Carcinogenesis is a complex interplay of genetic and epigenetic aberrations which lead to altered gene expression. Efforts to reverse these changes, in order to prevent and treat cancer, have been ongoing for several decades. Histone acetylation is one of the key epigenetic mechanisms involved in altering configuration of the chromatin structure, and is modulated by the opposing activities of histone acetyl-transferases and histone deacetylases. Histones whose lysine residues are heavily acetylated yield a more open chromatin structure, due to the repulsion of the negatively charged DNA strand by negatively charged acetylated lysine residues leading to transcriptional activation. Alternatively, histone deacetylation leads to gene repression, due to transcriptionally silent heterochromatin. Aberrant activity of histone acetyl-transferases and histone deacetylases leading to repression and activation of key genes has been documented in several cancers, including breast cancer. Hence, these enzymes are rational targets in cancer therapy to modulate gene expression, in particular genes involved in proliferation and differentiation. Novel histone deacetylase inhibitors have been developed, and preclinical and clinical data demonstrate their role in treatment and prevention of breast cancer. We present here the rationale for targeting histone deacetylases in breast cancer, and the preclinical and clinical data that support further development of these agents.

**Keywords:** breast cancer • endocrine resistance • epigenetics • histone deacetylase • histone deacetylase inhibitor

Epigenetics is a term used to describe nonsequence-specific gene regulation, which occurs via remodeling of chromatin following alteration of the histone code. There are two main known epigenetic mechanisms, DNA methylation and histone modification, which result in altered gene transcription. Histones are proteins that are integral to chromosome structure: the histone core, made up of eight histone protein subunits, is the unit around which the DNA strand is wound. When acetylated by enzymes called histone acetyl-transferases (HATs), histones become negatively charged, and repel the negatively charged nucleic acids. This repulsion results in a loosening of the chromosome coil, allowing for easier access to the DNA strand by transcription factors that mediate gene transcription. The level of histone acetylation is also modulated by histone deacetylases (HDACs), which, as the name suggests, mediate the deacetylation of histones. Deacetylation results in a tightening of chromatin structure, resulting in decreased gene transcription. This method of epigenetic regulation plays a pivotal role in tumor biology, as tumor cells tend to alter expression of selective genes to facilitate survival. Although histones are the primary targets for HDACs, more than 50 non-histone proteins that regulate key cellular functions, such as estrogen receptor-α, Hsp90 and others, have been identified as substrates for one of the HDACs [1,2]. Another mechanism by which HDACs regulate transcription is by catalyzing the deacetylation...
of sequence-specific DNA-binding transcription factors. The acetylation and deacetylation of sequence-specific transcription factors can either increase or decrease their DNA-binding activity and subsequently affect transcription (Figure 1) [1]. In this review, we briefly discuss the biology of the HDAC enzymes, various HDAC inhibitors (HDACi) in clinical development, and focus primarily on the therapeutic implications of the use of HDACi either as monotherapy, or in combination with cytotoxic chemotherapy or targeted therapies, in the treatment and prevention of breast cancer.

The emergence of HDACi as a novel class of anticancer agents is very exciting and promising. There are 18 HDAC isoenzymes and these are grouped by their

Figure 1. Impact of histone acetylation on gene transcription.
HAT: Histone acetyl-transferase; HDAC: Histone deacetylase.
homology to yeast proteins. The various HDACi down-regulate each of these enzymes to varying degrees \[2,3\]. HDACi are categorized based on their chemical structure and include short-chain fatty acids (sodium phenyl butyrate, sodium butyrate, valproic acid (VPA), OSU HDAC 42), hydroxamic acids (trichostatin A, vorinostat, panobinostat, belinostat), cyclic peptides (romidepsin) and benzamides (entinostat, MGCD-0103) \[4\]. The common mechanism of action of these drugs is binding to the critical zinc ion required for the catalytic function of HDAC enzymes \[5\]. This results in accumulation of acetylated histone and nonhistone proteins that are involved in gene transcription, cell proliferation, invasion and survival. Although all these compounds are capable of inhibiting histone deacetylation, they have widely varying potential, and HDAC isoenzyme specificity, as well as varying effects on acetylation of nonhistone substrates. Consequently, their therapeutic potential and toxicities may be different.

