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Preclinical studies on histone deacetylase inhibitors as therapeutic reagents for endometrial and ovarian cancers

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Abstract

Histone deacetylases (HDACs) remove acetyl groups from lysine residues of histones and the deacetylation allows for tighter electrostatic interactions between DNA and histones, leading to a more compact chromatin conformation with limited access for transactivators and the suppression of transcription. *HDAC* mRNA and protein overexpression was observed in endometrial and ovarian cancers. Numerous *in vitro* studies have shown that HDAC inhibitors, through their actions on histone and nonhistone proteins, are able to reactivate the tumor suppressor genes, inhibit cell cycle progression and induce cell apoptosis in endometrial and ovarian cancer cell cultures. Results from mou se xenograft models also demonstrated the potency of HDAC inhibitors as anticancer reagents when used as single agent or in combination with classical chemotherapy drugs.

Keywords

chemotherapy; gynecologic cancer; HDAC; HDAC inhibitor

It has been well established that coordinated epigenetic modifications in DNA methylation and histone modifications play a key role in the control of gene expression [1,2]. In mammalian cells, histones occupy the backbone of chromatin and undergo several post-translational modifications, including acetylation, methylation and phosphoryl ation [3]. The dynamic interplay between histone modifications affects the intrinsic properties of histones and modulates the interactions between DNA and transcription activators [4,5]. Among all

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the epigenetic modifications, acetylation is one of the better characterized. High levels of acetylation of histones in local chromatins are directly correlated with active gene expression [6–8].

Aberrant transcriptional silencing of tumor-suppressor genes via epigenetic modifications, such as DNA methylation and covalent modifications of histones, is a hallmark of cancer cells [5,9]. Histone acetyltransferases (HAT) and histone deacetylases (HDACs), catalyzing the addition and removal of acetyl groups, respectively, are the key enzymes controlling the extent of histone acetylation. The mammalian HDAC family comprises of 18 members and three major classes, and the expression, regulation and function of HDACs in cancer cells have been extensively studied [2]. Given their critical roles in cell cycling, apoptosis and differentiation, HDACs are considered important therapeutic targets for the treatment of gynecologic cancer and other malignancies [10].

It has been shown that transcriptional gene silencing can be effectively reversed by epigenetic derepression using HDAC inhibitors [11,12]. Because cancer cells contain multiple gene-expression alterations, targeting a single abnormality is often inefficient [4]. The simultaneous actions of HDAC inhibitors on multiple genes represent a major advantage for this class of drugs, which perhaps explains their versatility of effect and remarkable potency in different types of cancer cells [10,11]. Vorinostat was the first reagent to be approved by the US FDA for clinical use in 2006 [13,14]. Currently, a variety of established HDAC inhibitors and new compounds are being tested in cell culture and clinical trials of all phases [4,15–17]. While most reagents are pan-HDAC inhibitors with unselective activities, several compounds are able to target a specific HDAC class or isoform. Here, we provide an update in the following areas:

- Epigenetic alterations in gynecologic cancers;
- Mechanisms and actions of HDAC inhibitors in endometrial and ovarian cancer cell lines;
- Laboratory studies on the combined use of HDAC inhibitors and other anticancer reagents.

Anticancer mechanisms of HDAC inhibitors

The demonstration that small-molecule compounds with the capacity to interfere with HDAC activity have antitumor potential has led to the development of a series of structurally different HDAC inhibitors designed to release transcriptional repression of various targets, ultimately promoting cell cycle stability and/or inducing cell apoptosis [11–13,18]. Several actions by HDAC inhibitors have been suggested, including cell cycle arrest, activation of apoptotic pathways, induction of autophagy, reactive oxygen species (ROS) generation, Hsp90 inhibition and disruption of the aggresome pathway [19]. The HDAC inhibitor induces *p21* expression, leading to G1 cell cycle arrest, and frequently downregulates cyclin D and c-Myc [20]. HDAC6 has been shown to be a deacetylase of both tubulin and Hsp9046. Treatment with romidepsin, vorinostat, panobinostat or valproic acid (VPA) resulted in increased histone H3 acetylation or tubulin acetylation, depending on the cell lines [21]. Several *in vitro* and *in vivo* studies have suggested that the generation of ROS is a key event in cell death induced by HDAC inhibitors. ROS generated by HDAC inhibitors lead to DNA damage and the addition of *N*-acetyl cysteine and free radical scavenger has been shown to result in decreased ROS generation and decreased HDAC inhibitor-mediated cell death [22]. HDAC inhibitors have been shown to facilitate death by inducing the expression of TRAIL, DR-4, DR-5, Fas and FasL, as well as a decrease in c-FLIP, a protein associated with resistance to TRAIL-mediated apoptosis [23,24]. Moreover,

the miRNA expression profiles of different cell lines have been shown to rapidly change upon treatment with HDAC inhibitors [25].

Romidepsin, a natural product obtained from the bacteria *Chromobacterium violaceum*, is able to block HDAC activities and induces apoptosis in tumor cells [201]. In 2009, the FDA approved romidespin as an anticancer agent, and clinical trials have been performed in cutaneous T-cell lymphoma, peripheral T-cell lymphoma and a variety of tumors including prostate cancer, multiple myeloma, pancreatic cancer [26], breast cancer [202], ovarian cancer [203], melanoma [204], neuroendocrine tumors [205] and leukemias [206]. The most dramatic results were found in the treatment of cutaneous T-cell lymphoma and other peripheral T-cell lymphomas [26]. Moreover, romidepsin is one of the first HDAC inhibitors found to induce Hsp90 acetylation and cause degradation of the Hsp90 client p roteins EGFR, Her2 and Raf1 [27].

