activity, which leads to massive accumulation of ROS and apoptosis induced by c-Jun NH2-terminal kinase activation, p53 induction, and caspase-dependent cleavage of $p21^{CIP1}$, $p27^{KIP1}$, Bcl-2 and Mcl-1, X-linked inhibitor of apoptosis, and cyclin D1 downregulation (174).Moreover, the combination of tubacin, selective HDAC6 inhibitor, and bortezomib in an *in vitro* trial conducted by Hideshima et al. resulted in an accumulation of polyubiquitinylated proteins, activation of stress response and subsequent induction of apoptosis.

9.2. Combinations with irradiation

HDAC inhibitors have been reported to improve the therapeutic effects of irradiation, acting both as radiation sensitizers and radioprotectors from the side effects of gamma-radiation. Many HDACi have been found to enhance the effect of irradiation, including: NaBu (175-176), TsA (177), VPA (178), Vorinostat (179), entinostat, depsipeptide and hydroxamic acid analogues such as M344 (180). HDACi concomitantly suppress cutaneous radiation syndrome (CRS), one of major obstacles in radiotherapy of tumors. They have been observed to protect from fibrosis and secondary tumor formation and to reduce skin damage. This protecting activity is most probably a consequence of HDACi-mediated decrease in the expression of the inflammatory cytokines such as TNF and transfroming growth factors TGF-beta1 and TGF-beta2 (181).

10. RESISTANCE TO HDACi

Despite its success, HDACi-based therapy, as well as any new antitumor modality, meets with development of resistance. It seems that long-time exposure to HDACi leads to a selection of more aggressive phenotypes. Such a phenomenon have been reported by Fiskus et al. (182), who described a case of HL-60 cells resistant to NaBu,

vorinostat, LAQ824 and panobinostat that lacked expression of HDAC6. Another reason for HDACi resistance might be overexpression of antiapoptotic proteins Bcl-2 and Bcl-xl or deletion of proapoptotic Bim and Bid (88, 183). Overexpression of peroxiredoxins which correlates with reduction in ROS generation may also protect cells from HDACi activity (184). Resistance to some HDACi (depsipeptidine and apicidin) can be related to HDACi-induced increased acetylation in promoter region of gene encoding multidrug-associated protein (MRP1), which leads to overexpression of P-glycoprotein (Pgp) and efflux of HDACi (185-186). However, resistance to vorinostat is not related to MPR-1 expression (187) and correlates with activation of STAT-1,-3 and -5 (188). Therefore, given a variety of mechanisms engaged, resistance to HDACi needs further investigation.

11. CONCLUSIONS

HDACi have emerged as potent antitumor agents in preclinical models. So far, only vorinostat has been approved by the FDA for the treatment of cutaneous T-cell lymphoma. The potential use of HDACi is exciting, however they should be introduced into clinical use with great caution. Since many histone and non-histone proteins have been identified as targets for both acetyaltion and deacetylation, HDACi exert extremely pleiotropic effects. Elucidation of mechanisms of their action is critical for designing the most effective combinations for planning therapeutic schemes. Moreover, it can help to predict potential side –effects of those promising new drugs.

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