Vorinostat has been studied extensively in clinical trials and the most common side effects noted include fatigue, gastrointestinal symptoms, hyperglycemia, hypokalemia, anemia and thrombocytopenia \[6\]. The most frequent toxicities common to most HDACi include fatigue, nausea and diarrhea. Myelosuppression is relatively mild. Thrombocytopenia is common and is dose-limiting. One of the more serious adverse effects of certain HDACi noted in clinical studies is QT prolongation. Panobinostat and belinostat, which share the hydroxamate chemistry, have a dose-dependent QT interval increase. Depsipeptide, a cyclic peptide, was not developed further clinically because it induced significant QT prolongation, with reports of sudden cardiac deaths \[7,8\]. Valproic acid, which has been used more widely as an anti-epileptic agent, is associated with somnolence and thrombocytopenia. The various HDACi and their associated targets are listed in Table 1 \[9,10\]. Currently the most extensively studied HDACi clinically is vorinostat which is a pan-HDACi, and the only HDACi approved by the FDA for the treatment of relapsed, refractory cutaneous T-cell lymphoma.

**Table 1. Histone deacetylase inhibitors and their targets.**

<table>
<thead>
<tr>
<th>Class</th>
<th>Agent</th>
<th>Disease application</th>
<th>Molecular target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxamates</td>
<td>TSA</td>
<td>None, due to toxicity profile</td>
<td>HDACs 1, 2, 3, 4, 6, 7, 9 and telomerase</td>
</tr>
<tr>
<td></td>
<td>Vorinostat (TSA analog)</td>
<td>T-cell lymphoma, advanced solid tumors</td>
<td>HDACs 1, 2, 3, 4, 6, 7, 9 and nonhistone transcription factors</td>
</tr>
<tr>
<td></td>
<td>PCI-24781</td>
<td>Refractory advanced solid tumors</td>
<td>Broad-spectrum HDAC inhibition, p21 expression, induction of apoptosis via caspase-8 and FADD and production of reactive oxygen species</td>
</tr>
<tr>
<td></td>
<td>Panobinostat LBH-589</td>
<td>Acute leukemia’s and MDS</td>
<td>HDACs 1, 2, 3, 4, 7, 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H3 and H4 acetylation, p21 expression, inhibition of hsp90 chaperoning, halting of cell cycle at G1 phase in vitro</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H3 and H2B acetylation in B cells and blasts in vivo</td>
</tr>
<tr>
<td></td>
<td>Gavinstonat ITF-2357</td>
<td>Hodgkin’s lymphoma</td>
<td>Pan-HDAC inhibition</td>
</tr>
<tr>
<td></td>
<td>MGCD0103</td>
<td>Advanced solid tumors, leukemias and MDS</td>
<td>Pan-HDAC inhibition and H3 acetylation</td>
</tr>
<tr>
<td></td>
<td>Belinostat</td>
<td>Hematological and solid tumor</td>
<td>Pan-HDAC inhibition</td>
</tr>
<tr>
<td>Aliphatic acids</td>
<td>Valproic acid VPA</td>
<td>Cutaneous T-cell lymphoma, refractory solid tumors</td>
<td>Pan-HDAC inhibition, tubulin acetylation, decreased Akt activity and induction of apoptosis via caspase-3</td>
</tr>
<tr>
<td>Benzamides</td>
<td>MS-275 (SNDX-275)</td>
<td>Advanced solid tumors and lymphoma, acute leukemia</td>
<td>Preferential inhibition of HDACs 1, 2, 3 and 9 H3 and H4 acetylation in PBMCs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H3 and H4 acetylation, p21 expression and caspase-3 activation in bone marrow mononuclear cells</td>
</tr>
<tr>
<td>Cyclic peptides</td>
<td>Depsipeptide (FR901228)</td>
<td>Advanced solid tumors, CLL and AML</td>
<td>Pan-HDAC inhibition (little clinical effect as monotherapy)</td>
</tr>
</tbody>
</table>

