The Synergistic Effects of DNA-Targeted Chemotherapeutics and Histone Deacetylase Inhibitors As Therapeutic Strategies for Cancer Treatment[a]

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Abstract: Histone deacetylase (HDAC) inhibitors are a group of anticancer drugs which cause growth arrest and apoptosis of several tumor cells. HDAC inhibitors have been also found to increase the anticancer efficacy of several treatment modalities i.e. chemotherapy or radiotherapy. Here, we review the literature on combinations of HDAC inhibitors both with ionizing radiation and with other drugs, highlighting DNA-damaging compounds. The results of numerous studies with several types of cancer cells discussed in this review demonstrate that HDAC inhibitors enhance the effect of DNA damaging agents, such as inhibitors of topoisomerases, inhibitors of DNA synthesis, DNA-intercalators and agents covalently modifying DNA (i.e. doxorubicin, etoposid, 5-fluorouracil, cisplatin, melphalan, temozolomide and ellipticine) or of irradiation. Hence, the use of HDAC inhibitors combined with these antitumor drugs or ionizing radiation is a promising tool which may make treatment of patients suffering from many types of cancer more efficient. Several molecular mechanisms are responsible for the observed higher sensitivity of tumor cells towards therapeutic agents elicited by HDAC inhibitors. These mechanisms are discussed also in this review.

Keywords: Chromatin remodeling, histone acetylation, histone deacetylase, histone deacetylase inhibitor, anticancer drugs, DNA-damaging drugs, ionizing radiation, combined treatment modalities, mechanisms of combined treatment, apoptosis.

1. INTRODUCTION

The last few decades have seen the emergence of many novel antitumor drugs and combinations of these with established drugs or other therapeutic modalities. Epigenetic processes, i.e. posttranslational changes in chromatin, mRNA regulation or DNA methylation, have emerges as novel targets in numerous investigations. Among them, histone acetylation and deacetylation have been intensely investigated because of their importance in regulating gene expression and cell replication. A changed degree of histone acetylation leads to changes in chromatin condensation and these alterations influence gene transcription [1]. The balance between histone transacetylases and deacetylases is often upset in cancer leading to changed expressions of tumor suppressor genes and proto-oncogenes [1, 2], making them promising targets of cancer therapy.

Histone deacetylases (HDAC) seem to be most important [2] for gene espression since levels of HDAC vary greatly in cancer cells and differ according to tumor type. HDAC1 is highly expressed in prostate, gastric, lung, esophageal, colon and breast cancers [3-5]. High levels of HDAC2 were found in colorectal, cervical and/or gastric cancers [6, 7]. In addition, HDAC3 is over-expressed in colon and breast tumors [8], whereas HDAC6 is highly expressed in mammary tumors, HDAC8 is over-expressed in neuroblastoma cells and HDAC11 mainly in rhabdomyosarcoma [9-11]. Many HDACs contain Zn ions in the active site and many the most active inhibitors target these Zn ions. The substrates for HDAC are often acetylated lysine residues of histone molecules. As a result of suppressed histone deacetylation, chromatin is condensed and this process leads to reduced accessibility of DNA for gene expression or DNA synthesis (for overview see [2]). Published works show that decreased activities of HDACs are associated with suppressed tumor cell development and growth [12, 13]. Sirtuins (class III HDAC) play important roles in carcinogenesis. Some of them act as antioncogenes while others influence tumors by controling the metabolic state of the cell [14]. After promising results with HDAC inhibitors modifying epigenetic events *in vitro*, their rather low efficiency when used to treat certain types of tumors as a single drug was disappointing but led to examination of combinations of these compounds with other antitumor drugs. The results of various studies indicate that HDAC inhibitors increase the anticancer efficacy of conventional therapy modalities and may therefore be very efficient in the clinic, in combination with ionizing radiation or chemotherapy. Here, we describe different classes of HDAC inhibitors and mechanisms of their actions, as well as discuss combination therapies with anti-tumor drugs, highlighting DNA-damaging compounds.

2. HDAC INHIBITORS

HDAC inhibitors may act both specifically against only some HDACs (HDAC isoform-selective inhibitors) and against all types of HDACs (pan-inhibitors). HDAC inhibitors can be classified as members of at least four classes of compounds: (i) hydroxamic acids (hydroxamates), (ii) aliphatic acids, (iii) benzamides and (iv) cyclic tetrapeptides (structures are shown in Fig. (**1**)) (for overview see [2]).

(i) Trichostatin A (TSA), vorinostat (suberoylanilide hydroxamic acid, SAHA) which was approved by FDA as the first HDAC inhibitor for the treatment of relapsed and refractory T-cell lymphoma (CTCL) [15], belinostat (PXD-101) and panobinostat (LBH589) are examples of hydroxamates, and are pan-HDAC inhibitors.

(ii) The aliphatic acids, valproic acid (VPA), butyric acid and phenylbutyric acid, are known to be weak HDAC inhibitors [16-18].

(iii) Among the benzamides, entinostat (SNDX-275, MS-275) inhibits most HDACs such as class I HDACs. Another benzamide derivative, mocetinostat (MGCD0103) is a HDAC isoform-selective inhibitor against only classes I and IV HDACs [19].

(iv) The cyclic tetrapeptides include the bicyclic depsipeptide romidepsin (depsipeptide, FK228, FR901228), apicidin, trapoxin and the compounds that are cyclic hydroxamic acids containing peptides. Among the tetrapeptides, romidepsin is the most effective member, and was approved by the FDA and the European

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[a]*This work is dedicated to Professor Gustav Entlicher on the occasion of his 70th birthday.*

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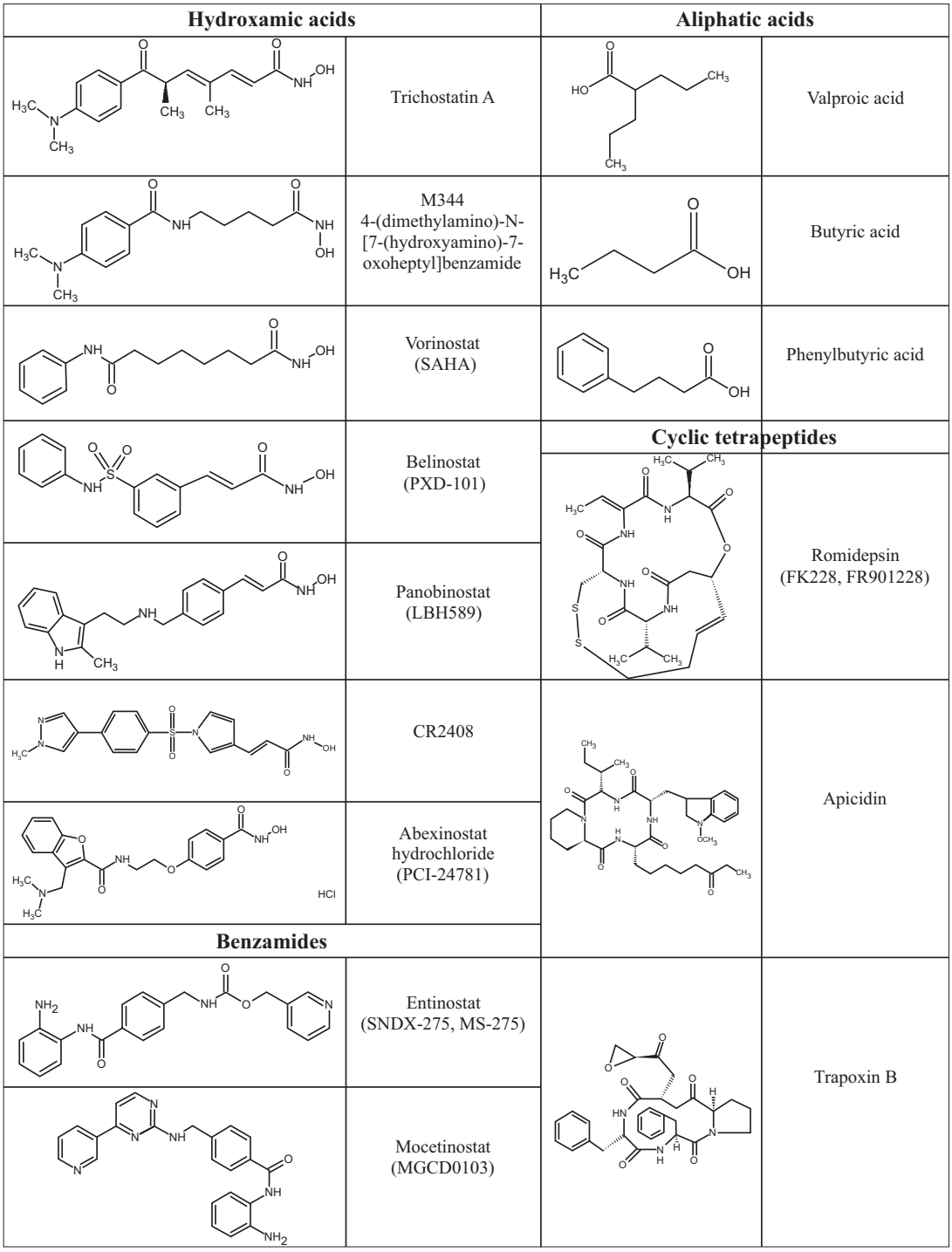


Fig. (1). Structures of major HDAC inhibitors.

Medicines Agency to treat CTCL [20, 21]. Romidepsin is a prodrug, which is reductively activated to a metabolite containing a thiol group that chelate the zinc ions in the active centre of the HDAC of class I, especially HDAC1 and HDAC2 [22].

3. MECHANISMS OF HDAC INHIBITION

Structurally very different HDAC inhibitors initiate cancer cell death by different processes, depending on the cell types, they have a variety of cellular targets. These mechanisms include changed gene expressions and alterations of histone but also or non-histone proteins by epigenetic and post-translational modifications. The shift to an enhanced acetylation status in a variety of tumors due to HDAC inhibition can result in modified expression of many genes such as those involved in cell signaling processes like the extracellular-regulated kinase (ERK) or Wnt pathways, can influence the proteasome system, activities of some protein kinases (i.e. protein kinase C) as well as DNA demethylation. Inhibition of HDACs results in changed expression of approximately 2-10 % of the genes investigated [23]. As a result, several biological

processes such as cell cycle arrest or apoptosis are induced. Indeed, many genes contributing to regulation of cell cycle (*p21, p27*^{KIP1}, and *p16*^{INK4a}) or apoptosis (*BAD, TRAIL, DR5,* and *FAS-L*) were found to be modified by HDAC inhibition [24-26]. In addition, HDAC inhibitors have antiangiogenic effects [27, 28].

3.1. HDAC Inhibitors and Cell Cycle Arrest

Several mechanisms are involved in the induction of cell cycle arrest by HDAC inhibitors; the most important one is an increased expression of cell cycle genes such as *CDKN1A* (*p21*) encoding the p21 protein [29, 30]. In a variety of tumor cells, inhibition of HDACs causes up-regulation of the *p21* gene, which blocks formation of dimers from cyclins and cyclin dependent kinases. These phenomena lead to cell cycle arrest and to inhibition of cell differentiation [30, 31]. The biosynthesis of p21, a member of the Cip/Kip family, is modulated by the tumor suppressor protein p53. Under stress conditions, the p53 protein interacts with a Sp1 site of the *p21* promoter, competing with HDAC1, which decreases transcription of *p21* [32]. When the cells are treated with HDAC inhibitors, the HDAC1 protein is released from the Sp1 site, and this process leads to an increase in *p21* expression. Furthermore, HDAC inhibition results in more acetylated p53 protein resulting in a longer half-life (hyperacetylation stabilizes the p53 protein [33]) and easier interaction with the *p21* promoter [34]. One of the HDAC inhibitors, VPA, not only enhances p53 acetylation status but also its nuclear re-localization in several cancer cells [35]. In addition, the interactions of the p53 protein with its activators like ASPPs (ankyrin-repeat-, SH3-domain- and proline-rich-regioncontaining proteins), 53BP1, TiP60/hMOF, hCAS/CSE1L, and HZF (hematopoietic zinc finger) seem to be regulated by the acetylation status [36, 37]. These interactions and the elevated acetylation of histones located close to the *p21* promoter facilitating the access of transcription factors, also enhance the HDAC inhibitor efficacies. Moreover, an increased stability of *p21* mRNA, as a results of HDAC inhibition was demonstrated [38]. Finally, the p21 levels are increased, thereby mediating cell cycle arrest and apoptosis [30, 39, 40]. HDAC inhibitors can also repress expression of genes coding cyclin D and cyclin A resulting in the absence of activities of the corresponding kinases, CDK2 and CDK4 [31, 41]. In addition, the HDAC inhibitors may increase the stability and transcriptional activities of RUNX3 (runt-related transcription factor 3), which mediates induction of p21 and Bim, again leading to cell cycle arrest and apoptosis of cancer cells [42-45].