Furthermore, the original model used to explain the antiproliferative effects of these agents suggested that chromatin remodeling resulting from hyperacetylation of core histones altered gene expression [11,12]. It is now clear that HDAC inhibitors induce a wide spectrum of biologic changes resulting from their impact on both transcription and nontranscription levels, as well as their impact on histone and nonhistone proteins (see Figure 1 of [10]). Remarkably, the anticancer effects of HDAC inhibitors were first suggested as a result of their ability to induce differentiation of erythroleukemia cells [10].

To date, several classes of HDAC inhibitors have been proven to have potent and specific anticancer activities in preclinical studies. These data have justified their introduction into clinical trials for the treatment of both hematological malignancies and solid tumors [12,16,28]. Several natural and synthetic small molecular compounds have been employed as bioactive reagents in an attempt to elucidate the mechanism by which HDACs influence cell proliferation [29]. Trichostatin (TSA) is a natural HDAC inhibitor that inhibits almost all class I and II HDACs. It binds to HDACs by its long aliphatic side chain and inhibits enzyme activity by interacting with a Zn motif and other active-site residues. Compounds with HDAC inhibitory effects can be divided into six groups based on their structures:

- Hydroxamic acids (e.g., TSA) and suberoylanilide hydroxamic acids (e.g., SAHA)
- Cyclic tetrapeptides (e.g., trapoxin, apicidin and HC-toxin)
- Depsipeptides (e.g., FK228)
- Short chain fatty acids (e.g., butyrate and VPA)
- Synthetic pyridyl carbamate (e.g., MS-275)
- Synthetic benzamide derivatives (e.g., tacedinaline) and trifluoromethyl ketone

These reagents exhibit varied potencies in terms of upregulating the expression of tumor suppressor genes and blocking cell cycle p rogression [1,10,30].

HDAC inhibitors are small molecules capable of inducing differentiation and preventing cell cycle progression in transformed cells of varying tissue morphology, including acute myeloid leukemia, erythroleukemia and neuroblastoma carcinomas [31]. In recent years, the importance of epigenetics in cancer development has become increasingly appreciated, as is evident by its recent rising popularity in the literature. HDAC inhibitors are currently being investigated in Phase I/II trials for patients with hematologic and solid malignancies, and most data indicate generally tolerable toxicity profiles [2,16]. In regards to endometrial carcinoma (EC), epigenetic defects have been documented for multiple genes, including *hMLH1*, progesterone receptor-B and *PTEN* [32]. Silencing of *hMLH1* and/or *MSH2* by epigenetic mechanisms has been associated with microsatellite instability, invasive growth

and acquired resistance to cisplatin [33,34]. Epigenetic reactivation of *MLH1* gene expression restores normal DNA repair function [31]. Similarly, progesterone receptor-B silencing occurs commonly in high-grade EC, rendering these tumors recalcitrant to progestational therapy. Treatment with epigenetic-modifying reagents results in re-expression of progesterone receptor-B and, potentially, r esensitization of EC to hormonal therapy [35].

The effect of HDAC inhibitors on ovarian carcinoma (OC) has not been examined as extensively as it has in EC. One study indicated that sodium butyrate (NaB) had a significant growth-suppressing effect on human OC cells, irrespective of their *p53* gene status [36]. The authors examined the effects of a wide array of HDAC inhibitors (SAHA, VPA, TSA and NaB) on nine OC cell lines (SK-OV-3, OVCAR-3, TOV-21G, OV-90, TOV-112D, OVCA420, OVCA429, OVCA432 and OVCA433) and found that HDAC inhibitors were able to reduce the non-functional form of the p53 tumor-suppressor protein. The molecular pathways were not investigated. Takai *et al*. observed that HDAC inhibitors exhibit antiproliferative activity and potently induces apoptosis in human OC cells [37]. These events are accompanied by the induction of $p21^{WAF1}$ and $p27^{KIP1}$, and the downregulation of several antiapoptotic and cell cycle-related proteins (Bcl2, cyclin D1 and cyclin D2). VPA also significantly inhibited tumor growth in nude mice without any apparent toxicity. The tumor sections from VPA-treated mice exhibited necrosis and histological changes suggestive of apoptosis, including the formation of apoptotic bodies. Fibrosis accounted for approximately 30% of the tumor area. Moreover, VPA-treated mice showed expression of $p21^{WAF1}$ using immuno-histochemical ana lysis. These findings suggest that HDAC inhibitors may be effective in the treatment of OC.

Expression of HDACs in endometrial & ovarian cancer

HDACs regulate gene transcription through the removal of acetyl groups from histone tails and DNA sequence-specific transcription factors [4,5]. Examples of nonhistone targets include tumor-suppressor protein p53, E2F and Sp3, where HDAC-based deacetylation has been linked to reduced DNA binding and transcriptional activity [38,39]. Through these mechanisms, HDACs are emerging as critical regulators of such pathways as apoptosis, cell growth and differentiation. Indeed, HDAC inhibitors including NaB, TSA, SAHA and VPA, induce cell cycle arrest, differentiation and apoptosis in colon cancer cell lines *in vitro* [40]. Takai *et al*. [37] and Dowdy *et al*. [41] have independently shown that treatment with HDAC inhibitors dramatically increased the number of apoptotic cells in ovarian and EC cell lines, respectively. These observations suggest a physiologic role for transcriptional repression mediated by HDACs in maintaining cell proliferation and survival. Conversely, aberrant changes in the HDAC-mediated transcriptional repression function may be related to t umorigenesis [3,42].