FADD: Fas-associated death domain; HDAC: Histone deacetylase; MDS: Myelodysplastic syndrome.
Reversing endocrine resistance
Approximately two-thirds of breast cancers express hormone receptors, and targeting these receptors to achieve tumor response forms a mainstay of the management of these tumors. Resistance to endocrine therapy develops in approximately one-third of patients receiving these agents, and is a major challenge in the management of breast cancer. The role of HDACi is not only being explored as a strategy to improve response to estrogen therapy, but also as a possible means to induce re-expression of estrogen receptor-α (ESR 1), in ER-negative tumors. The two main classes of anti-estrogen drugs are selective estrogen receptor modulators and aromatase inhibitors (AIs). A number of possible mechanisms for endocrine resistance have been described, which include activation of growth factor-receptor pathways, overexpression of ER co-activators, ER gene silencing and metabolic resistance due to polymorphisms in metabolizing enzymes [11]. While many of these changes are caused by genetic alterations, there is also increasing evidence to implicate epigenetic regulatory mechanisms in the development of endocrine resistance, especially in the form of gene silencing. Since epigenetic modifications are easier to reverse than genetic mutations, they are appealing therapeutic targets [12].

The loss of ER-α expression is partly attributed to epigenetic alteration as a result of hypermethylation of the CpG islands within the ESR1 promoter. The epigenetic regulation of ESR1 is mediated through the recruitment of protein complexes containing HDAC1 and DNMT1 that are recruited to the promoter region along with N-CoR and SMRT [13,14]. Treatment of ER-negative cell lines with methyltransferase inhibitor, 5-aza-2′-deoxycytidine (Aza-C) led to partial demethylation of the ER CpG island, re-expression of ER mRNA, and synthesis of a functional ER protein. HDACs also interact with ER-α and suppress ER transcriptional activity. Treatment of ER-negative breast cancer cells with the HDACi, TSA, induced re-expression of ER-α mRNA and protein [15]. Co-treatment with DNMTi and HDACi such as TSA or scriptaid is an alternative strategy to synergistically induce ER gene expression in ER-negative cell lines. The combination of treatment with the DNMTi and HDACi functions by reducing DNMT1 expression and activity, yielding partial demethylation of the ER promoter CpG island, and increasing acetylation of histones H3 and H4 [15,16]. This provides the rationale for exploring such strategies in the clinical setting.

In addition, the expression of progesterone receptor (PR) may be epigenetically regulated in breast cancers, as the PR gene is hypermethylated in 40% of tumors that are PR negative [17]. Combined treatment of PR-negative tumors with DNMT and HDACi result in re-expression of PR mRNA [15,17]. Re-expression of PR is also seen with HDAC-mediated modulation of ER in ER-negative cell lines, demonstrating the functional activation of ER. Although this PR re-expression may not directly predict response to antihormone therapy, PR may play a role in HDACi-induced sensitization of breast cancer cells to hormone therapy [18].

The understanding of epigenetic regulation of hormone receptors led to treatment of ER-negative cell lines with a combination of HDAC and DNMT inhibitors, demonstrating the ability to restore sensitivity to tamoxifen, the most widely used selective estrogen receptor modulator [14,19]. In addition, a xenograft study comparing the combination of the HDACi entinostat and letrozole resulted in better inhibition of ER-negative tumor growth in vivo, compared with treatment with letrozole alone [20]. Inhibition of migration, as well as a significant reduction in the number of both visible and micrometastases was observed in the combination group. The same group showed that treatment with entinostat led to upregulation of ER-α, aromatase expression and aromatase activity in a dose-dependent manner in cell lines and xenografts. This provided the proof of concept that upregulation of ER-α and aromatase results in sensitization of tumors to hormonal therapy with letrozole, yielding significant inhibition of growth, cell migration and formation of micrometastases.