3.2. HDAC Inhibitors and Apoptosis

HDAC inhibitors are capable of inducing apoptosis in tumor cells by regulating the expression of genes dictating apoptosis (proapoptotic and antiapoptotic genes) (for a review, see [2, 26, 46]). Mechanisms by which different HDAC inhibitors induce apoptosis include activation of the death-receptor (extrinsic) pathway or the mitochondrial (intrinsic) apoptotic pathway.

The participation of HDAC inhibitors in initiation of the extrinsic apoptotic pathway was proven in many *in vitro* investigations. HDAC inhibitors have been demonstrated to influence death receptors such as TRAIL, DR5, Fas and TNF or TNF-related ligands like Fas-L, LIGHT and TLA1 [26, 47]. Such findings have been confirmed by inhibiting death receptors and their ligands resulting in loss of apoptosis induction mediated by HDAC inhibitors [48, 49]. Furthermore, experiments in which TRAIL and Fas were suppressed with small interfering RNA (siRNA) in a mouse model showed a significant decrease in apoptosis when mice were treated with VPA [50].

HDAC inhibitor-induced apoptosis has also been demonstrated to be associated with the intrinsic pathway. Indeed, these inhibitors regulate on the transcriptional level the expression of proapoptotic BH3-only proteins such as Bid, Bad and Bim that function as activators of the intrinsic apoptotic pathway [29, 46, 51, 52].

In a summary, it can be concluded from studies investigating the profiles of gene-expression in tumor cells exposed to inhibitors of HDACs that proapoptotic genes involved in the extrinsic (e.g. *TRAIL*, *DR5*, *FAS*, *FAS-L*, and *TNF-*α) and/or intrinsic apoptotic pathways [*BAX* (gene of Bcl-2–associated X protein), *BAK* (gene of Bcl-2-associated death promoter**)** and *APAF1* (gene of apoptotic protease activating factor 1)] are up-regulated, while down-regulation of antiapoptotic genes [i.e. *BCL-2* and *XIAP* (gene of X-linked inhibitor of apoptosis protein)] occurs [7]. HDAC inhibitors, *via* activation of ERK, can however enhance the levels of some antiapoptotic proteins such as Bcl-2 [53]. This does not seem to influence the activities of proapoptotic factors like Bid, since their expression seem to be sufficient to initiate apoptosis even though Bcl-2 is expressed at high levels [7].

Besides these effects on gene expression, increased amounts of reactive oxygen species (ROS) that can induce apoptosis were found in cancer cells when they were treated with HDAC inhibitors [51, 54, 55]. Higher levels of ROS can precede changes in mitochondrial membrane potential [55], indicating an association of HDAC inhibitors, ROS, BH3-only proteins with activation of the intrinsic apoptosis pathway. At least two mechanisms that might be responsible for the inductions of oxidative stress by HDAC inhibitors are suggested; either damage to mitochondria, or modulation of the amounts of cellular antioxidants.

Two HDAC inhibitors, vorinostat and entinostat, have been found to regulate thioredoxin binding protein-2 (TBP-2) that inhibits thioredoxin [56]. Thioredoxin is a compound with a wide spectrum of functions, where its predominant role is to act as an intracellular antioxidant. Indeed exposure of tumor cells to these HDAC inhibitors resulted in ROS-dependent apoptosis [57, 58]. On the contrary, nonmalignant cells are not affected by HDAC inhibitors, and no formation of ROS was found, but instead increased thioredoxin levels. The protection of normal cells was due to a HDAC inhibitor-induced thioredoxin expression because siRNA against the antioxidant increased oxidative stress and sensitivity towards HDAC inhibitors [59].

3.3. HDAC Inhibitors and Cell Signaling Pathways

An important feature of HDAC inhibitors, which could find its place in cancer therapy, is regulation of cell differentiation by activation of some of protein kinases (i.e. ERK), because protein kinases are essential modulators of biological processes like cell growth, differentiation and apoptosis. At least three different mitogen-activated protein kinases (MAPK) such as ERK, c-Jun N-terminal kinase (JNK) or p38 were identified. HDAC inhibitors were found to increase DNA binding and transactivation activity of AP-1 transcription factor by ERK activation. HDAC inhibitors increased expression of c-Jun and its phosphorylation in several cancer cells [53, 60]. Although it is not yet clear how HDAC inhibitors act, the current accepted hypothesis for the effect on ERK is not caused by inhibition of HDACs [61]. One of the suggestions is that HDAC inhibitors can mediate synthesis of a still unknown factor that might activate the ERK signaling pathway [53] and/or is incorporated into larger phospholipid molecules that activates ERK *via* the phosphatidylinositol 3-kinase (PI-3K)/Janus kinase 2 (JAK 2)/MEK-1-dependent- and the tyrosine kinase-Ras-dependent-pathways [62, 63]. Inhibitors of HDAC have also been demonstrated to increase the expression of genes that are involved in regulation of ERK/AP-1 signaling, for example growth-associated protein-43 (GAP-43) and Bcl-2, and hence they might increase cancer cell growth (e.g. in SH-SY5Y neuroblastoma cells) [53, 64].

VPA also affects Wnt signaling that is due to phosphorylation of serine 9 in the glycogen synthase kinase-3β (GSK-3β) [28]. This

process resulted in prevention of subsequent adenomatosis polyposis coli (APC)-dependent degradation of β-catenin [65, 66]. The Wnt signaling pathway with β-catenin as the predominant effector is important in various pathologies including colon, breast, ovarian, prostate and endometrial cancers as well as medulloblastoma, melanoma and skeletal pathologies in humans [67]. Inactivation of APC functions as well as activation of β-catenin results in overexpression of the HDAC2 enzyme, which suggest this enzyme to be a target of APC/β-catenin [7]. Moreover, increased expression of the HDAC2 enzyme was found to stop death of colon carcinoma cells. Despite the fact that HDAC inhibitors cause an increase in β-catenin, these inhibitors also decrease polyp generation in a colon carcinoma model of APC-deficient mice, probably in association with the enhanced damage of the HDAC2 enzyme caused by its degradation in proteasomes [7]. Furthermore, VPA was found to increase proliferation and self renewal of normal hematopoietic stem cells by inhibition of GSK-3β that results in activation of the Wnt pathway [68].

HDAC inhibitors also induce the expression of a variety of enzymes involved in the proteasomal degradation pathway. Acetylation of p53 decreases generation of a complex formed from p53 and Mdm2 E3 ligase, making the p53-mediated stress response possible regardless of its phosphorylation status, whereas hypoacetylation of p53 increases its degradation by the proteasome system and cancels p53-mediated growth arrest and apoptosis [37]. In addition, the expression of several enzymes involved in the proteasomal degradation pathway seems to be influenced by acetylation. For example, the Ubc8 E2 ubiquitin conjugase enzyme, the levels of which are a limiting factor dictating proteasomal degradation, as well as expression of RLIM, a subunit of the SCF E3 family of the ubiquitin ligase enzymes, are induced by HDAC inhibitors, which suggests the mechanism of HDAC2 degradation [69].

3.4. HDAC Inhibitors and Angiogenesis

HDAC inhibitors can affect development and survival of cancer cells by decreasing cancer angiogenesis as well as by inhibiting cellular stress response pathways and thereby interfere with the metastatic process. The antiangiogenic effects seem to be associated with down-regulation of proangiogenic genes such as vascular endothelial growth factor (VEGF) or endothelial nitric oxide synthase (eNOS) [12, 13, 70]. Changes in levels of eNOS and its enzymatic activity are mediated by phosphorylation of this enzyme catalyzed by protein kinase Akt and numerous hemodynamic and hormonal stimuli [71-73]. HDAC inhibitors decrease the stability of eNOS mRNA, which is associated with the enhanced transcription of a protein causing destabilization of this mRNA by its binding to the 5′- untranslated region of eNOS mRNA [74]. In addition to VEGF, HDAC inhibitors decrease the levels of the VEGF receptors [75]. They impair the handling of proteins that are not properly folded, by affecting endoplasmic reticulum stress responses [55, 76]. Changes in acetylation of the chaperone Hsp90 (heat shock protein 90) produce the changes in its functions. Indeed, when this protein is hypoacetylated, it protects client proteins such as Bcr-Abl, epidermal growth factor receptor and ErbB2 from degradation [77]. Hyperacetylation of Hsp90 in response to HDAC inhibition reduces the chaperone association with its client proteins resulting in polyubiquitination and proteasomal degradation of a number of cancer-related client proteins, which belong to Hsp90 substrates [77].

In addition, after treating cells with HDAC inhibitors, HIF-1α (hypoxia inducible factor), a proangiogenic transcription factor, is hyperacetylated, and this process results in its degradation [78]. HDAC inhibitors are supposed to negatively influence the stability of this factor because a decrease in activity of HDAC6 due to trichostatin A produces Hsp90 acetylation, accumulation of a complex between heat shock protein 70 and HIF-1α, and proteasomal degradation of HIF-1α [79]. VPA decreases angiogenesis and vasculogenesis by enhancing expression of the antiangiogenic proteins thrombospondin-1 and activin A, by down-regulation of proangiogenic factors such as basic fibroblast growth factor (bFGF) [80]. We found that VPA and trichostatin A decrease formation of capillary tubes of HUVEC (human vascular endothelial cells) in an *in-vitro* model, but they do not induce apoptosis of HUVEC (unpublished results), therefore antiangiogenic therapies combined with inhibitors of HDACs might increase their efficiency against tumors.

4. HDAC INHIBITORS AND OTHER THERAPEUTIC REGIMENS

Even though inhibitors of HDACs were found to exhibit promising antitumor effects when used alone, their rather lower efficiencies have stimulated numerous researchers to investigate combining these compounds with additional antitumor drugs.

The results found in experiments utilizing various tumor cells have demonstrated that combination of HDAC inhibitors with a variety of anticancer therapies produced synergistic or at least additive effects [81]. Such combinations have also been investigated in clinical trials (for a review, see [82]).

Several types of therapies have been investigated for combined treatment with HDAC inhibitors.

i) HDAC inhibitors were combined with other epigenetic modifiers. Inhibitors of DNA methyl transferases [i.e. 5-azacytidine (azacitidine) and 5-aza-2'-deoxycytidine (decitabine)] had increased antitumor effects when used with HDAC inhibitors (VPA, trichostatin A, phenylbutyrate, depsipeptide, vorinostat) [36, 42, 45, 83-85]. Decitabine and VPA both induce apoptosis, enhance expression of inhibitors of cyclin dependent kinases $p57^{KIP2}$ and p21 in several cancer cells at concentrations ranging between 1 – 1000 μM. Combining these two drugs resulted in an increase in their potency to inhibit cell growth, to enhance apoptosis and reactivate $p57^{KIP2}$ and p21 [86]. These additive effects of decitabine and VPA were found also *in vivo* in a heterozygous *PTCH* knockout mouse model. These mice develop medulloblastoma and rhabdomyosarcoma that are similar to those in humans. When mice were treated with VPA and decitabine, tumor development decreased, probably because of reduced methylation of the *PTCH* promoter as well as by acetylation of histones [87] leading to reactivated *PTCH* expression.

ii) Co-treatment of prostate [45] and pancreatic tumor cell lines [42] with trichostatin A in combination with decitabine resulted in reduction of cell proliferation that was associated with the enhanced apoptosis of these cancer cells. Analogous findings were also made in other cancer cells; increased apoptosis in an AML1/ETO-positive acute myelogenous leukemia (AML) cell line [88], and decreased cell proliferation in non small cell lung cancer (NSCLC) cells [89] exposed to depsipeptide and 5-aza-2'-deoxycytidine. In addition, an inhibitor of histone demethylases (tranylcypromine) and the HDAC inhibitor vorinostat showed a synergistic enhancement of apoptosis in glioblastoma cells [46].