Normal ovarian surface epithelium showed weak nuclear expression of HDAC-1, -2 and -3 proteins [43,44]. The transcriptional co-repressors mSin3, NCoR, NuRD and SMRT are multiprotein complexes that recruit HDACs to specific chromatin domains [45]. HDAC3 is a component of the NCoR–SMRT corepressor complex, which is distinct from corepressor complexes that typically contain HDAC1 and HDAC2 [46]. On the other hand, HDAC2 and HDAC1 appear to co-exist together in multiprotein complexes and many transcription activators target HDAC1 and HDAC2 to specific p romoters to repress the transcription machinery [47].

High levels of class I HDACs were observed in OC in one study, with HDAC-1, -2 and -3 being positive in 61, 93 and 84% of the cases, respectively [44]. HDAC-1 and -2 are primarily found in the nucleus, whereas HDAC3 is found in the nucleus, cytoplasm and cell

membrane [48,49]. Khabele and coauthors also reported an overexpression of all three isoforms of class I HDACs in OC [43]. As in most other entities, class I HDAC expression levels were correlated with each other. Expression levels were significantly different in specific tumor subtypes, with the following values in a decreasing order: mucinous (71%) > serous (64%) > clear cell (54%) > endometrioid (36%). In addition, expression levels were usually higher in highly proliferative tumors [43,44,50]. Class I HDAC protein expression had no statistically significant correlation with patient survival in cases of mucinous, clear cell and serous entities. By contrast, in endometrioid ovarian cancer, stronger class I HDAC expression is associated with shorter patient s urvival (Table 1) [43].

Expression of class I HDAC-1, -2 and -3 proteins in normal endometrium varies with the cell cycle [51]. Weichert and coworkers have observed that the majority of EC showed elevated expression of class I HDAC isoforms in the nuclei of tumor cells, with the following levels of expression in decreasing order: HDAC2 (95%) > HDAC3 (83%) > HDAC1 (61%) [44]. However, a loss of HDAC1 protein expression in EC has also been reported [51]. Similar to OC, clear cell and serous subtypes showed significantly higher expression rates for all three HDACs when compared with endometrioid carcinomas [44]. The authors correlated the expression of HDAC-1, -2 and -3 within individual cells and also between cells of varying proliferative capacity. Strong HDAC1 protein expression was associated with poor prognosis in endometrioid endometrial carcinoma, analogous to its ovarian counterpart [50,51]. Notably, other isoforms that were studied failed to d emonstrate such a relationship between endometrioid s ubtype and poor prognosis [44].

Changes in HDAC expression levels may play a role in the underlying mechanisms involved in cell cycle dysregulation [52–54]. Kawai *et al*. reported that HDAC1 may affect breast cancer progression by promoting cell proliferation through inhibition of ER-α expression [52]. Silencing of HDAC3 expression in colonic cell lines resulted in growth inhibition, decreased cell survival and increased apoptosis. Similar effects were observed for HDAC-1 and -2 [46]. Jin *et al*. reported increased expression of *HDCA1*, *HDCA2* and *HDCA3* mRNA in 83, 67 and 83%, respectively, and overexpression of HDAC-1, -2 and -3 proteins in 94, 72 and 83%, respectively, in ovarian cancer tissue samples, compared with normal tissue samples [55]. The relative densities of *HDAC1* and *HDAC3* mRNA in serous, mucinous and endometrioid cancer tissues and *HDAC2* mRNA in serous cancer subtypes were significantly higher than those found in benign tissues [55]. These findings suggest that class I HDAC-1, -2 and -3 are upregulated in OC and may play a significant role in ovarian carcinogenesis.

The class I HDACs play an important role in steroid hormone-dependent gene expression by directly interacting with proteins recruited to the steroid hormone receptor complex after ligand binding [56,57]. Recently, Hrzenjak *et al*. studied the expression of HDAC-1 and -2 in endometriotic and endometrial cell lines using TaqMan® gene expression assays [58]. The expression of both *HDAC1* and *HDAC2* genes is significantly higher in diseased cells when compared with normal endometrial cells. Steroid hormone treatment induced an upregulation of HDAC-1 and -2 in endometrial stromal cells. Moreover, HDAC1 expression was increased by progesterone, whereas HDAC2 expression was increased by both estrogen and progesterone.

Preclinical studies on HDAC inhibitors in gynecologic cancer cells

Research demonstrating that inappropriate recruitment of HDACs contributes to tumorigenesis has provided a strong mechanistic rationale for applying HDAC inhibitors to cancer therapy regimens (Table 2) [10,16,41]. Acetylation of histones may enhance or inhibit the function of transcription factors, as well as chaperone proteins such as p53,

GATA1, E2F, BCL6, Ku70, Hsp90, RelA, c-Jun and STATs. Therefore, enhancing the degree of acetylation by cell treatment with an HDAC inhibitor can either increase or repress gene expression [59]. It has been found that structurally diverse compounds can bind to and inhibit HDAC catalytic activity. Currently, more than 50 naturally occurring or synthetic HDAC inhibitors have been developed [10,60]. Initial clinical trials indicate that HDAC inhibitors from several different structural classes are well tolerated and exhibit clinical efficacy against a variety of human malignancies [59].