Most recently, Chen et al. demonstrated that LBH589, a pan-HDACi that inhibits HDAC 6, downregulates aromatase expression by selectively suppressing its promoter, 1.3/II, which is primarily responsible for driving the expression of aromatase in breast cancer tissue [21]. Furthermore, in cell co-culture models, the combination of LBH589 and letrozole was superior to letrozole alone in suppressing proliferation of hormone-responsive breast cancer cells.

In summary, treatment with selective and non-selective HDACi potentiates the effects of tamoxifen in ER-positive cell lines, and potentially reverses endocrine resistance in ER-negative cell lines by re-establishing ER expression [14,15,22]. Preclinical data also demonstrates that combining HDACi with AIs improves outcomes in ER positive and negative breast cancers [20,21]. This has led to several clinical trials combining HDACi with antiestrogen therapy, which will be discussed in later sections.

Enhancing response to chemotherapy & trastuzumab
Many HDACi, including TSA, vorinostat, panobinostat and LAQ 824 (dacinostat), have been shown to work synergistically with several chemotherapeutic
agents in cell lines. One of the mechanisms for HDACi resulting in improved sensitivity to cytotoxic agents include a more open chromatin, allowing for increased binding of Topo II inhibitors to DNA substrates, and inhibition of nonhistone proteins such as α-tubulin, resulting in increased sensitivity of tumor cells to taxanes [23]. Furthermore, acetylation of Hsp 90 inhibits its chaperoning ability, and promotes depletion of Her2-neu, Akt and/or c-Raf-1, leading to increased sensitivity to gemcitabine, taxanes, epithelones and trastuzumab [24]. One of the mechanisms of resistance to trastuzumab that has been identified includes Her2-independent increased activity of AKT [25]. This increase was seen as a result of the loss of PTEN or increased levels and activity of insulin-like growth factors. Since treatment with HDACi LAQ 824 attenuates the levels and activity of AKT and c-Raf-1, it is possible that resistance to trastuzumab based on Her2-independent increased activity of AKT may be overcome by combination treatment with an HDACi and trastuzumab [24].

LAQ 824-mediated decline in the mRNA levels of Her2 neu was associated with the depletion of intracellular Her2 neu protein. LAQ 824 was also shown to deplete the cell membrane associated, tyrosine phosphorylated levels of Her2 protein. This led to a decline in the dimerization of Her2–Her3. Her2–Her3 dimers have been recognized as the most potent Her2 signaling complex leading to activation of PI3K/AKT and Ras-Raf-Erk1/2, which is well known to be involved in cell proliferation, survival and chemoresistance of breast cancer cells [26,27]. This may prove to be of significant clinical value since the PI3K/AKT pathway, as discussed above, has been found to be involved in conferring resistance to trastuzumab [1,23,24,28].

Enhancing response to radiation therapy

HDACi enhance the sensitivity of the cancer cells to the effects of ionizing radiation and are under clinical investigation as radiosensitizers in cancer therapy [29]. Several potential mechanisms for this property of HDACi have been discussed in the literature. HDACi can modulate the effects of ionizing radiation by altering gene expression leading to cell-cycle arrest in the G1-phase, and via inhibition of DNA synthesis in the S-phase, resulting in induction of cell death. Alternatively, at a lower dose, HDACi could simply affect the expression of genes involved in DNA damage response, such as BRCA1, MGMT and hMLH1 [30–35]. The effect of vorinostat on tumor cell radiosensitivity was investigated in a breast cancer brain metastasis model using MDA-MB-231 BR cells. Cells exposed to vorinostat for 16 h before, and maintained in the medium after irradiation, had an increase in radiosensitivity (as measured by clonogenic assay), with a dose enhancement factor of 1.57 [29,36]. DNA damage was increased, as measured by γ H2AX foci per cell. Irradiation of subcutaneous MDA-MB-231-BR tumors in mice treated with vorinostat resulted in an increase in radiation-induced tumor growth delay. Furthermore, animals with intracranial tumor implants lived the longest after combination treatment. These results indicate that vorinostat enhances tumor cell radiosensitivity in vitro and in vivo [36].