iii) Promising preliminary results have also been reported for combining HDAC inhibitors with ROS-generating agents. One such agent, adaphostin, can increase anticancer efficiencies of HDAC inhibitors (i.e. entinostat and vorinostat), by enhancing DNA fragmentation and inducing the apoptotic processes in leukemia cells [46]. In addition, β-phenylethyl isothiocyanate that causes depletion of GSH [90], increases sensitivity of

acute myeloid leukemia cells to vorinostat [91]. This natural product was found to induce cell cycle arrest as well as to initiate apoptosis of tumor cells [92]. In addition to GSH depletion, β-phenylethyl isothiocyanate inhibits phase I drug-metabolizing enzymes (i.e. cytochromes P450), can induce NAD(P)H:quinone oxidoreductase, microsomal epoxide hydrolase, the phase II metabolizing enzymes, UDP-glucuronosyl transferase and glutathione transferase [92, 93]. The combination of HDAC inhibitors with β-phenylethyl isothiocyanate led to ROS accumulation and initiation of apoptotosis in acute promyelocytic leukemia cells that exhibit resistance to HDAC inhibitors, by stimulation of NADPH oxidase. This increase in ROS results in a transfer of the transcription factor Nrf2 to the cell nucleus, which enhances transcription of genes involved in the glutathione system [91].

iv) Other drugs that have been combined with HDAC inhibitors are drugs inhibiting tumor growth by microtubule stabilization (i.e. paclitaxel) [94, 95]. The HDAC inhibitor VPA increases toxic effects of paclitaxel in CAL-62 and ARO anaplastic thyroid carcinoma cells due to interaction with the tubulin β subunit; it enhances tubulin hyperacetylation that results in stabilization of microtubule structures. Such a microtubule stabilization adversely influences cellular proliferation, since it prevents microtubules plasticity, and this process finally leads to apoptosis [94]. Similarly enhanced apoptosis was observed in Ark2 and KLE endometrial tumor cell lines treated with trichostatin A and oxamflatin together with paclitaxel. Apoptotic effects of trichostatin A when used in combination with paclitaxel result from activation of the intrinsic mitochondria-dependent pathway. Trichostatin A also stabilizes microtubules *via* α-tubulin acetylation *in vitro* and also *in vivo*, in mouse xenografts [95]. In addition, trichostatin A enhances anticancer effects of paclitaxel in human gastric tumor cells, OCUM-8 and MKN-74 [96].

v) Another combination that might increase effects of HDAC inhibitors in cancer treatment is that with proteasome inhibitors. These drugs target enzymes associated with proteasomal proteolytic activities. An increase in cancer cell death caused by combination of proteasome and HDAC inhibitors is caused by induction of oxidative stress, ER (endoplasmic reticulum) stress and stimulations of JNK (Jun NH2-terminal kinase). Mechanistic studies of HDAC inhibitors combined with novel, more efficient proteasome inhibitors are identifying new potential mechanisms. Bortezomib, marizomib (NPI-0052) and carfilzomib are proteasome inhibitors which have been combined with HDAC inhibitors. Treatment of multiple myeloma cells with bortezomib made the cells more sensitive to vorinostat and sodium butyrate, increasing apoptosis [97]. In addition, early-phase clinical trials of vorinostat in patients suffering from multiple myeloma demonstrated an increase in its antitumor effects in combinations with bortezomib [98-100]. Mitochondrial damage, stimulations of JNK and caspases or enhanced oxidative stress were increased in cells exposed to proteasome and HDAC inhibitors used in combination. Furthermore, treating cells with antioxidants prevented the stimulation of JNK and the apoptotic processes, which suggests contribution of ROS to such effects [97]. Likewise, induction of apoptosis mediated by reactive oxygen species has been found when a Bcr/Abl$^+$ leukemia cell line was exposed to proteasome inhibitors combined with inhibitors of HDACs. Furthermore, cell growth was inhibited when a K562 (Bcr/Abl$^+$CML) cell line resistant to imatinib and also a patient-derived CD134$^+$ cell line, which was refractory to imatinib therapy, were treated with bortezomib combined with HDAC inhibitors [101]. Another mechanism that is supposed to be important in the synergistic effects between HDAC inhibitors and proteasome inhibitors is disruption of aggresome formation. In addition, both oxidative stress and interfering with aggresome formation leading to induction of ER stress are important pathways described that are considered to contribute to the synergy observed between bortezomib and HDAC inhibitors [102].

Miller *et al.* [103] have demonstrated that the proteasome inhibitor, marizomib in combination with HDAC inhibitors vorinostat or entinostat increased apoptosis in several leukemia cell lines. These authors then showed that caspase 8 and oxidative stress contributed to the synergistic effects [46]. Marizomib also influenced epigenetic processes, because acetylation of histone H3 was found to be increased by this drug. This phenomenon might explain the higher synergistic effects of marizomib combined with HDAC inhibitors than of bortezomib and HDAC inhibitors. Studies using diffuse large B cell lymphoma (DLBCL) cells showed that another proteasome inhibitor, carfilzomib, increased the effects of an HDAC inhibitor vorinostat [104, 105]. DNA-damage and apoptosis were enhanced *in vitro* and tumor growth was decreased in mice. This combined treatment was also active in DLBCL cells resistant to bortezomib [104, 105].

vi) An important approach for cancer therapy is the combination of HDAC inhibitors with DNA damaging agents. Because of the importance of these combinations, the molecular mechanisms of DNA-damaging drugs or ionizing radiation (radiotherapy) combined with HDAC inhibitors are discussed in more detail below.

4.1. HDAC Inhibitors and DNA-Damaging Agents or Ionizing Radiation

Numerous investigations showed synergisms or at least additive effects combining HDAC inhibitors and DNA-damaging agents such as inhibitors of topoisomerases, DNA-intercalators, inhibitors of DNA synthesis and agents covalently modifying DNA (i.e. doxorubicin, epirubicin, etoposid, cisplatin, 5-fluorouracil, mephalan, temozolomide and ellipticine, Fig. (**2**)) and ionizing radiation in many cancer cell lines [36, 81, 106-127]. The use of HDAC inhibitors permits utilizing of lower amounts of therapeutics and would probably lead to less negative side effects due to the DNA-damaging drugs. The molecular mechanisms that can explain how HDAC inhibitors enhance the effects of antitumor agents and radiation against tumors have not been fully understood as yet and this fact blocks a development of more efficient therapeutic regimens. Generally, the HDAC inhibitor-mediated sensitization of cells to DNA-damaging drugs is explained by the impact of HDAC inhibitors on chromatin structure, because HDAC inhibitors facilitate decondensation of chromatin by increased acetylation of several histones and this relaxed chromatin is easily attacked by drugs which damage DNA Fig. (**3**). A pioneering study of Kim *et al.* [119] strongly supports this hypothesis. They found that exposing several human cancer cells to the hydroxamate HDAC inhibitors trichostatin A or vorinostat prior to etoposide, ellipticine, doxorubicin and cisplatin increased the cytotoxicity of these drugs. The levels and activities of a target enzyme, topoisomerase II, were not influenced after such an exposure, however expression of p53, p21, and Gadd45 proteins was significantly increased [119].

An additional mechanism for HDAC inhibitor synergy with DNA damaging agents and radiation has been suggested, implicating abrogation of DNA double-strand break (DSB) repair. This hypothesis was confirmed by the finding of higher expression of γ-H2AX that is a marker of DNA double strand breaks and activation of the checkpoint protein Chk2, when HDAC inhibitors were combined with DNA-targeted drugs and ionizing radiation [111, 128].

In addition to impairing DNA repair, HDAC inhibitors can sensitize cells to DNA damaging agents by interfering with genes important for survival [36, 129]. HDAC inhibitors increase levels of some proapoptotic proteins, for example Bim and Bmf [129].

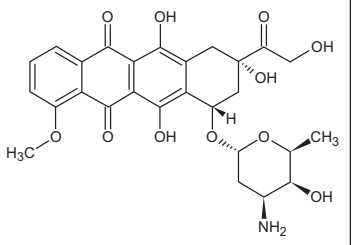
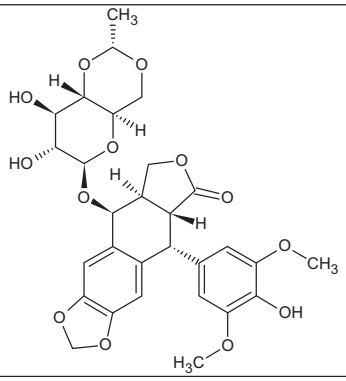
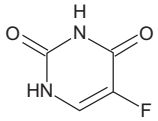
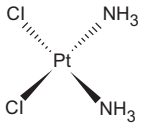
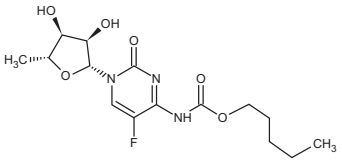
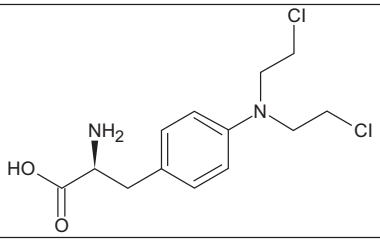
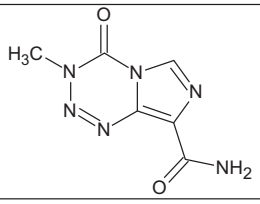
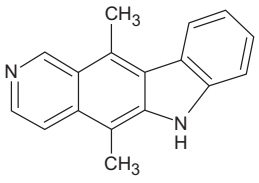
	Doxorubicin		Etoposide
	5-Fluorouracil		Cisplatin
	Capecitabine		Melphalan
	Temozolomide		Ellipticine

Fig. (2). Structures of DNA damaging drugs tested in combination with HDAC inhibitors.

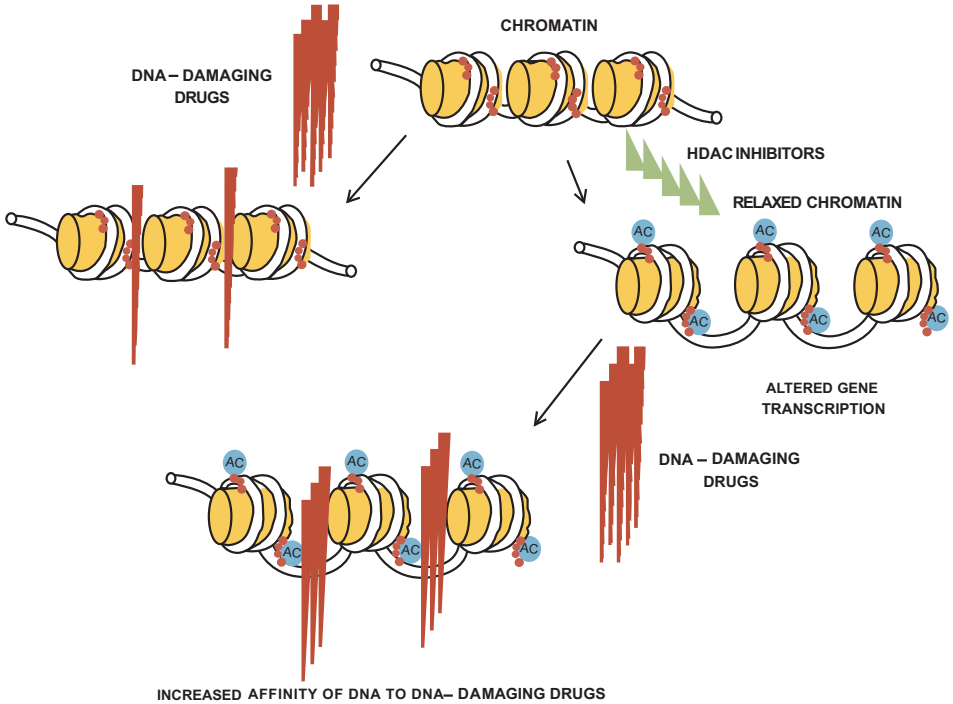


Fig. (3). A proposed mechanism of the HDAC inhibitor action causing an increase in DNA damage mediated by DNA-damaging drugs.

4.1.1. DNA Intercalators and Inhibitors of Topoisomerase II

4.1.1.1. Anthracyclines

Doxorubicin Fig. (**2**), one of the chemotherapeutic anthracyclines, is an effective antineoplastic drug used to treat cancer for more than five decades [130]. It is a DNA intercalator that inhibits topoisomerase II resulting in DSBs in DNA followed by induction of apoptosis [131]. It was also shown that doxorubicin can covalently bind to DNA, and DNA adducts formed in this process induce higher toxic effects in cells than the inhibition of topoisomerase II [130, 132-138].