In proliferating endothelial cells, HDAC inhibitors upregulate gene expression of $p21^{WAF1/CIP1}$, which induces cell cycle arrest and downregulates gene expression of survivin, an inhibitor of apoptosis [61]. TSA inhibits the VEGF-induced expression of VEGF receptors VEGFR1, VEGFR2 and Nrp1 (Table 2). Furthermore, TSA and SAHA upregulate the expression of SEMA3, a VEGF protein competitor, at both mRNA and protein levels [62]. Downregulation of endothelial nitric oxide synthase (eNOS) in endothelial cells has also been shown to be critical for the antiangiogenic activity of VPA. Moreover, VPA was also shown to inhibit angiogenesis both *in vitro* and *in vivo* via a mechanism involving diminished expression of eNOS [63]. Depsipeptide was shown to suppress the expression of proangiogenic factors, including VEGF and bFGF [61]. Consistently, TSA and SAHA directly inhibit VEGF family member D (VEGFD) and bFGF-stimulated endothelial cell proliferation, migration, invasion, vascular sprouting and n eovascular f ormation [64].

The HDAC inhibitor NVPLAQ824 blocks expression of proangiogenic tyrosine kinase receptors Tie-2, Tie-2 ligand and Ang2, at both mRNA and protein levels (Table 2). However, HDAC inhibitor NVPLAQ824 exerts no effect on the Tie-1 receptor [61]. Butyrates upregulate endothelial cell adhesion molecules, including ICAM-1 and E-selectin. Downregulation of eNOS in endothelial cells has also been shown to be critical for the anti-angiogenic activity of VPA [63]. A study by Takai *et al*. demonstrated both antiproliferative and proapoptotic activities of HDAC inhibitors in human OC cells [65]. Furthermore, soft agar colony formation assays and 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide MTT assays showed that many endometrial and ovarian cancer cell lines were sensitive to the growth inhibitory effect of HDAC inhibitors, although normal endometrial epithelia were viable after the treatment with the same concentrations of HDAC inhibitors. VPA significantly inhibited human endometrial and ovarian tumor growth without toxic side effects [36,66]. Terao *et al*. indicated that NaB had a significant growth-suppressing effect on human EC and OC cells, irrespective of their p53 status [36].

Takai *et al*. used six EC cell lines to investigate the antiproliferative effects of SAHA, VPA, TSA and NaB [37]. All cancer cell lines were sensitive to the growth inhibitory effect of HDAC inhibitors. Cell cycle ana lysis indicated that treatment with HDAC inhibitors decreased the proportion of cells in S phase and increased the proportion of cells in the G0–G1 and/or G2–M phases of the cell cycle. These effects were accompanied by the altered expression of genes related to malignant phenotypes, including an increase in $p21^{Waf1}$, $p27^{Kip7}$ and E-cadherin, as well as a decrease in Bcl2, cyclin D1 and cyclin D2 (Table 3). Similarly, apicidin, CBHA [66] and scriptaid [67] were also reported to increase the proportion of cells in G0/G1 and/or G2/M phases and to decrease the proportion of cells in S phase. Several studies indicated that HDAC inhibitor-induced apoptosis is associated with the loss of mitochondrial transmembrane potential. Accompanying cell apoptosis is the altered expression of $p21^{WAF1}$, $p27^{KIP1}$, p16, cyclin A and E-cadherin [68]. It was confirmed that apicidin, CBHA and scriptaid treatments indeed increased the acetylation of H3 and H4 histone tails [66–68]. These results suggest that HDAC inhibitors exert antiproliferative effects through selective induction of genes influencing cell growth, malignant phenotype and apoptosis [68]. The observation of a remarkable increase in

acetylated histones associated with the *p21* promoter after suberoyl anilide bishydroxamine treatment has provided evidence for the decreased HDAC activity following HDAC inhibitor administration [31]. Besides cell cycle inhibition and induction of apoptosis, an anti-inflammatory action could be an additional activity contributing to the overall anticancer effects of HDAC inhibitors. It was reported that HDAC inhibitors can suppress the expression of key adhesion molecules, such as VCAM-1, thus leading to a reduction in the number of activated monocytes recruited to inflamed endothelium [69].

In addition to synthetic HDAC inhibitors, natural HDAC inhibitors have also been shown to induce cell cycle arrest in various cancer cell lines. PsA is a phenolic natural product that has been isolated from marine sponges and may be a promising HDAC inhibitor [70,71]. Ahn *et al*. reported that PsA induces cell cycle arrest and apoptosis and increases the proportion of human EC cells in the G1 phase and G2/M phases of the cell cycle (Table 3) [71]. This PsA-induced cell cycle arrest was associated with the downregulation of cyclins/CDKs and pRb, as well as the induction of $p21^{WAF1}$ through a p53-independent pathway. Recently, it was reported that PsA has antibacterial and antitumor properties and inhibits various enzymes including FPT, topoisomerase II, leucine amino chitinase and peptidase [28,70,72]. There is evidence that PsA inhibits both HDACs and DNMTs, although the molecular mechanism for the dual inhibitory effects is not clear [70]. Recently, Berry *et al*. proposed that PsA induces apoptosis in endometrial cancer cells through increased expression of FOXO1 [15], a member of the Forkhead/winged helix family that plays a role in cell survival, cell cycle progression and oxidative-stress resistance [73]. These observations show diversified and dynamic activities of HDAC inhibitors on a broad spectrum of regulatory factors/pathways. Sulforaphane (SFN), a compound found in cruciferous vegetables, inhibits HDAC activity in human colorectal and prostate cancer cells [74]. Myzak *et al*. demonstrated that SFN acted as an HDAC inhibitor in the prostate, causing enhanced histone acetylation, derepression of *p21* and *Bax* and induction of cell cycle arrest/ apoptosis, leading to cancer prevention [75]. The ability of SFN to target aberrant acetylation patterns, in addition to effects on phase 2 enzymes, may make it an effective c hemoprevention agent.