Clinical trials of HDACi in breast cancer

Clinical trials with HDACi as a single agent and in combination with other conventional therapies are currently ongoing in various subtypes and stages of breast cancer. We present below the results of the published and ongoing studies that illustrate the role of HDACi therapy in breast cancer (Table 2).

Single agent

Phase I studies of intravenous and oral vorinostat in advanced solid tumors have demonstrated accumulation of acetylated histones both in tumor biopsies and in peripheral blood mononuclear cells [6]. Vorinostat was well tolerated with minimal toxicities. A total of 73 patients were treated on the Phase I study with oral vorinostat, of which two had breast cancer. The maximum tolerated dose was 400 mg once a day and 200 mg twice a day (b.i.d.) on a continuous daily dosing and 300 mg b.i.d. for 3 consecutive days per week schedule. The studies also demonstrated consistent accumulation of acetylated histones in peripheral blood mononuclear cells post-therapy, and ELISAs demonstrated a trend towards dose-dependent accumulation of acetylated histones in the dosing range of oral SAHA 200–600 mg. Preliminary activity was seen with one complete response, three partial responses and 30% of patients remaining on therapy for 4 to >37 months. This led to the Phase II trial of vorinostat in patients with metastatic breast cancer [37]. A total of 14 patients received vorinostat 200 mg b.i.d. orally for 14 days of a 21 day cycle. Response was evaluated using RECIST criteria. The median age of the patients was 60.5 years (range 37–88 years). Patients had received a median of 1.5 prior (range 0–2) chemotherapeutic regimens for metastatic disease. The median number of cycles delivered was two (range 1–20). No complete or partial responses were noted. The study was terminated after the first stage because of the lack of objective response. However, four patients had stable disease with time to progression ranging from 4 to 14 months. The most frequently noted clinically significant adverse events were fatigue, nausea, diarrhea and lymphopenia.
<table>
<thead>
<tr>
<th>Trial</th>
<th>Setting</th>
<th>n</th>
<th>Regimen</th>
<th>Primary end point(s)</th>
<th>Common AEs</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broccoli sprout extract in DCIS or atypical ductal hyperplasia (NCT00843167)</td>
<td>Phil I Precancerous</td>
<td>66</td>
<td>Broccoli extract t.i.d. every 2–8 weeks</td>
<td>Biomarkers and prognostic value</td>
<td>NA</td>
<td>Ongoing</td>
<td>[28]</td>
</tr>
<tr>
<td>SAHA + trastuzumab, for patients who failed trastuzumab</td>