Several HDAC inhibitors (i.e. trichostatin A, VPA, entinostat, panobinostat, sodium butyrate, vorinostat, abexinostat) were shown to potentiate the effects of doxorubicin in many cancer cell lines *in vitro*, in studies *in vivo*, and also in clinical trials. The HDAC inhibitor VPA significantly increased doxorubicin-mediated apoptosis in anaplastic thyroid cancer cells CAL-62 and ARO. VPA also potentiated the effect of doxorubicin on induction of apoptosis, in malignant mesothelioma cell lines [125]. The sensitizing effect of VPA, caused by acetylation of histones, led to enhanced apoptosis of anaplastic thyroid cancer cells by doxorubicin. This increased apoptosis was also demonstrated by enhanced caspase 3 activity and elevations in doxorubicin-mediated G2 cell cycle arrest [107]. The authors of this study reported that acetylation of histones due to a changed chromatin structure potentiates accessibility of DNA to binding of doxorubicin Fig. (**3**), which finally leads to better effects of this drug [107]. Similar findings were reported by other authors [139], which investigated the effects of one derivative of butyric acid (AN 9). They also found an increase in toxicity of doxorubicin caused by this HDAC inhibitor, whereas cellular doxorubicin uptake was not affected. The combined exposure of canine and human osteosarcoma cells to VPA and doxorubicin produced a decrease in cell growth and potentiated apoptosis due to enhancement of doxorubicin accumulation in DNA of these cells. The same combined treatment was also highly efficient in the therapy in canine osteosarcoma xenografts [140].

Trichostatin A, vorinostat (SAHA) and entinostat (MS-275) sensitized DU-145, LNCaP and PC-3 prostate cancer cell lines to doxorubicin due to acetylation of Ku70. This protein is an important constituent of the nonhomologous end joining (NHEJ) repair system of DNA double strand breaks. Exposure of these cell lines to the HDAC inhibitors resulted in enhanced Ku70 acetylation accompanied by reduced DNA-binding affinity without disrupting the Ku70/Ku80 heterodimer formation. The enhanced levels of histone H2AX (γ-H2AX) phosphorylated at serine 139 indicated that impaired Ku70 function decreased the ability of cells to repair DNA double strand breaks caused by doxorubicin, thus enhancing the cytoxicity of this drug. The best effect was achieved when cells were pretreated with the HDAC inhibitor before the cytostatic drug [110]. Trichostatin A also promoted doxorubicin-induced apoptosis in the 293T human embryonic kidney epithelial and A549 type II alveolar adenocarcinoma cells.

Here, the sensitizing effect was by induction of Egr-1 expression, acetylation of core histones at the promoter of the tumor suppressor gene phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*), and consequently induction of *PTEN* transcription [141].

The sensitizing influence of panobinostat (LBH589) on doxorubicin action in acute myeloid leukemia (AML) cells was mediated by an increase in apoptosis. This combined treatment produced cellular injury by changing the permeability of the outer mitochondrial membrane, which results in release of cytochrome c. These features resulted in caspase-dependent apoptosis and were also accompanied by the up-regulation of *BAX, BAK* and particularly, *BAD*. This combined treatment also stimulated a DNA damage response, which implies the initiated cellular death that is caused by an increase in DSBs in DNA [142]. Panobinostat also potentiated the anticancer effects of doxorubicin in multiple myeloma cells and a human multiple myeloma LAGλ-1cell xenograft model in mice. Treatment of multiple myeloma cell lines with panobinostat and doxorubicin led to an increase in acetylation of histones, caspase cleavage, a decrease in cell division as well as to enhanced anti-multiple myeloma effects both *in vitro* and *in vivo* (multiple myeloma xenograft) [124]. An increase in doxorubicin-induced apoptosis by entinostat was also found in glioblastoma cells. Pretreatment of glioblastoma cells with this HDAC inhibitor (entinostat) caused modulation of histone H3 functions by its acetylation and finally enhanced doxorubicin-mediated apoptosis. Moreover, pretreatment of cells with entinostat (MS-275) enhanced apoptosis caused by temozolomide, etoposide, and cisplatin [143]. Another HDAC inhibitor, sodium butyrate, in combination with doxorubicin also potentiated apoptosis. This HDAC inhibitor inhibited myeloma cell growth independent of p53 and caspase 3 and 7 stimulation [112], because apoptosis corresponded to translocation of apoptosis-inducing factor (AIF), which suggests potentiation of caspase 3 and 7 independent pathways. This result corresponded to a finding that combining sodium butyrate with doxorubicin enhanced the cytoplasmic cathepsin B activities, because a decrease in these activities reversed apoptosis caused by sodium butyrate and doxorubicin [112].

Combined exposure to vorinostat with doxorubicin also increased apoptosis in fibrosarcoma *in vitro* and, most importantly, decreased growth of xenografts in nude mice *in vivo* more effectively than when these drugs were used as single agents [144]. A study using an HDAC inhibitor abexinostat hydrochloride (PCI-24781, Fig. (**1**)) to sensitize bone sarcoma cells to doxorubicin demonstrated that this process was also caused by doxorubicin-induced apoptosis. In this case, activities of caspases 3 and 7 were enhanced after exposure of the cells to doxorubicin with PCI-24781 [145]. Vorinostat also potentiated the activity of doxorubicin in retinoblastoma cells [123]. The mechanism of its effect in these cells has, however, not been investigated in detail.

Häcker and her coworkers described multiple mechanisms which are responsible for the synergy of HDAC inhibitors and doxorubicin in cancer cells [108]. These authors reported that entinostat (MS-275), VPA or vorinostat sensitize medulloblastoma to doxorubicin-mediated DNA damage due to increasing p53-dependent, mitochondrial apoptosis. Furthermore, studies investigating the mechanisms of this process revealed that exposure to entinostat alone mediates acetylation of the Ku70 protein resulting in release of Bax from this non-histone protein, while doxorubicin triggers an increase in levels of acetylated p53 in the cells. Exposure to entinostat with doxorubicin led to increased formation of a binary complex of p53 with Bax and p53-dependent Bax activation, which resulted in elevated loss of mitochondrial membrane potential, cytochrome c release and increased apoptosis mediated by caspases. It should be, however, noted that enhanced expression of Bcl-2 abolished sensitization of cells to doxorubicin caused by entinostat. This underlines the significance of the mitochondrial-mediated pathway in initiation of the apoptotic processes. In addition, entinostat cooperated with doxorubicin to decrease long-term clonogenic survival. Moreover, entinostat increased the doxorubicin-mediated apoptotic processes in primary medulloblastoma cells and cooperated with doxorubicin to decrease their growth *in vivo* [108].

The synergistic effects of a novel pan-HDAC inhibitor CR2408 Fig. (**1**) in multiple myeloma cells treated with doxorubicin might also be caused by the above mechanisms [146]. This inhibitor induced hyperacetylation of histone H4, reduced growth of multiple myeloma (MM) cells and strongly increased apoptosis in these cells and primary MM cell lines. It led to fragmentation of cells associated with a moderate decrease in amounts of cyclin D1 and Cdk4 and strongly decreased levels of Cdc25a, retinoblastoma protein (pRb) and p53. Treatment with CR2408 also led to higher

expression of Bim and pJNK proteins and down-regulation of Bad and Bcl-xL as well as to activation of caspases 3, 8 and 9 [146].

A study utilizing a combination of VPA and epirubicin, another anthracycline, indicated that pre-treatment with VPA followed by epirubicin decreases growth of human breast cancer xenografts in a mouse model, whereas the reverse order of administration exhibited a lower effect [36]. VPA also increased epirubicin-mediated apoptosis *in vitro,* in a MCF-7 breast adenocarcinoma cell line, which was due to an enhanced interaction of epirubicin with DNA. This synergistic effect might be due to depletion of topoisomerase IIα, therefore only tumor cells that express topoisomerase IIα, would be sensitive to this potentiation [147]. Enhanced apoptosis due to epirubicin in a MCF-7 breast cancer cell line was caused also by vorinostat. This apoptosis was again accompanied by a down-regulation of topoisomerase IIα protein expression, but not of topoisomerase IIβ [148].

4.1.1.2. Etoposide

Etoposide (Vepesid, VP-16, Fig. (**2**)) belongs to the anti-cancer drugs that inhibit topoisomerase II and DNA synthesis that is mediated by formation of a binary complex of this enzyme with DNA. The resulting DSB in proliferating DNA are not repaired by topoisomerase II in cells exposed to etoposide. This disruption of DNA finally leads to cell cycle arrest and cell death. Tumor cells rely on topoisomerase II more than healthy cells, because cancer cells proliferate more intensively. Etoposide is usually combined with other drugs also with HDAC inhibitors..

One of the first studies investigating the combination of HDAC inhibitors with etoposide was carried out by Kurz and coworkers [149] who showed that the HDAC inhibitor sodium butyrate increased sensitivity of HL-60 myeloid leukemia or K562 erythroleukemia cells to etoposide-induced DNA damage and cell death. This effect was associated with increased apoptosis induced by etoposide in the HL-60 cells, and growth arrest of K562 cells and poly(ADP-ribose)polymerase (PARP) cleavage [149].

The study of Valentini *et al.* [150] showed that the VPA-mediated sensitization of M14 human melanoma cells to etoposide treatment was caused by initiation of cell cycle arrest and apoptosis. This HDAC inhibitor induced the apoptotic processes in M14 cells both alone and when combined with etoposide, inducing G1 arrest and up-regulation of p16, p21 and cyclin-D1 related to retinoblastoma protein phosphorylation [150]. Similar mechanisms of VPA to sensitizisation to etoposide were found in U87, U251, and LN18 glioblastoma cells.

Induction of differentiation and up-regulating the expression of p21 and both isoforms (α- and β- isoforms) of topoisomerase-II were detected in these cells after exposure to VPA and etoposide [24]. Exposure of cells to both drugs increased the fraction of cells with a G2/M and sub-G1 (apoptotic) DNA content. In addition, an increased activity of caspase 3 and apoptosis were produced by combining VPA with etoposide compared with exposure of cells to either drug as a single agent [24]. The influence of VPA on the epigenetic regulation of gene expression causing an increase in toxic effects of etoposide was studied by the same research group also in neuroblastoma cells [151]. Growth of human neuroblastoma SK-N-AS and SK-N-SH cells was more impaired when VPA and etoposide were combined than if cells were treated with each of these drugs alone. The SK-N-AS cell line was more sensitive to such treatment than the cell line SK-N-SH. An enhanced acetylation of histone H3 and expression of p21 were found in both neuroblastoma lines, but up-regulation of expression of the topoisomerase IIβ gene and increased PARP cleavage were found only in the more sensitive SK-N-AS line. The results found in this study indicate that epigenetic mechanisms of modulation of the topoisomerase IIβ gene expression are probably responsible for these effects [151].

Trichostatin A, with etoposide also increased the apoptotic processes in drug-resistant non-small cell lung carcinoma (NSCLC) cells [152]. This co-treatment increased apoptosis in a caspase-dependent pathway with significant repression of Bcl-xL content that allows *BAX* activation and the following initiation of the apoptosis inducing factor (AIF)-dependent death pathway. AIF was found to be essential for the effects of trichostatin A plus etoposide because RNA knockdown of AIF abolished trichostatin A/etoposide-mediated apoptosis. Apoptosis induced by both caspase-dependent and caspase-independent pathways is required to kill drug-resistant NSCLC cells by trichostatin A/etoposide. They also imply that combining HDAC inhibitors with DNA damaging agents might be crucial for the treatment of NSCLC as well as for other cancers where Bcl-xL is highly expressed [152].

The pretreatment of hepatocellular carcinoma (HCC) cells with trichostatin A prior to etoposide also increased apoptosis in these cells. Here, apoptosis was increased by inhibition of ERK phosphorylation, reactivation of the caspase and PARP enzymes, and induction of translocation of p53 and Bid to cytoplasm [153].

Another HDAC inhibitor, panobinostat (LBH589), alone and combined with etoposide sensitized various lung cancer and mesothelioma cells. Small cell lung cancer (SCLC) cells were found to be most sensitive. In lung cancer and mesothelioma animal models, panobinostat reduced growth of these tumors, by about 62%. This HDAC inhibitor exhibited the same effect in immunocompetent and in severe combined immunodeficiency mice, which indicates that reduced tumor growth due to this inhibitor was not caused by direct immunologic effects. Panobinostat has been also found to be highly efficient in SCLC xenografts, and its combination with etoposide increased anticancer activity. Protein expression patterns of tumor biopsies were analyzed and indicated that increased levels of modulators of cell cycle such as p21 and factors promoting apoptosis (caspases 3 and 7, and cleaved PARP) were associated with lower amounts of antiapoptotic factors Bcl-2 and Bcl-xL in mice treated with the combination [154].