Takai and coworkers discovered two putative tumor-suppressor genes, *Tig1* and CCAAT/enhancer binding protein-α (c/ebp-α), that are stimulated by demethylating agents and/or HDAC inhibitors in endometrial cancers [31]. In additional studies, HDAC inhibitors were found to inhibit the growth of human endometrial cancer cells both *in vitro* and in a mouse xenograft model [31]. Jiang *et al*. examined the effects of HDAC inhibitors oxamflatin and HDAC inhibitor-1 in endometrial cancer cells and observed significant growth inhibition and morphologic changes (Table 3) [76]. Sensitivity to individual agents appears to be cell type specific, with oxamflatin having a stronger growth inhibitory effect than HDAC inhibitor-1 in the Ark2 cell line, while the reverse is true in the AN3 cell line. With respect to the specific apoptotic pathways involved, both caspase-8 and caspase-9 are activated by oxamflatin in the Ark2 cell line. Furthermore, loss of mitochondrial membrane potential occurs upon treatment with the agents. These results suggest that the intrinsic apoptotic pathway may play an important role in the induction of cell death by oxamflatin [76]. Clinically speaking, these findings suggest that HDAC inhibitors could have an important impact on the treatment of the most aggressive subset of EC.

Uchida *et al*. found that HDAC inhibitors, in particular SAHA, enhanced single and collective cell migration, and HDAC inhibitor-induced glycodelin expression played an essential role in promotion of Ishikawa cell migration (Table 3) [77]. Glycodelin is the progesterone-induced glycoprotein secreted into the uterine luminal cavity by secretory/decidualized endometrial glands [78]. A study by Mandelin *et al*. showed that chemotherapy-treated serous OC patients with glycodelin-expressing tumors have longer

survival time than those with glycodelin-negative tumors [79]. Thus, HDAC inhibitor treatment appears to alter some of the malignant phenotypes and improve OC patient survival. However, it is not clear how the glycodelin-induced cell migration is related to the longer survival and the HDAC inhibitor therapeutic effects or if the anticancer effects of HDAC inhibitors may rely on different pathways. It is also noteworthy that not all the diversified effects by HDAC inhibitors are solely therapeutic. Rather, it is highly possible that some effects may be antitherapeutic or even constitute a source of toxicity, as frequently observed in HDAC inhibitors.

Tumor growth and metastasis depend on the development of a neovasculature within and around the tumor [80]. Angiogenesis is regulated by the balance between inhibitory factors, for example, angiostatin, IL-10 and interferon, as well as stimulatory factors, for example, bFGF, IL-8, MMP-2, MMP-9, TGF-β1 and VEGF, that are released by tumor cells and surrounding stroma [81]. HDAC inhibitors modulate angiogenesis in a manner that has potential therapeutic implications. HDAC1 downregulates the expression of p53 and the von Hippel–Lindau tumor suppressor gene and stimulates angiogenesis of human endothelial cells. Correspondingly, HDAC inhibitors inhibit endothelial cell proliferation and angiogenesis by downregulating angiogenesis-related gene expression [63,82]. Phenyl butyrate, LBH589, LAQ824 and TSA are able to exert antiangiogenic activity both *in vitro* and *in vivo* [61,83]. Other HDAC inhibitors, such as SAHA, FK228, VPA and apicidin, have also been shown to possess antiangiogenic activities [63,84,85]. Angiogenesis inhibition, induced by HDAC inhibitors, was associated with modulation of angiogenesis-related genes in both neoplastic cells; for example, inhibition of HIF-1α and VEGF, and benign endothelial cells; for example, inhibition of Tie-2 and survivin. Downregulation of endothelial cell migration and proliferation [85] may also contribute to the angio-suppressive effect. Furthermore, LBH589 was shown to inhibit human umbilical vein endothelial cell Matrigel™ invasion and endothelial tube formation *in vitro* [83].

Studies on combination therapy for gynecologic cancers

Many investigators feel that HDAC inhibitors are more likely to be effective in solid tumors if used in combination with other cytotoxic reagents. Conceivably, a HDAC inhibitor-mediated increase in histone acetylation produces a more open chromatin conformation that facilitates the re-expression of silenced tumor-suppressor genes. This may result in restoration of apoptotic and/or cell cycle control mechanisms, enhancing the chemo-sensitivity of tumor cells [86,87]. For example, SAHA, depsipeptide, MS-275 and TSA enhance the activity of carboplatin, docetaxel gemcitabine, cisplatin, etoposide, doxorubicin and paclitaxel in ovarian as well as endometrial cancer cells (Table 4). HDAC inhibitor-induced apoptosis was greatly enhanced in the presence of the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine, suggesting that DNA methylation status plays an important role in the efficacy of HDAC inhibitors [88]. Interestingly, HDAC inhibitors enhanced paclitaxel-induced cell death in ovarian cancer cell lines, independent of p53 status [89]. While these results indicate clinical value for the combined administration of HDAC inhibitors with other treatment, one precaution for HDAC inhibitor application is that pretreatment of tumor cells with HDAC inhibitors may also cause initial cell damage, resulting in increased side effects.