<td>Phil I/II metastatic/locally advanced</td>
<td></td>
<td>SAHA 200 mg b.i.d. + trastuzumab 6 mg/kg, every 21 days</td>
<td>Objective response</td>
<td>Thrombocytopenia, neutropenia, nausea, vomiting, diarrhea</td>
<td>Her2+ (n = 11): no objective response Her2- (n = 5): 20% response</td>
<td>[39]</td>
</tr>
<tr>
<td>SAHA + lapatinib, for patients who failed trastuzumab (NCT01118975)</td>
<td>Phil II metastatic/locally advanced</td>
<td>47</td>
<td>SAHA 300 or 400 mg, 4 days on, 3 days off + lapatinib 1250 mg/day</td>
<td>Response, EMT biomarkers + breast cancer stem cells</td>
<td>Thrombocytopenia, neutropenia, nausea, vomiting, diarrhea, hand–foot syndrome</td>
<td>Ongoing</td>
<td>[28]</td>
</tr>
<tr>
<td>Panobinostat + trastuzumab, for patients who failed trastuzumab (NCT00567879)</td>
<td>Metastatic</td>
<td>33</td>
<td>LBH: iv. days 1 and 8, every 3 weeks, at 10, 15 and 20 mg/m² or p.o. TIW at 15, 20, 30 and 40 mg + trastuzumab 2 mg/kg weekly</td>
<td>Response, EMT biomarkers, breast cancer stem cells</td>
<td>Thrombocytopenia, neutropenia, nausea, vomiting, diarrhea</td>
<td>32% SD</td>
<td>[41]</td>
</tr>
<tr>
<td>Panobinostat (LBH589) + trastuzumab, + paclitaxel, for patients who failed trastuzumab (NCT007888931)</td>
<td>Phil I/II metastatic</td>
<td>52</td>
<td>LBH: iv. or p.o. – MTD to be determined + trastuzumab every 21 days + paclitaxel every 21 days</td>
<td>MTD for iv. and oral panobinostat Response</td>
<td>Thrombocytopenia, neutropenia, nausea, vomiting, diarrhea</td>
<td>Ongoing</td>
<td>[28]</td>
</tr>
<tr>
<td>Panobinostat + capecitabine + lapatinib (NCT00632489)</td>
<td>Metastatic/locally recurrent</td>
<td>15</td>
<td>LBH: 20, 30, 45 and 60 mg, BiW + Cap: 825 mg/m² (n = 4) or 1000 mg/m² (n = 11) for 14/21 days</td>
<td>MTD and DLT of LBH + Cap combination Response</td>
<td>Thrombocytopenia, anemia, hand-foot syndrome, dehydration, fatigue, peripheral edema</td>
<td>Ongoing; 27% SD LBH MTD = 20 mg</td>
<td>[40]</td>
</tr>
<tr>
<td>SAHA + tamoxifen, for patients who failed prior endocrine (NCT00365599)</td>
<td>Metastatic/locally advanced</td>
<td>42</td>
<td>SAHA 400 mg, days 1–21, every 28 days Tamoxifen 20 mg/day</td>
<td>Response TTP, OS Tolerability</td>
<td>Thrombocytopenia, neutropenia, nausea, vomiting, diarrhea, arthralgia, hot flashes</td>
<td>Ongoing</td>
<td>[28]</td>
</tr>
<tr>
<td>SAHA + radiation therapy for brain metastases</td>
<td>Phil I metastatic</td>
<td>24</td>
<td>Dose escalation: SAHA 100–400 mg/day</td>
<td>Safety and efficacy</td>
<td>Nausea, vomiting, diarrhea, fatigue</td>
<td>Ongoing</td>
<td>[28]</td>
</tr>
</tbody>
</table>