An increase in apoptosis by HDAC inhibitors was also found to improve the efficacy of the usual first-line chemotherapy of SCLC, etoposide combined with cisplatin. VPA in SCLC cells induced apoptosis that proceeded through mitochondrial and death receptor pathways. This increase in apoptosis included higher expression of p21, inhibition of Bcl-xL, destroying of Bid as well as phosphorylation of ERK and γ-H2AX. Furthermore, due to VPA, Bax was moved from the cytoplasm into the mitochondria and changed to its 18 kDa form, while cytochrome c was released from the mitochondria to the cytosol. In these cells, VPA modulated transcription of genes (Na^+/K^+ ATPase, Bcl-xL) involved in chemoresistance of cells to etoposide and cisplatin. The combination therapy of VPA plus etoposide with cisplatin was very effective in the treatment of SCID mice with SCLC cells engrafted [155]. The potency of VPA and also trichostatin A to sensitize various multi-drug resistant tumor cells to etoposide was found to be associated with an enhanced acetylation of several lysines in histones H3 and H4 such as lysine 16 in histone H4 [156]. Efficacy of etoposide combined with cisplatin in SCLC cells, was also enhanced with two other HDAC inhibitors, belinostat and romidepsin [157]. Treatment with the HDAC inhibitors led to normalization of cell cycle processes and decreased PARP destruction. The higher amounts of phosphorylated γ-H2AX corroborated that co-treatment regimens enhanced DNA double-strand breaks. Changes in a DNA structure such as its relaxation are not exclusively necessary to synergize the effects of studied HDAC inhibitors with DNA damaging agents in SCLC cells [157].

Similar to sensitization of DU-145, LNCaP and PC-3 prostate cancer cell lines to doxorubicin by trichostatin A, vorinostat and entinostat (see chapter 4.1.1.1.), these HDAC inhibitors sensitized these cells also to etoposide, by targeting Ku70 acetylation. The

mechanism of the synergistic effects, which is the same also for doxorubicin, is described in Chapter 4.1.1.1. Briefly, as proven by an enhanced phosphorylation of serine 139 in histone γ-H2AX, disturbed functions of Ku70, decreased the ability of cells to repair DNA double strand breaks caused by DNA-damaging drugs, which finally increased anticancer effects of these drugs [110].

4.1.2. 5-Fluorouracil (5-FU)

5-Fluorouracil (5-FU, Fig. (**2**)) is a pyrimidine antagonist that inhibits DNA synthesis and blocks the production of RNA. It is utilized to treat many cancers (esophageal, gastric, pancreatic, colorectal, anal, hepatocellular, and breast, head and neck, and ovarian carcinomas). It is a suicide inhibitor and works by irreversible inhibition of thymidylate synthase (TS). Several studies demonstrated that the antitumor effects of this drug might be increased by HDAC inhibitors by at least two predominant mechanisms: (i) a synergistic increase in apoptosis and (ii) reduced expression of the 5-FU celullar target, thymidylate synthase (TS).

Mitochondrial apoptosis induction has been proposed to be the reason for vorinostat-mediated sensitization of hepatocellular carcinoma (HCC) cells (HepG2, Hep1B and MH-7777A cells) to an otherwise ineffective 5-FU dose combined with irinotecan. Adding of only 1 μM vorinostat enhanced apoptosis in HCC cells, up to 92%. An increase in apoptosis correlated with loss of the membrane potential of mitochondria, a decrease in Bcl-2 expression and activation of caspase 3 [158].

Entinostat (MS-275) sensitized human colorectal cancer cells to 5-FU affecting cell cycle progression and initiating apoptosis [159].

HDAC inhibitors have also been shown to influence additional molecular pathways involved in colon cancer carcinogenesis and growth. These include down-regulation of cyclin B1 in a p21 and transcriptional dependent manner, suppression of Cox-activation and repression of Src family kinase members [160].

The proof of a direct association of thymidylate synthase (TS) down-regulation with sensitization to 5-FU by HDAC inhibitors was demonstrated by Fazzone *et al.* [161]. They found that the HDAC inhibitors vorinostat and panobinostat Fig. (**2**) decrease expression of TS in several colon tumor cells and that these effects were not dependent on amounts of p53, p21 and HDAC2. A strong repression of TS expression by HDAC inhibitors was found also *in vivo*, in mouse xenograft models. Furthermore, combined treatment of 5-FU or its prodrug 5-fluorodeoxyuridine (Floxuridine, FUdR) with vorinostat or panobinostat increased cell cycle arrest, while it decreased cell growth. The downstream effect of TS inhibition was increased by this combination including the inhibition of acute TS induction and the enhanced accumulation of the cytotoxic nucleotide intermediate dUTP [161].

The same mechanism was demonstrated to be responsible in combined exposures of NSCLC and breast adenocarcinoma MCF-7 cells to low-dose of vorinostat (SAHA) and 5-FU. SAHA enhanced the effect of 5-FU in these cells and led to a synergism of the two drugs, predominantly in cell lines resistant to 5-FU. A study analyzing amounts of mRNAs and proteins of TS, dihydropyrimidine dehydrogenase and orotate phosphoribosyl transferase, enzymes that are connected with 5-FU sensitivity /response indicated that lung tumor cell lines resistant to 5-FU showed high levels of TS mRNA and protein. Vorinostat down-regulated amounts of TS mRNA and protein, when all cell types tested in the study of Noro *et al.* [162] (A549, PC9, PC9/f14 and MCF-7) were treated with 5-FU. The authors of this study also investigated the status of the Rb-E2F1 pathway, with vorinostat up-regulating expression of *p21* gene through promoter histone acetylation; this, in turn, blocked the Rb-E2F1 pathway [162]. In addition, another HDAC inhibitor, VPA, augmented the anti-tumor effects of 5-FU in human pancreas tumor cells (SUIT-2) as well as cholangiocarcinoma cells (HuCCT1) [163].

Trichostatin A enhanced the chemosensitivity of human gastric tumor cells (OCUM-8, MKN-74 cells) to 5-FU. The up-regulation of *p53*, *p21*, *DAPK-1* and *DAPK-2* genes can explain this observed synergism [96]. Trichostatin A also reverses resistance of human colon cancer cells to 5-FU. This process was found to be caused by down-regulation of TS [164]. In addition, other HDAC inhibitors, vorinostat, panobinostat and entinostat were found to cause an increase in the antiproliferative effects of 5-FU *in vitro* and also *in vivo*, in models bearing human colorectal cancer. Moreover, it caused resensitization of cells resistant to 5-FU [159-161]. Vorinostat and panobinostat mediated a down-regulation of TS expression by suppressing its transcription in colon cancer cells *in vitro,* as well as in mouse xenografts [160, 161]. This indicates that a combined treatment of HDAC inhibitors with 5-FU is an important chemosensitization strategy reversing TS-associated resistance of the tumors to 5-FU. Vorinostat also mediates up-regulation of *p53* gene expression in wild-type p53 colorectal cancer cells and down-regulation in mutant p53 cells, indicating another antiproliferative mechanism for the synergistic effects found in the cells treated with this HDAC inhibitor and 5-FU [165].

In the study of Lee and collaborators [166] the authors demonstrated that the HDAC inhibitor trichostatin A also overcomes 5-FU resistance by down-regulating TS. This protein interacts with the Hsp complex, and exposure to trichostatin A mediated acetylation of Hsp90 chaperone and subsequently increased Hsp70 interaction with the TS leading to proteasomal degradation of the TS protein. Therefore, exposure of 5-FU-resistant cancer cells to low concentrations of trichostatin A increased toxic effects of 5-FU in these cells, due to lower TS protein levels [166].

Interestingly, another mechanism might be responsible for synergistic effects of HDAC inhibitors and a pro-drug of 5-FU, capecitabine Fig. (**2**). Exposure of breast MCF-7 and MDA231 cells to HDAC inhibitors, trichostatin A or vorinostat, enhanced expression of thymidine phosphorylase (TP) mRNA and protein. Such an increase in TP expression seems to be a predictive factor for the enhanced anticancer effect of capecitabine, because TP is essential for bioactivation of this 5-FU precursor Fig. (**4**). Hence, increased amounts of TP determine sensitivity of cells to capecitabine. Interestingly, the efects of these HDAC inhibitors is not mediated by the cytokine TNF that efficiently induces TP [167].

4.1.3. Alkylation Agents

4.1.3.1. Cisplatin

Cisplatin [cis-diammine dichloroplatinum (II), Fig. (**2**)] is a chemotherapeutic drug that binds to DNA, and crosslinks DNA leading to induction of apoptosis. Cisplatin is a mustard which is activated intra-cellularly by the aquation of one chloride releasing group, finally generating covalent DNA adducts [168]. The resulting cellular outcome of these adducts is induction of apoptosis thought to occur by stopping many cellular processes resulting in G2 cell-cycle arrest, deregulation of signal transduction [169].

The anticancer effect of cisplatin has also been found to be increased by HDAC inhibitors. Since 2003, the synergistic action of HDAC inhibitors and cisplatin has been studied in greater details. All mechanisms described above to be responsible for HDAC inhibitor-mediated potentiation of the effects of DNA-damaging drugs may also be effective in combinations with cisplatin. Among them, the changes in chromation structure, which make DNA more accessible to covalent modification by cisplatin or changing DNA interaction with transcriptional regulators, seem to be the major mechanism responsible for sensitizing cancer cells to cisplatin. Such changes in DNA can finally lead to many cellular responses causing elevated initiation of cell cycle arrest and apoptosis. Indeed, these mechanisms were described by Kim and coworkers [119] to be responsible for trichostatin A- and vorinostat-mediated

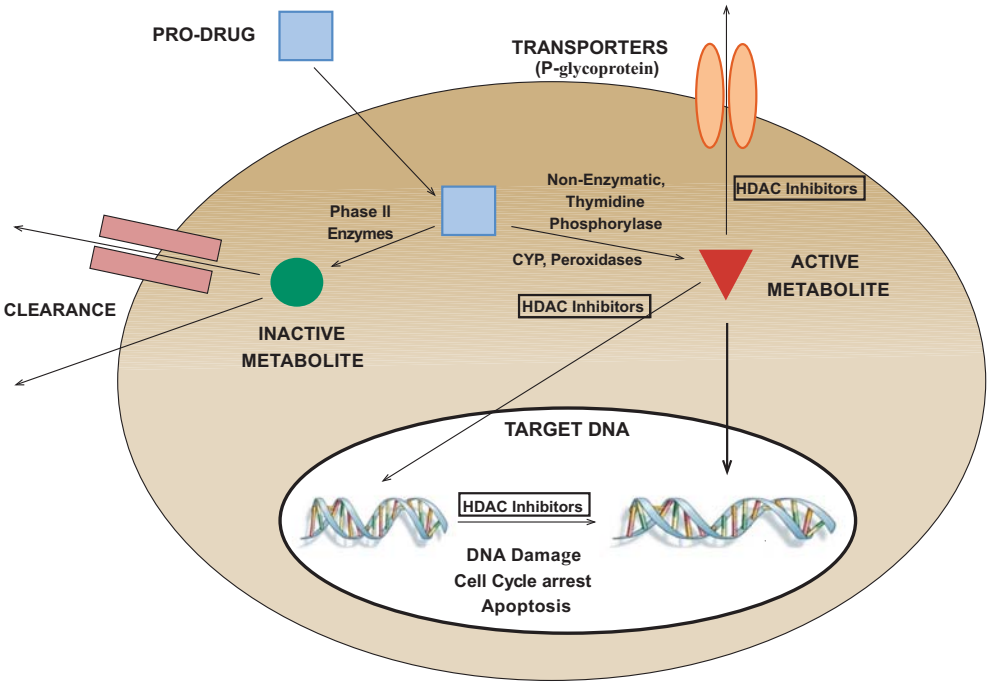


Fig. (4). Scheme of the fate of HDAC inhibitors and DNA damaging drugs and their synergistic effects in cancer cells. Several anticancer drugs can be metabolized (both activated and inactivated) either non-enzymatically or by enzymatic catalysis. The expression and activities of the enzymes participating in such catalysis are modulated by several HDAC inhibitors. The products of drug metabolism enter the nucleus and cause processes leading to cell death. Various membrane proteins eliminate drugs from the cells, transporting them across the cell membrane. These transporters such as P-glycoprotein are induced by HDAC inhibitors such as VPA.

sensitizing of several human tumor cells (D54, U118, MCF-7) towards cisplatin.