Uterine papillary serous carcinomas, with their propensity for metastasis and recurrence, present difficult therapeutic challenges for clinicians. Paclitaxel showed limited efficacy for patients suffering from this type of diseases, with a median progression-free interval of only 7.3 months [90]. Improved results come from a combined treatment with carboplatin and paclitaxel. In the adjuvant setting, this treatment increased the median progression-free survival to 30 months [91]. Dowdy *et al*. performed preclinical studies in type II endometrial

cancer cells and found that while treatment of Ark2 and KLE cells with a single reagent, paclitaxel, doxorubicin, carboplatin or the HDAC inhibitor TSA all inhibited cell proliferation, significant synergistic action was detected in the combination of TSA/paclitaxel [41]. Cell death was accompanied by the activation of the cell apoptotic cascade. Dramatic morphological changes were also observed following treatment with TSA/paclitaxel [41]. The synergism most likely resulted from a common pathway involving the inhibition of HDAC activity since similar effects were detected when TSA was substituted by diversified HDAC inhibitors such as HDAC inhibitor-1 and oxamflatin. Indeed, further experiments demonstrated that tubulin acetylation plays a central role for the synergistic actions of the two drugs. While treatment with a single drug led to an increase in acetylated tubulin and microtubule stabilization, combined treatment with TSA/paclitaxel resulted in much enhanced effects. Moreover, in a mouse xenograft model, TSA/paclitaxel treatment resulted in a greater tumor weight reduction than the single agent regimens. Thus, the TSA/paclitaxel combination seems to hold promise for the treatment of EC and other malignancies that are resistant to paclitaxel [41].

Sonnemann *et al*. investigated the sensitivity of ovarian cancer cells to SAHA and paclitaxel in a pilot study employing three established cell lines (OVCAR-3, SK-OV-3 and A2780) as well as cancer cell primary cultures [92]. All five isolates were sensitive to SAHA, whereas four out of five were resistant to paclitaxel. These *ex vivo* findings support the idea that SAHA might be effective in the treatment of paclitaxel-resistant ovarian cancer *in vivo*. Cooper *et al*. examined the effects of the SAHA/paclitaxel combination in ovarian cancer cell line 2774 using *in vitro* culture and mouse xenograft models [93]. They found that while a combination of the two did not generate significantly different results as compared with single agents, paclitaxel followed by SAHA and paclitaxel alone increased survival compared with SAHA alone or SAHA followed by paclitaxel. The authors concluded that adding SAHA to ovarian cancer chemotherapy regimens could increase the drug efficacy and that sequential administration of the drugs is critical to achieving synergism.

PXD101 is a novel hydroxamate-type HDAC inhibitor [94]. A Phase I trial of PXD101 was performed in patients with advanced solid tumors [95]. Phase II study results of PXD101 were reported by Ramalingam *et al*. for the treatment of advanced malignant pleural mesothelioma [96]. In this study, 30 patients with metastatic or recurrent and refractory pleural mesothelioma were enrolled. Two out of the 30 patients had stable diseases. While recruitment was still ongoing, initial results appear to be promising. No reported data regarding PXD101 effects for gynecologic cancer are currently available. It will be of great interests to examine how effective this potent reagent may be when applied to treat gynecologic cancers.

Qian *et al*. examined the anticancer effect of the combined application of PXD101 with docetaxel, paclitaxel and carboplatin [83]. In the A2780 ovarian cancer xenograft model, PXD101 as a single agent showed moderate antitumor activity. In the same system, the combination of PXD101/carboplatin resulted in much increased effects. Interestingly, it was found that PXD101 was able to increase the acetylation of α-tubulin induced by docetaxel as well as the phosphorylation of H2AX induced by carboplatin. The efficacy of PXD101 alone or in combination therapy for the treatment of OC requires c linical evaluation [83].

The nonsteroidal anti-inflammatory drug aspirin has shown promise as an antineoplastic agent. Sonnemann *et al*. found that HDAC inhibitors SAHA, NaB and aspirin cooperated synergistically to induce cell death in the OC cell line A2780 [97]. However, histone acetylation was not affected by aspirin neither in the absence or presence of HDAC inhibitors. The exact molecular pathway responsible for the observed synergism could not be fully explained by the currently available data.

It was thought that the antitumor effects of HDAC inhibitors are mediated by the reactivation of silenced tumor-suppressor genes. However, it is clear that a variety of nonhistone proteins are acetylation targets. The regulation mechanisms and cellular effects of nonhistone acetylation are not fully understood. As described above, one HDAC target is tubulin. Tubulin acetylation modulates microtubule assembly and disassembly and the disturbance of the balanced actions has destructive effects on cellular functions, ultimately leading to cell death. Zhang *et al*. reported that HDAC6 is capable of interacting with purified tubulin and microtubules *in vitro* and HDAC6 colocalizes with microtubules in NIH-3T3 cells [98]. Tubulin acetylation is increased following TSA treatment. Moreover, knockout of the *HDAC6* gene also led to elevated levels of α-tubulin acetylation. Blagosklonny *et al*. demonstrated an immediate acetylation of the Lys40 residue in α-tubulin following TSA treatment, and that this process was also accompanied by the initiation of cell apoptosis [20]. Paclitaxel has also been shown to bind to and stabilize microtubules, eventually precipitating apoptosis [99]. As discussed above, Dowdy *et al*. observed a pronounced synergistic effect of the TSA/paclitaxel combination on α-tubulin acetylation, microtubule stabilization and endometrial cancer cell apoptosis in cell culture and mouse xenograft models [41], thereby suggesting a significant role for nonhistone p roteins in HDAC-mediated cell function.