AE: Adverse event; b.i.d.: Twice daily; BiW: Twice weekly; Cap: Capecitabine; DCIS: Ductal carcinoma in situ; DLT: Dose-limiting toxicity; EMT: Epithelial to mesenchymal transition; iv.: Intravenously; LBH: LBH589 (panobinostat); MTD: Maximum tolerated dose; Ph: Phase; p.o.: Per orem; SAHA: Suberoylanilide hydroxamic acid (vorinostat); t.i.d.: Three times daily; TIW: Three times weekly.
In summary, these single-agent studies demonstrate that vorinostat is well tolerated and results in acetylation of histones as measured in PBMCs, but has minimal single-agent activity in breast cancer.

**Biomarker studies**

Biomarker studies with biopsies before and after short-term administration of vorinostat prior to surgery are currently ongoing in ductal carcinoma *in situ* and invasive cancers (NCT00788112 and NCT00262834). Stearns *et al.*, presented the data from the biomarker study in invasive cancer, where newly diagnosed breast cancer patients received six doses of vorinostat (300 mg b.i.d. × six doses), with the last dose being administered 2 h prior to surgery or biopsy. Pre- and post-vorinostat tumor specimens were analyzed for gene methylation and expression by RT-PCR using the Oncotype Dx 21 gene assay and IHC for Ki-67 and cleaved caspase-3 [38]. Tissues were also obtained from untreated controls. Among 25 evaluable matched sample sets for both IHC and RT-PCR, 76% had ER-positive tumors by both assays (100% concordance). In the vorinostat group, a statistically significant decrease in Ki-67 and STK 15 by RT-PCR, but not in Ki-67 by IHC was reported. No significant changes were observed for ER expression or in gene methylation. Contrary to the expected outcome, no change in the level of ER expression was noted, but the study did demonstrate that short-term vorinostat is associated with a decrease in the expression of proliferation-associated genes. This was perhaps the result of direct effect of vorinostat as opposed to any modulation of ER signaling.

Another pilot study to determine the molecular effects of VPA, administered for approximately 2 weeks before definitive surgery is currently ongoing, and may shed some further light on the HDAC activity of VPA and the resultant impact on the proliferative activity in the tumor (NCT01007695: Molecular signature of VPA in breast cancer with functional imaging assessment – pilot (VAST)) [101].

A Phase 0, nonrandomized, single-arm, proof-of-principle trial of oral panobinostat with a biological end point is currently ongoing. Eligible patients who had failed prior tamoxifen and chemotherapy will be given five 30 mg doses of panobinostat over a period of 2 weeks. Tumor biopsies obtained before and after therapy will be evaluated for the primary end points, with immunohistochemical staining for ERB-B4, induction of which has been shown to be favorable. Secondary end points include the evaluation of apoptosis with staining for DNA breaks by TUNEL assay (NCT00993642) [101].

In summary, these biomarker studies, when completed, will help us better understand the molecular changes in breast tumors exposed to HDACi, and will help in rationally designing correlative studies for future clinical trials.

**Combination studies**

With chemotherapy

A Phase I trial of vorinostat and doxorubicin in solid tumors was recently conducted by Munster *et al.* at the University of California (CA, USA) [39]. The primary goal was to assess the Phase II recommended dose, toxicity profile and tolerability of vorinostat in combination with weekly doxorubicin. A total of 32 patients were enrolled and received vorinostat at 400, 600, 800 or 1000 mg/day on days 1–3 followed by doxorubicin on day 3, for 3 of 4 weeks. The maximum tolerated dose was 800 mg/day. Of the enrolled patients in this study, five had advanced breast cancer, of which one patient had a partial response as measured by RECIST criteria. The response ceased when the doxorubicin was stopped. Two patients with malignant melanoma had partial responses. The investigators also showed that the histone acetylation in the peripheral blood and tumor was comparable, and that HDAC2 expression in the primary tumor was predictive of histone acetylation. Furthermore, the investigators demonstrated reduced expression of the chromatin remodeling proteins HP-1 and Topo II-α by immunofluorescence in eight out of 12 patients. Several patients withdrew consent after one cycle because of toxicities, mainly fatigue. Another frequent toxicity seen in this study was thromboembolic events (four out of 32 patients). Although the combination did not result in high response rates, the study demonstrated that higher doses of vorinostat can be administered with weekly doxorubicin, but resulted in more toxicity. This was also one of the studies that showed expression of particular HDAC isoenzymes may predict response to HDAC inhibition.