In the study of Jin and collaborators [170], HeLa cells were found to be sensitized to cisplatin by an HDAC inhibitor vorinostat, which down-regulated Bcl-2 and X-linked inhibitor of apoptosis protein (XIAP). Such processes can be caused by relaxation of chromatin by vorinostat, which improves the accessibility of cisplatin to DNA and transcriptional regulators [170].

Another HDAC inhibitor, sodium butyrate, also sensitized HeLa cells to cisplatin. [171]. Cells exposed to this drug were about two-fold more sensitive to cisplatin than cells exposed to cisplatin alone. Interestingly, exposure to sodium butyrate initially reversed cytotoxicity of cisplatin, which was due to enhanced acetylation of histone H4 in euchromatin regions responsible to the G1/S phase transition and DNA synthesis induction. This process abolished the cisplatin-mediated cell cycle arrest and the cells traversed S phase with damaged DNA. The prolonged effects of this HDAC inhibitor were a high histone acetylation in eu- and hetero-chromatin, a decrease in DNA replication and induction of the apoptotic processes [171]. More importantly, the life span of an albino mouse model bearing Ehrlich ascites tumor was extended nearly two-times when the animals were treated with cisplatin and butyrate compared to mice exposed to cisplatin only.

In the work of Mutze *et al.* [122], sequential treatment of human cancer cell lines (GS, KATOIII, MKN28 and MKN45) with vorinostat and cisplatin showed synergistic effects irrespective of the initial cisplatin sensitivity [122]. In addition, trichostatin A was found by Zhang and coworkers [96] to have a positive effect on sensitivity on human gastric tumor cells, OCUM-8 and MKN74, to a platin derivative oxaliplatin [96].

This HDAC inhibitor showed an additive effect to cytotoxicity of these cells to oxaliplatin. The up-regulation of *p53*, *p21*, *DAPK-1* and *DAPK-2* genes can explain the mechanism of an increased cytotoxicity [96]. More importantly, the synergistic effects of SAHA and cisplatin were supported by the preliminary results found in a study with the patients suffering from gastric cancer [122].

The mechanisms of the increased effects of HDAC inhibitors on cisplatin-mediated apoptosis in human oral squamous cell carcinoma (HSC-3) cells were investigated using vorinostat as a model HDAC inhibitor by Rikiishi *et al.* [172]. Vorinostat activated caspases 3, 8 and 9, therefore a mitochondrial-dependent amplification loop seems to initiate apoptosis in these cells. Exposure of cells to vorinostat followed by cisplatin is much more effective than any other sequence of exposure. Moreover, another mechanism of the effect of vorinostat was found in this study. Cells treated with this HDAC inhibitor showed a decrease in amounts of GSH. Since an inhibitor of GSH synthesis, diethyl maleate, diminished GSH and increased also the cisplatin-mediated apoptotic processes in HSC-3 cells, vorinostat seems to impair the redox balance in these cells, mediating highest apoptosis at the G0/G1 phase arrested by cisplatin. It is evident that vorinostat not only causes an alteration of the histone acetylation status, but it is also capable of affecting apoptosis at several levels, and GSH is a key player that governs a vorinostat-mediated increase in apoptosis caused by cisplatin [172].

The mechanisms causing synergistically induced cytotoxicity and apoptosis by co-administration of vorinostat and cisplatin were also examined in oral squamous cell carcinoma (OSCC) cells, the Tca8113 and KB lines. Combined exposure to vorinostat increased the effects of subtoxic amounts of cisplatin. Furthermore, up-regulation of the expression of apoptotic proteins such as p53, Bid, cytochrome c and caspase 3 by vorinostat enhanced cisplatin-induced cytotoxicity and the induction of apoptosis [173]. Vorinostat also enhanced cisplatin-induced apoptosis in another OSCC cell line, HSC-3. The complex, multifunctional mechanisms of this process were studied by Suzuki and collaborators [174]. Treatment with vorinostat/cisplatin increased significantly apoptotic

processes in this OSCC cell line, producing enhanced activities of caspases 4 and 9. Vorinostat as a single agent also caused initiation of sustained phosphorylation of translation initiation factor 2 (eIF2)α that is up-regulated during ER stress. A decrease in ER stress due to salubrinal, the compound that inhibits eIF2α dephosphorylation, abolished the vorinostat induced increased sensitivity of HSC cells to cisplatin. In the cells exposed to vorinostat, lower amounts of phospho-Akt were produced, connected to enhanced enzymatic activity of protein phosphatase 1 (PP1), the phosphatase upstream of Akt. The results found in this study demonstrated that up-regulation of selective-ER stress-associated events play an important role in the mechanism of the influence of vorinostat on cisplatin-mediated apoptotic processes [174].

VPA augmented also apoptosis initiated by cisplatin, in mesothelioma cells and in tumor cells from patient biopsies. Induction of apoptosis in cells involved extrinsic and intrinsic pathways that require the activities of caspases 8 and 9, respectively. Furthermore, VPA efficiently stimulated the production of ROS in these cells. The ROS scavenger *N*-acetylcysteine inhibited apoptosis, which demonstrates that ROS were the main mediators of the VPA effects. VPA as a single drug as well as in combination with cisplatin increased acetylation of histone H3.

Bid protein processing in truncated t-Bid and cytochrome c release from mitochondria were increased in the presence of VPA, explaining the enhancement of apoptosis caused by cisplatin. VPA administered together with cisplatin prevented growth of malignant epithelioid mesothelioma in tumor bearing mice [175].

The combined exposure of several cancer cell lines (i.e. A549, PC3, SK-OV3 and MCF-7 cell lines) to a derivative of trichostatin A, M344 {4-(dimethylamino)-*N*-[7-(hydroxyamino)-7-oxoheptyl]-benzamide, Fig. (**1**)}, and cisplatin also enhanced the cytotoxic effects of cisplatin by the increased induction of apoptosis [176]. This co-treatment led to increased induction of the proapoptotic factor, Activating Transcription Factor (ATF) 3, compared with cisplatin alone. This factor is a key regulator of the cellular integrated stress response the expression of which has also been correlated with proapoptotic activities in tumor cells. The mechanism of ATF3 induction by M344 is not dependent on the MAPKinase pathway, but depends on ATF4, a known regulator of ATF3 expression. These findings indicate that ATF3 is a novel target of M344, as well as a mediator of the co-operative efficacies of cisplatin and M344 induced cytotoxicity [176].

Another mechanism was responsible for the enhanced cisplatin-induced cytotoxicity by M344 produced in various breast (MCF-7, T-47D and HCC1937) and ovarian (A2780s, A2780cp and OVCAR-4) cancer cell lines [177]. The enhanced sensitivity of HDAC inhibition to platinum seems to be mediated through a Breast Cancer 1 (BRCA1)-dependent mechanism in these cancer cells. Upon addition of M344, the breast and ovarian cancer cell lines that displayed relatively high BRCA1 protein levels demonstrated significant potentiation of cisplatin cytotoxicity associated with a reduction of BRCA1 protein. A2780s cells subjected to combination of platinum and M344 treatment also demonstrated increased DNA damage as assessed by the presence of phosphorylated H2AX foci in comparison to either treatment alone. Using chromatin immunoprecipitation, A2780s and MCF-7 cell lines treated with M344 as a single drug or combined with cisplatin, did not show enhanced acetylated histone H4 at the *BRCA1* promoter, suggesting an indirect effect on this promoter [177].

HDAC inhibitors were also found to overcome cisplatin-mediated resistance or hypoxia-mediated resistance of tumor cells. VPA that exhibited synergistic cytotoxicity with cisplatin in several ovarian carcinoma cells (SK-OV-3, OVCAR-3, TOV-21G, A2780 and A2780/cp70), also resensitized the cell lines, which were resistant to cisplatin. In accordance with the enhanced toxicity, co-treatment with VPA up-regulated DNA damage due to cisplatin shown by phosphorylation of ataxia telangiectasia mutation as well as H2AX. In addition, ROS accumulated and higher expression of tumor suppressor phosphatase and tensin homolog (PTEN) that might participate in the increased cytotoxicity, were induced by VPA. Since PTEN knockdown by siRNA or exposure to antioxidants might decrease cisplatin-induced cytotoxicity, it seems that up-regulation of PTEN or ROS by VPA participates in increased toxic activity of cisplatin to the cancer cells [178]. VPA also synergized cisplatin in UKF-NB-3 neuroblastoma cells under hypoxic conditions. Cells exposed to a combination of VPA and cisplain showed a higher degree of apoptosis under hypoxic conditions, suggesting not merely a synergistic effect for VPA and cisplatin, but the added ability of VPA to overcome hypoxia-induced resistance to cisplatin [179]. In addition, pretreatment of resistant A2780 CIS ovarian cancer cells with trichostatin A overcame apoptosis resistance to cisplatin by restoration of expression of p73 and Bax, but not of p53 expression. An important role of histone deacetylase activation in the contribution to chemotherapy resistance in human ovarian tumor cell lines was also found [180].

Likewise, trichostatin A induced apoptosis in the human lung adenocarcinoma A549 cells and also in cisplatin resistant cells (A549/cisplatin). This HDAC inhibitor increased the sensitivity of the resistant A549/cisplatin cells to cisplatin, together with DAPK (death-associated protein kinase) up-regulation. After over-expression of DAPK, an A549/cisplatin cell line was resensitized to cisplatin and the cytotoxicity of trichostatin A could be increased. Furthermore, toxic effects of trichostatin A could be alleviated by a decrease in DAPK activity by expression of a recombinant C-terminal fragment of DAPK or RNA interference [181]. Trichostatin A also synergistically enhanced the antitumor effect of cisplatin and resensitized the cisplatin resistant human bladder cancer cell line T24R2. The mechanism could be synergistic cell cycle arrest, initiation of the caspase mediated apoptotic processes or up-regulated expression of proapoptotic Bad and Bax [182].

4.1.3.2. Melphalan

Melphalan hydrochloride Fig. (**3**) is an anticancer chemotherapeutic agent that is a member of a group of nitrogen mustard alkylating drugs. This drug alkylates the nitrogen 7 of guanine residues in DNA. This antitumor drug is predominantly used for melanoma treatment. Several studies showed that anticancer action of this drug might be intensified with HDAC inhibitors, again mediated by various mechanisms.

For example, the combination of entinostat (SNDX-275) with melphalan synergistically inhibited growth of multiple myeloma (MM) cells, MM1.S and RPMI8226 cell lines. Entinostat synergistically enhanced the melphalan-mediated apoptotic processes in MM cells by increased DNA damage. The combination of both types of drugs compared to either drug alone significantly increased caspase-mediated apoptosis in exposed cells. Entinostat practically did not influence cell cycle progression of a MM1.S cell line, however enhanced the percentage of RPMI8226 cells in S phase of the cell cycle connected with up-regulation in p21 and repression of cyclin D1 and E2F1. Melphalan as single drug caused arrest of MM1.S and RPMI8226 cells in S phase and increased levels of p53 and p21. Moreover, investigation of DNA damage response showed that levels of H2AX (γ-H2AX), a marker of DSBs in DNA, together with those of phosphorylated Chk1 (P-Chk1) and Chk2 (P-Chk2) were significantly increased with entinostat and melphalan. The amounts of γ-H2AX and P-Chk1 were significantly higher when the cells were exposed to the combined drugs compared to either drug alone. Such alterations corresponded to the enhancement of cell death caused by mitotic catastrophe [183].

Panobinostat (LBH589) combined with melphalan increased the anti-multiple myeloma (MM) action in MM cells and the human MM xenograft model LAGλ-1. Exposure to panobinostat led to enhanced acetylation of histones, initiation of caspase cleavage, a decrease in cell proliferation as well as a synergism of anti-MM activity of melphalan *in vitro*. Furthermore, panobinostat combined with melphalan significantly increased anti-myeloma effects *in vivo* [124].

The HDAC inhibitor depsipeptide was more potent when used in combination with melphalan in the MM cells, U266 [interleukin (IL)-6 dependent], and RPMI8226 again by modifying expression of proteins involved in apoptotic processes. The expression of antiapoptotic proteins Bcl-2, Bcl-xL and Mcl-1, were suppressed in cells exposed to depsipeptide [184].