Conclusion

Accumulated evidence supports the idea that acetylation of histone and nonhistone proteins plays a critical role in a variety of cell functions, including gene expression, microtubule assembly, cell apoptosis and cell cycle regulation. The significant alterations of HDAC expression levels and frequent epigenetic silencing of tumor-suppressor genes in ovarian and endometrial cancers further corroborates the value of HDACs as therapeutic targets for the treatment of gynecologic malignancies. Numerous *in vitro*, *ex vivo* and clinical trials have been performed to investigate the effects, regimen designs, molecular mechanisms, therapeutic values and side effects of HDAC inhibitors as a new class of chemotherapy reagents. Relatively high response rates and low toxicity profiles of available HDAC inhibitors have been observed in several studies. Despite the promising develop ment, there are several issues that need to be addressed before moving forward. A better understanding of the molecular basis behind the antiendometrial and antiovarian cancer activities of HDAC inhibitors is essential. Translational studies are also required to correlate protein acetylation and gene transcription to tumor response and patient survival for more objective data interpretation and improvement of regimen arrangement in terms of dosing, scheduling and formulations. Owing to the need for constant drug exposure to achieve *in vivo* tumor mass reduction, a more detailed study and comparison of the pharmacokinetic profiles for various HDAC inhibitor is needed. Rationally designed combinations of HDAC inhibitors with conventional chemotherapy drugs for treatment of gynecologic cancers are showing encouraging results in tumor cell culture and animal models. However, the efficacy of these combination therapies has yet to be proven in clinical trials. Despite these difficulties, through coordinated efforts in basic, translational and clinical fields, the use of HDAC inhibitors is expected to open up a new avenue in mechanism-based therapy for gynecologic cancers.

Future perspective

The FDA has approved vorinostat (SAHA) and romidepsin (Istodax®) for the treatment of cutaneous lymphoma. These drugs are actively being tested for application in solid tumors. Representing a new class of anticancer reagents, HDAC inhibitors will be more extensively investigated, setting the stage for the discovery and development of novel compounds with improved inhibitory potency or HDAC isoform specificity. In 5–10 years, additional HDAC

inhibitors will be subjected to clinical trials. It is also expected that the combination treatment strategy will allow for a significant decrease in the doses of both drugs without sacrificing efficacy. Future clinical trials may provide evidence for the efficacy of HDAC inhibitors in sensitizing hormone- and drug-resistant gynecologic cancers to radiation and chemotherapy.

While the major cellular effects of HDAC inhibitors, including those affecting cell cycle progression and apoptosis, have been well recognized, the detailed molecular mechanisms remain to be elucidated. Further characterization of tissue-specific control, hormonal regulation and the oncogenic alterations in *HDAC* gene expression will provide insight into how HDAC inhibitors affect cell function. Studies on the regulation of nonhistone protein acetylation will enrich our knowledge on the diverse acetylation targets and pathways. The development of new proteomics and DNA sequencing technologies has opened the door for in-depth analysis of the mechanistic aspects attributable to the observed effects. These studies may lead to a better understanding of the cellular response to HDAC inhibitors and, ultimately, to an i ndividualized regimen for gynecologic cancer patients.

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Executive summary

Expression of histone deacetylases in endometrial & ovarian cancer

- Overexpression of class I histone deacetylases (HDACs) is detected in ovarian carcinoma (OC) and endometrial carcinoma (EC); increased HDAC expression levels correlate with a poor prognosis of OC and EC patients.
- HDACs participate in regulation of the cell cycle and may also play a role in the control of steroid hormone-dependent gene expression.

Preclinical studies on HDAC inhibitors in gynecologic cancer cells

- HDAC inhibitors (HDACi) induce dramatic changes in gene expression. HDACi are potent inhibitors for the proliferation of gynecologic cancer cells.
- HDACi inhibits angiogenesis by downregulating the expression of angiogenesis-related genes.

Studies on combination therapy for gynecologic cancers

- Combination approaches using HDACi and conventional reagents have been tested in cell culture, as well as mouse xenografts. Improved drug effects have been observed in many experiments.
- The HDAC inhibitor/taxol combination has been shown to be very effective for EC and OC cells.

Table 1

Multivariate prognostic impact of histone deacetylase expression in gynecologic entities.

Gynecologic entity	Isoform	HR	95% CI	p-value	End point	Material	Ref.
Endometrial endometrioid carcinoma (n = 123)	HDAC1	2.19	0.71–6.83	0.18	DSS	Protein	[44]
	HDAC2	1.14	0.25–5.10	0.87	DSS	Protein	
	HDAC3	1.53	0.35–6.80	0.58	DSS	Protein	
Ovarian serous carcinoma (n = 176)	HDAC1	0.93	0.59–1.48	0.81	DFS	Protein	[44]
	HDAC2	1.00	0.05–20.00	0.02	DFS	Protein	
	HDAC3	1.07	0.46–2.47	0.09	DFS	Protein	
Ovarian endometrioid carcinoma (n = 114)	HDAC1	6.10	1.69–21.98	<0.01	DSS	Protein	[44]
	HDAC2	1.20	0.14–10.47	0.87	DSS	Protein	
	HDAC3	4.13	0.64–26.85	0.14	DSS	Protein	

Data were calculated on the basis of the cohorts given in the respective references. HRs are given for high isoform expression; to match this, data from references were partially transformed. p-values < 0.05 were considered significant.

DFS: Disease-free survival; DSS: Disease-specific survival; HDAC: Histone deacetylase; HR: Hazard ratio.

Table 2

Histone deacetylase inhibitors modulate gene transcription in endothelial cells to inhibit tumor-driven angiogenesis.

Gene	Function on angiogenesis	Target cell	Direction of modulation	Ref.
Tie2	Promotes	Endothelial	↓	[61]
Ang2	Promotes	Endothelial	↓	[61]
Nrp1	Promotes	Endothelial	↓	[62]
SEMA3	Suppresses	Endothelial	↑	[62]
VEGFR1	Promotes	Endothelial	↓	[62]
VEGFR2	Promotes	Endothelial	↓	[62]
eNOS	Promotes	Endothelial	↓	[63]
VEGFD P	Promotes	Endothelial	↓	[64]

Table 3

Published studies on histone deacetylase inhibitors in gynecologic cancers.