In the study of Kaiser and collaborators [185], the potency of vorinostat was examined to increase the antitumor effects of several drugs including melphalan in MM cells *in vitro* and mouse xenografts *in vivo*. Synergistic effects were found when RPMI8226 and U266 cells were treated with vorinostat and melphalan. This HDAC inhibitor combined with melphalan led also to reduction in tumor growth *in vivo*. The mechanism of this effect has, however, not been evaluated in this study [185]. Another HDAC inhibitor, VPA, also initiated death of myeloma cells mediated by caspase-dependent and –independent pathways, and enhanced the anti-myeloma activities of melphalan and dexamethasone [186].

4.1.3.3. Temozolomide

Temozolomide (brand names Temodar and Temodal, Fig. (**2**)), an oral alkylating agent, is frequently used to treat glioblastoma multiforme and other glial tumors and is tested in melanoma [187]. The antitumor pro-drug temozolomide leads to methylation of guanine residues in DNA, generating O^6-methylated and N^7-methylated adducts. The activation of temozolomide proceeds through ring-opening to 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) that further decomposes to a diazonium ion at guanine-rich sequences in DNA. The proximate methylating agent is the reactive methyldiazonium ion. Runs of guanine residues represent an accessible nucleophilic microenvironment in DNA site-specific conversion of the pro-drug temozolomide to MTIC possibly *via* an activated water molecule in the major groove [188]. The O^6-methylguanine-DNA methyltransferase (MGMT) enzyme demethylates the O^6-methylguanine residue to unmodified guanine [189]. MGMT methylation was found to be connected with prolonged progression-free and overall survival in patients suffering from glioblastoma multiforme, when they were treated with temozolomide [190].

Results showing synergistic effects of HDAC inhibitors on other DNA-damaging drugs would suggest that HDAC inhibitors might also improve the cytotoxicity of temozolomide. Investigations of the effects of HDAC inhibitors upon glioblastoma cells indicated the best effects by combining the HDAC inhibitor AN-9 with temozolomide or radiation [191]. Another HDAC inhibitor, entinostat (MS-275), sensitized glioblastoma cell lines to temozolomide-mediated induction of apoptotic processes [143]. This increased potency of temozolomide by HDAC inhibitors resulted in numerous clinical trials, predominantly for gliomas (http://www.clinicaltrials.gov/).

VPA also sensitized malignant melanoma to temozolomide used as first-line therapy. In malignant melanoma cells expressing wild-type p53, the temozolomide-mediated formation of O^6MeG in DNA induces up-regulation of the Fas/CD95/Apo-1 receptor without activation of the apoptotic cascade. This phenomenon is caused by silencing of procaspase 8. This important function of procaspase 8 in potentiating drug effects in melanoma cells was proven by blocking the death receptor pathway by expressing dominant-negative FADD, siRNA knockdown of procaspase 8, and stimulation with Fas/CD95/Apo-1 activation antibody. The levels of procaspase 8 might additionally be increased further by exposure to VPA that sensitized melanoma cell lines *in vitro*. Moreover, potentiation of the effects of temozolomide by VPA in melanomas was found in a xenograft mouse model [192].

4.1.3.4. Ellipticine

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Fig. (**2**)) and its derivatives are efficient anticancer compounds that function through multiple mechanisms leading to cell cycle arrest and initiation of apoptosis (for a summary see [193-196]). Ellipticine was found (i) to arrest cell cycle progression due to modulation of levels of cyclinB1 and Cdc2, and phosphorylation of Cdc2 in human mammary adenocarcinoma MCF-7 cells, (ii) to initiate apoptosis by toxic free radicals, stimulation of the Fas/Fas ligand system and modulation of proteins of the Bcl-2 family in several tumor cell lines, (iii) to induce the mitochondria-dependent apoptotic processes (for a summary see [138, 194, 196]). The predominant mechanisms of the biological effects of ellipticine were suggested to be (1) intercalation into DNA [197, 198] and (2) inhibition of topoisomerase II [194, 197, 198]. In addition we showed that this antitumor agent forms covalent DNA adducts after its enzymatic activation by cytochromes P450 (CYP) and peroxidases [193-196, 199], which suggests another DNA-damaging effect of ellipticine.

The potentiating of cytostatic effects of ellipticine by HDAC inhibitors was described by Kim *et al.* [119]. Preincubation of human glioblastoma, breast, and colon tumor cells with either trichostatin A or vorinostat before adding ellipticine (or etoposide, doxorubicin or cisplatin) made cells more sensitive to these agents (see also Chapter 4.1.). Alterations in the chromatin structure by acetylated histone molecules was proposed to increase the efficiency of ellipticine to intercalate into DNA or inhibit topoisomerase II Fig. (**3**). These changes additionally led to induction of p53, p21, and Gadd45 proteins [119].

Our recent study has demonstrated that trichostatin A and also VPA increased cytotoxicity of ellipticine to human neuroblastoma UKF-NB-3 and UKF-NB-4 cells [106]. We found that a higher sensitivity of these neuroblastoma cell lines to ellipticine corresponded to enhanced levels of covalent DNA adducts generated by activated ellipticine in these cells. Even though this most effective DNA damage by ellipticine [200], could be mediated by the relaxation of chromatin that making DNA more accessible to activated ellipticine, we found another reason for the observed increase in ellipticine-derived DNA adducts caused by both HDAC inhibitors. In both neuroblastoma cell lines VPA and trichostatin A modulate expression of cytochromes P450 (CYP) and peroxidases and of cytochrome b_5, thereby influencing reactions that activate or detoxify ellipticine. Exposure to trichostatin A or VPA resulted in higher amounts of reactive ellipticine metabolites (13-hydroxy- and 12-hydroxyellipticine) that bind to DNA. Most of the enhancing effects of VPA and trichostatin A on ellipticine cytotoxicity was found to be attributed to enhanced ellipticine-DNA adduct formation caused by increased levels of cytochrome b_5, CYP3A4 and CYP1A1. Lower responses of UKF-NB-4 cells to the combined effects of ellipticine with VPA or trichostatin A than of UKF-NB-3 cells is also attributable to high levels of P-glycoprotein in the former cells. This transporter regulates drug concentrations in the cells, is expressed in UKF-NB-4 cells and is induced by HDAC inhibitors. The results of this work warrant further studies and may help to design of new protocols geared to the treatment of high risk neuroblastomas [106].

4.1.4. HDAC Inhibitors and Ionizing Radiation

Numerous carcinoma are treated by radiotherapy. Ionizing radiation damages DNA, causing DSBs that are considered to be the predominant defect in DNA associated with cell survival and changes in genomic integrity. The major responses of cells to DSBs involve modulation of cell-cycle, changes in repairing this DNA

lesion, modulation of important genes (involving genes connected with DNA repair) at the transcriptional and post-transcriptional levels as well as initiation of apoptosis. The response of cells to DSBs was suggested to be a classical signal-transduction cascade [201, 202], because ionizing radiation activates signaling pathways, which modulate repair of damaged DNA, and interrupt cell cycle progression [203].

In order to increase the therapeutic efficiencies of radiation, a longstanding goal has been the development of chemical agents that can sensitize cancer cells (radiation sensitizers) or protect healthy cells (radioprotectors) from the action of ionizing radiation. Interestingly, HDAC inhibitors were found to act both as effective radiation sensitizers and radiation protectors (for a summary see [113-115]). Among the combinations of HDAC inhibitors with several types of DNA damaging agents, those combining these inhibitors with ionizing radiation are the oldest ones [204-206]. Indeed, the utilization of the HDAC inhibitor sodium butyrate to sensitize cancer cells to ionizing radiation has already been described more than 25 years ago [204]. Several studies carried out in the 1980s showed that almost nontoxic concentrations of this HDAC inhibitor increased the efficiency of radiotherapy in colon cells [204-206]. Following these studies, many different HDAC inhibitors were investigated using various cancer cells and found to increase the therapeutic effects, leading to an increased interest in potential clinical utilization of HDAC inhibitors in combinations with classical radiation therapies. Now, many different HDAC inhibitors combined with radiation are studied in clinical trials as potential therapeutic regimens for treatment of several cancer diseases (http://www.clinicaltrials.gov/, http:// www.cancer.gov/ clincialtrials).

Also here several mechanisms for this increased efficiency of radiation by HDAC inhibitors have been suggested; (i) HDAC inhibitors might have additive effects, since they act through a variety of cytotoxic mechanisms, or (ii) a synergistic effect may be a consequence of the ability of HDAC inhibitors to modulate chromatin structure and to regulate gene expression by histone acetylation [115]. Packing and accessibility of DNA in chromatin appear to be the major factors that influence the radiation sensitivity. The capability of HDAC inhibitors to modify chromatin structure, to bind to key signal transduction proteins associated with cellular response to DNA damage as well as modulating transcription, especially of genes involved in repairing DSB in DNA, were suggested as reasons for the synergistic effects with radiation [120, 207-211].

Sodium butyrate, which was the first HDAC inhibitor combined with ionizing radiation [204], but also other HDAC inhibitors (trichostatin A, phenylbutyrate and tributyrin) radio sensitized human melanoma cells (A375 and MeWo) by reducing the cells repair of the DNA damage caused by ionizing radiation. Sodium butyrate induced hyperacetylation of histone H4 in these melanoma cells. This inhibitor increased apoptosis initiated by radiation, which correlated with functional impairment of DNA repair. It repressed levels of the repair-related proteins Ku70 and Ku86 and the catalytic subunit of DNA-dependent protein kinase. In addition, histone γ-H2AX levels remained high in cells pre-treated with sodium butyrate additionally exposed to radiation [120].

Recently, sodium butyrate has also been found to radio sensitize HeLa cells, studied by Koprinarova *et al.* in detail [212]. FACS analysis demonstrated that this HDAC inhibitor does not abrogate the ionizing radiation imposed G2 cell cycle arrest. The dynamics of γ-H2AX foci disappearance in the presence and in the absence of butyrate, however, demonstrated that butyrate inhibited DSB repair. In order to explain which of the above processes affect the DSB repair, the authors of the study synchronized a HeLa cells in G1 phase and analyzed the repair of the DSBs after radiating cells. Because homologous recombination (HR) is not functional in G1 phase, DSBs in DNA are repaired by non-homologous end-joining (NHEJ) only. The data found in their study indicated a decrease in NHEJ when cells were treated with sodium butyrate. The dynamics of RAD51 disappearance in irradiated HeLa cells by their treatment with sodium butyrate were also examined. Because RAD51 takes part in homologous recombination (HR) only, this study allowed evaluation of the influence of sodium butyrate on the DSB repair by HR; and HR was also blocked by this HDAC inhibitor. These findings were corroborated using host cell reactivation assays that determined the repair of plasmids containing a single DSB by NHEJ or HR. Based on these results, a mechanism of the butyrate effect on sensitization of HeLa cells to ionizing radiation was suggested. When a DSB is generated in cells, the HDAC enzymes deacetylate core histones located close to these DNA breaks in order to compact the chromatin architecture and prevent the broken DNA ends from moving apart from each other, thereby assuring efficient repair [212]. HDAC inhibitors prevent this repair.

Trichostatin A, vorinostat, a derivative of trichostatin A, M344, and depsipeptide made radiation resistent human squamous cell carcinoma SQ-20B and SCC-35 cells, sensitive to ionizing radiation. Depsipeptide was most efficient in SQ-20B cells, whereas M344 had the highest effect on sensitizing SCC-35 cells. Furthermore, exposure of SQ-20B cells to radiosensitizing amounts of trichostatin A produced cell cycle arrest in G1 phase and a decrease in DNA synthesis [211].

In the study of Karagiannis *et al.* [116], trichostatin A was found to modulate the radiation sensitivity of human erythroleukemic K562 cells [116] by caspase 3 and 7 mediated apoptosis. The modulation of radiation effects observed at low trichostatin A concentrations was associated with histone hyperacetylation and changes in phosphorylated γ-H2AX formation on euchromatin. At higher trichostatin A concentrations drug-mediated cytotoxicity and G1 cell cycle arrest, contributed to the sensitization effect [116].

Romidepsin (FK228) pretreatment augmented ionizing radiation-induced cell death of human squamous cell carcinoma SAS cells, by apoptosis. This inhibitor increased levels of *BMF* gene and knockdown of *BMF* transcription by siRNA decreased significantly the romidepsin effect on ionizing radiation-mediated cytotoxicity, disruption of mitochondrial membrane potential and breaks in DNA, while Bmf overexpression increased ionizing radiation-mediated death. These effects of romidepsin were also found in a squamous carcinoma HSC2 line. These results demonstrated that acetylation of histones might increase ionizing radiation-mediated death through stimulation of *BMF* transcription, thus implying Bmf to be an important molecule for the romidepsin-induced increasing effect on radiation-mediated cell death [127].

Vorinostat significantly radiosensitized human melanoma A375, MeWo and A549 cells [213], again by multiple mechanisms that may involve antiproliferative growth inhibition and a decrease in DNA repair after exposure to ionizing radiation. Whereas vorinostat and ionizing radiation as single agents were not able to initiate the apoptotic processes in melanoma cells, their combination led to apoptosis. Vorinostat exhibited a significant inhibitory effect on the non-homologous end-joining (NHEJ) pathway of DNA repair when A375 cells were irradiated. A detailed examination of the involvement of the DNA repair pathway following vorinostat treatment showed that this HDAC inhibitor reduced the expression of the repair-related genes *Ku70*, *Ku80*, and *Rad50* in A375 cells. The combination of vorinostat and radiation caused a prolongation of expression of DNA repair proteins (i.e. γ-H2AX) [213].

VPA also sensitized myelogenous leukemia K562 cells to ionizing radiation-induced cell death. The radio sensitizing effect was correlated with VPA-mediated histone hyperacetylation, chromatin decondensation and enhanced formation of radiation-

induced γ-H2AX preferentially on euchromatic alleles. Heterochromatin was much more resistant to histone tail modification, changes in chromatin architecture and DNA damage [109]. Exposure of colorectal tumor cells to non-toxic amounts of VPA and its effects on radiation induced damage were studied by Chen *et al.* [111] on LS174T cells and an isogenic pair of HCT116 cells with and without wild-type p53 [111]. Radio sensitization by VPA depended on p53 status. VPA and ionizing radiation repressed clonogenic survival, enhanced apoptosis and elevated amounts of γ-H2AX only in cells expressing wild-type p53 Treatment with VPA increased in ionizing radiation-mediated mitochondrial locations of Bax and Bcl-xL, disruption of mitochondrial membrane potential and cytochrome c release, exclusively in wild-type p53 cells. In addition, VPA decreased tumor growth after irradiation only in wild-type p53 xenografts. Such results indicate that VPA might significantly increase radiotherapy response in colorectal tumors, with the wild-type p53 genotype [111]. Exposure of Y79 and WER1-Rb1 human retinoblastoma cells to VPA or depsipeptide (FK228) increased induction of apoptosis mediated by irradiation, increased caspase 3 activity and cleavage of PARP. These two HDAC inhibitors increased ionizing radiation-mediated phosphorylation of serine 139 in H2AX that precedes apoptosis. Treatment of cells with ionizing radiation together with VPA or depsipeptide increased levels of p53 that was acetylated at lysine 382 and phosphorylated at serine 46 *via* a decrease in binding affinity to Mdm2 and Mdmx [214].

Trichostatin A radiosensitized human NSCLC A549 and H1650 cells, by enhancing G2/M cell cycle arrest, promoting apoptosis through multiple pathways and interfering with DNA damage repair processes. In A549 cells this inhibitor enhanced ionizing radiation-induced accumulation of cells in G2/M phase, with upregulated expression of p21. In addition, trichostatin A treatment caused pronounced apoptosis in irradiated cells, which was accompanied by p21 cleavage to a 15 kDa protein. The enhanced apoptosis was by the mitochondrial pathway, as indicated by a disrupted mitochondrial membrane potential and cytochrome c. Caspase 3 activity significantly increased, more PARP was cleaved, associated with the repression of X-linked inhibitor of apoptosis protein (XIAP). Furthermore, trichostatin A impaired DNA repair capacity after ionizing radiation by down-regulation of Ku70, Ku80 and DNA-PKCs, reflected by enhanced and prolonged expression of γ-H2AX [215].

In another study, exposure of neuroblastoma cells to vorinostat resulted in a decrease in cell viability and led to increased effects of ionizing radiation. In a mouse metastatic neuroblastoma model, vorinostat combined with ionizing radiation caused a more pronounced decrease in tumor volumes compared to single modality. Since DNA repair enzyme Ku86 was repressed in neuroblastoma cells exposed to vorinostat, this inhibitor potentiated anti-cancer effects of ionizing radiation by impairing DNA repair [216].

The molecular mechanisms connected with the radiosensitizing influence of trichostatin A and vorinostat (SAHA) were also investigated by Seo and coworkers [217] in human lung cancer cells [217]. They showed that both HDAC inhibitors and ionizing radiation synergistically triggered cell death in human NSCLC cells. Cellular viability as well as clonogenic survival was repressed in cultures exposed to HDAC inhibitors in combinations with ionizing radiation. Furthermore, exposure of cells to combined therapies resulted in enhanced acetylation of p53 at lysine 382, while at the same time c-Myc expression was down-regulated. Radio sensitization by HDAC inhibitors was decreased upon transfection with siRNA against *p53* and c-*Myc* overexpression, which indicated the involvement of p53 and c-Myc in this process. In addition, c-Myc down-regulation and apoptotic cell death mediated by ionizing radiation combined with HDAC inhibitors were decreased in cells transfected with mutant K382R p53 or C135Y p53 with either no lysine at position 382 or no DNA-binding activity. Hence, these findings demonstrated that the efficacy of HDAC inhibitors to increase sensitivity of cancer cells to ionizing radiation was affected by acetyl p53-mediated c-Myc down-regulation [217].

5. PESPECTIVES

The data summarized in this review demonstrate that many HDAC inhibitors have synergistic or at least additive effects on antitumor activities of other anticancer drugs or ionizing radiation. Many of the mechanisms were elucidated in *in-vitro* studies, it is not clear if these also apply *in vivo* particularly in humans. Many studies have shown that exposure HDAC inhibitors and anti-cancer drugs lead to increased cytotoxicities. Further *in-vivo* studies in animal models but mainly clinical trials will show which are the most suitable combination of drugs, the sequence of their administration, and their dosages. Susceptibility to therapy is dependent both on mitotic activity of the tumor cells, on their differentiation status and on various genetic alterations. In addition both HDAC inhibitors and chemotherapeutic drugs assessed in combination treatments may be metabolized by biotransformation enzymes. This is the case of pro-drugs such as doxorubicin, temozolomide, etoposide and ellipticine, which are activated to pharmacologically more effective metabolites or detoxified and eliminated from cancer cells or the body [194, 218, 219] Fig. (**4**). Furthermore, expression levels of transporters that mediate drug efflux can protect cancer cells against damaging agents Fig. (**4**). The activities and substrate specificities of these enzymes and transporters and their expression are regulated by genetic and epigenetic modifications leading to individual variability among patients. Because HDAC inhibitors are epigenetic modifiers the expression of these proteins can be changed by the HDAC inhibitors themselves. Indeed, VPA, trichostatin A, sodium butyrate and vorinostat (SAHA) are inducers of cytochrome P450 (CYP) 1A1/2, 2B1/2/6 and 3A4 [106, 220-226] which metabolize drugs used in cancer therapy such as paclitaxel [227, 228], bortezomib [229], 5-FU [230], etoposide [219] and ellipticine [194] and HDAC inhibitors themselves like VPA [231], romidepsin [232] and panobinostat [233]. Furthermore, one transporter protein that mediates drug elimination from cells, P-glycoprotein, is induced by VPA and trichostatin A [106, 220, 234]. Therefore, precise patient selection and defined protocols for therapies combining HDAC inhibitors with other drugs is necessary. To reach the most suitable drug combination for a special therapy, more *in vitro* and *in vivo* studies as well as clinical trials should be and are carried out.

6. SUMMARY AND CONCLUSIONS

The data reviewed in this paper show that HDAC inhibitors are promising substances suitable to be combined with other antitumor drugs. In spite of the fact that these HDAC inhibitors provide anticancer benefits, the multitude of possible cellular targets and down-stream effects make their clinical use difficult. Therefore, additional knowledge of these processes is necessary to increase efficacy of these compounds and to develop them as therapy regimens to be included in combinations with other anticancer drugs or radiotherapy. Most of the studies investigating HDAC inhibitors looked at changes in gene expression mediated by increased histone acetylation as a consequence of inhibition of histone deacetylation. Results of recent studies open interesting new targets of HDAC inhibitors, because an increased acetylation of non-histone proteins was found as a consequence of exposure of tumor cells to HDAC inhibitors [235]. The non-histone proteins p53, HIF-1 and E2F1 that regulate transcription, the signaling protein Smad 7, the

Table 1. Clinical Trials Considering Combined Treatment with HDAC Inhibitors and Other Antitumor Drugs Described in the Review

HDAC Inhibitor	Other Therapies	Tumor Type
Vorinostat	Azacitidine, Bortezomib, Bortezomib, dexamethasone, Cisplatin, Cisplatin, radiotherapy, Doxorubicin, Fluorouracil, Melphalan, Marizomib, Paclitaxel, Temozolomide, Radiation therapy	AML, DLBCL, Lymphoma, MM, NSCLC, Myelodysplastic Syndrome Non-Hodgkin's Lymphoma Lymphoma MM Gastric Cancer NSCLC, head and neck squamous cell carcinoma Refractory Lymphoma, MM Pancreatic Adenocarcinoma MM NSCLC, Pancreatic Cancer, Melanoma, Lymphoma NSCLC Glioblastoma Multiforme and other Central Nervous System Tumors, Melanoma NSCLC, Squamous Cell Carcinoma, Brain Metastases, Pancreatic Cancer, Pelvic Cancer, Pancreatic Adenocarcinoma
Valproic Acid	Azacitidine, Decitabine, Radiotherapy, Bevacizumab, Radiotherapy, Temozolomide, Temozolomide	Advanced Cancers, AML, Myelodysplastic Syndrome AML Children with High Grade Gliomas High Grade Gliomas, Other Brain Tumors Brain Metastases
Panobinostat	Azacitidine, Bortezomib, Bortezomib, Dexamethasone, Decitabine, Decitabine, Temozolomide, Radiotherapy	AML, Chronic Myelogenous Leukemia Myelodysplastic Syndrome Peripheral T cell Lymphoma, NK/T Cell Lymphoma, MM, Pancreatic Cancer MM AML, Myelodysplastic Syndrome Melanoma Prostate Cancer, Esophageal Cancer, Head and Neck Cancer
Entinostat	Azacitidine	Leukemia, Colorectal Cancer, Myelodysplastic Syndrome, NSCLC
Mocetinostat	Azacitidine	Myelodysplastic Syndrome
Abexinostat (PCI-24781)	Doxorubicin	Sarcoma
Romidepsin	Bortezomib	Myeloma
Belinostat	Etoposide, Cisplatin	Small Cell Lung Carcinoma Malignant Epithelial Neoplasms

(Source: http://www.clinicaltrials.gov/, http://www.cancer.gov/clincialtrials).

Hsp90 chaperone and proteins of the cytoskeleton such as tubulin are all substrates of the HDAC enzymes.

Because of the limited activity of HDAC inhibitors as single agents against cancer these compounds were combined with other cancer therapies. This paper reviewed suitable therapeutic candidates for such combined treatments as are (i) other epigenetic modifiers such as inhibitors of DNA methyl transferases or histone demethylases, (ii) compounds mediating formation of ROS, (iii) drugs inhibiting tumor growth by microtubule stabilization, (iv) proteasome inhibitors, (v) agents that cause DNA damage, and (vi) radiotherapy.

Because of the promising results found in preclinical studies utilizing HDAC inhibitors combined with other anticancer agents or radiotherapy, many clinical trials in solid tumors and leukemias were carried out. Most of these trials have shown promising results, particularly if the HDAC inhibitor was administered before the cancer drug or radiation. Many synergistic or additive effects of HDAC inhibitors when combined with other antitumor drugs were observed also in clinical settings. Currently over hundred clinical trials are examining combinations of HDAC inhibitors with other chemotherapeutics or ionizing radiation (http:// www.clinicaltrials.gov/, http://www.cancer.gov/ clinicaltrials; [82]). Even though summarizing the data of clinical trials of combined treatment with HDAC inhibitors and other therapeutic regimens was beyond the scope of this review, a limited overview of HDAC inhibitors tested in combined trials is shown in (Table **1**). The drugs listed in (Table **1**) are focused to those described in this review.

In conclusions, although most research is focused at developing novel HDAC inhibitors for clinical use as single agents, the possibility of utilizing HDAC inhibitors in combination with other chemotherapeutics or as radiation sensitizers or protectors warrants further investigation on the basis of preclinical and clinical findings.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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