HDACi	Class	Entity	Function	Ref.
TSA	Hydroxamic	Endometrial cancer	Inhibit VEGF receptors VEGFR1, VEGFR2 and Nrp1 Induce glycodelin expression	[31,100]
Valproic acid/ valproate	Short-chain fatty acids	Endometrial cancer	Downregulation of eNOS in endothelial cells Inhibit G1/S transition and influence expression of $p21^{WAF1}$ and cyclin D1	[31,63]
SAHA (vorinostat, zolinza)	Hydroxamic	Endometrial and ovarian cancer	Decrease the proportion of cells in S phase and increase the proportion of cells in the G0/G1 and/or G2/M phases Induce glycodelin expression	[77]
		Ovarian cancer	Had minimal activity in a Phase II study Induce apoptosis	[93,101]
R306465	Hydroxamic	Ovarian cancer	Induce apoptosis and inhibit angiogenesis	[102]
Butyrates	Short-chain fatty acids	Endometrial and ovarian cancer	Regulate ICAM-1 and E-selectin	[102]
PsA	A phenolic natural product isolated from marine sponges	Endometrial and ovarian cancer	Induce the expression of acetylated H3 and H4 histone proteins Upregulate the expression of cyclin-dependent kinase inhibitor, p21WAF1 and downregulate the expression of pRb, cyclins and cyclin-dependent kinases	[28,71]
PsA	Marine sponge compound	Endometrial cancer	Induce apoptosis in endometrial carcinoma cells	[15]
NVP-LAQ824 (dacinostat)	Hydroxamic	Endometrial cancer	Block tyrosine kinase receptors Tie2 and Tie2 ligand, Ang2, mRNA and protein expression	[61]
Butyrate	Short-chain fatty acids	Endometrial and ovarian cancers	Induce p53 expression	[36,37]
Oxamflatin	Hydroxamic	Endometrial cancer I and II	Loss of mitochondrial membrane potentials consistent with the induction of apoptosis	[76]
HDACi-1, HDAC-I1		Endometrial cancer I and II	Induction of apoptosis	[76]
CI-958	Benzamide	Ovarian cancer	Potently inhibit DNA and RNA synthesis	[103]
PXD101(Belinostat)	Hydroxamic	Epithelial ovarian cancer and micropapillary/ borderline ovarian tumors	Platinum-resistant epithelial ovarian cancer and micropapillary/borderline Histone H4 hyperacetylation	[104,105]
Scriptaid	Hydroxamic	Endometrial and ovarian cancers	Induce apoptosis and increase acetylation of histone tails Decrease the proportion of cells in the S phase and increase the proportion in the G0/G1 and/or G2/M phases	[67]
FK228 (romidepsin/ depsipeptide)		Endometrial cancer	Modulation of angiogenesis-related genes Inhibition of endothelial cell migration and proliferation	[63]

HDACi	Class	Entity	Function	Ref.
Apicidin	Tetrapeptides	Endometrial and ovarian cancers	Increase acetylation of H3 and H4 histone tails Induce apoptosis	[68]
Biphenyl-4-yl-acrylohydroxamic acid derivatives	Hydroxamic	Ovarian cancer	Induce acetylation of p53 and tubulin	[106]
CBHA	Hydroxamic	Endometrial and ovarian cancers	Increase acetylation of histone proteins and induce apoptosis	[66]
LBH589 (panobinostat)	Hydroxamic	Endometrial cancer	Endothelial tube formation and Matrigel™ invasion	[83]
M344	Hydroxamic	Endometrial and ovarian cancers	Acetylation of histones, induction of apoptosis and increase the proportion of cells in the G0/G1 and/or G2/M phases	[107]
Valproate	Short-chain fatty acids	Endometrial cancer	Inhibit G1/S transition and influence expression of p21WAF1 and cyclin D1	[58]

eNOS: Endothelial nitric oxide synthase; HDACi: Histone deacetylase inhibitor; SAHA: Suberoylanilide hydroxamic acid; TSA: Trichostatin.

Table 4

Combination of histone deacetylase inhibitors with other antitumor agents in gynecologic cancers.

HDACi	Class	Other agents	Disease	Function	Ref.
TSA	Hydroxamic	5-aza-2′-deoxycytidine	Endometrial cancer	Inhibit VEGF receptors VEGFR1, VEGFR2 and Nrp1-1.85 Downregulate DNMT3B mRNA and protein expression	[31,62,108]
PXD101 (belinostat)	Hydroxamic	Carboplatin Paclitaxel	Ovarian, epithelial and fallopian tube cancers	Histone H4 hyperacetylation	[12,104]
TSA, NaB		Paclitaxel	Ovarian cancer	Induction of p53 protein	[89]
TSA	Hydroxamic	Paclitaxel	Endometrial cancer	Induction of p21 Increase acetylation of tubulin and microtubule stabilization	[41]
SAHA (vorinostat), NaB	Hydroxamic, short-chain fatty acids	Aspirin	Ovarian cancer	Induction of apoptosis	[97]
PXD101 (belinostat)	Hydroxamic	Docetaxel Paclitaxel Carboplatin	Ovarian cancer	Enhance carboplatin anticancer effect in xenograft Increase the phosphorylation of H2AX induced by carboplatin Enhance induction of histone H4 hyperacetylation	[83]
SAHA	Hydroxamic	Paclitaxel	Ovarian cancer	Slightly induce apoptosis	[103

HDACi: Histone deacetylase inhibitor; SAHA: Suberoylanilide hydroxamic acid; TSA: Trichostatin.

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