Preliminary data from a Phase I and II study with vorinostat in combination with paclitaxel 90 mg/m² on days 1, 8 and 15 and bevacizumab 10 mg/m² on days 1, 8 and 15 and vorinostat (dose level 1: 200 mg and dose level 2: 300 mg) as first-line therapy in patients with recurrent chest wall or metastatic breast cancer was presented in 2009 [40]. No prior chemotherapy for advanced disease was allowed on the study. The recommended vorinostat dose from the Phase I study was 300 mg b.i.d. and sufficient responses were seen in the first stage to proceed with accrual to the second stage. A total of 50 patients were enrolled on the study with a median age of 55.5 years and with 36 patients (72%) having ER-positive breast cancer. Among the 34 evaluable patients, 31 were evaluable for response. Median number of cycles was 8.5 (range 2–18). Of the three patients in the first dose level, two had partial
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response, and 15 out of 28 patients, overall. Five out of 34 patients had prolonged stable disease. The toxicity profile was comparable to that of paclitaxel and bevacizumab alone. Grade 3/4 toxicities were primarily neutropenia (21%), thrombosis in three patients (9%), hypertension in one patient (3%), cardiac ischemia (3%), sensory neuropathy in seven patients (21%), fatigue in six patients (18%), dyspnea in four patients (12%) and arthralgia in two patients (6%). The effect of vorinostat on HSP 90 and AKT in vivo was studied by obtaining sequential tumor biopsies and peripheral blood mononuclear cells, collected before and 4 h after the third vorinostat dose in two patients. Western blot analysis demonstrated increased acetylation of the K69 lysine residue of HSP 90, upregulation of HSP 70 (indicating HSP 90 inhibition) and downregulation of AKT after vorinostat administration, providing evidence of HSP 90 inhibition in tumor and PBMCs. Considering the preliminary activity seen with this regimen and recognizing the need to confirm the molecular impact of vorinostat with more tissue biopsies, the NCI allowed for expansion of this study to include eight more patients with mandatory biopsy before and after vorinostat.

This study is now closed to accrual and final analyses of the results are awaited. From the preliminary results, this study demonstrated that the combination of vorinostat with paclitaxel and antiangiogenic therapy was feasible with limited toxicity, in addition to demonstrating clinical activity, and elucidating pharmacodynamic effects of vorinostat on the tumor. Clearly, the lack of randomized design makes it difficult to confirm the additional benefit of adding vorinostat to standard of care (paclitaxel and bevacizumab in first-line setting), but provides the rationale to perform further studies with the combination.

A Phase I/II study of VPA in combination with epirubicin/5-fluorouracil and cyclophosphamide (FEC 100) to study the clinical and biological effects of adding VPA to anthracycline-based therapy was performed by Munster et al. [41]. The Phase I part was performed in 44 patients with advanced solid tumors, with dose escalation of VPA given on days 1 to 3 followed by epirubicin (day 3). This was followed by a cohort expansion study of VPA (120 mg/kg/day) combined with FEC 100 in 15 breast cancer patients. Objective responses were seen in nine of 14 (64%) evaluable breast cancer patients at the dose expansion, with a median number of six administered cycles. Grade III and IV nondose-limiting, treatment-related toxicities included nausea and vomiting (grade 3: 20% of patients), grade III thrombocytopenia (one out of 15 patients, 7%) and febrile neutropenia (three of 15 patients, 20%) in any cycle (median 6). Unlike the side effects usually seen with long-term administration of VPA, no electrolyte imbalances or liver function changes (+grade 1) were seen. Dose adjustments for VPA at the dose expansion were required for the loading dose (120 mg/kg) in the post-DLT period for 20% of the patients (three patients). Two patients experienced somnolence and one patient was unable to tolerate the excessive number of tablets. Valproic acid plasma levels were associated with short-term, reversible depletion of WBCs and neutrophils within 48 h. Histone acetylation in tumor samples and in PBMCs correlated with VPA levels. This study also showed HDAC2 expression in tumors at baseline was predictive of response and histone acetylation. A follow-up Phase II study of VPA in combination with FEC 100 for primary therapy, in patients with locally advanced or primary metastatic breast cancer is currently ongoing (NCT00437801) [41].

A Phase I/II study of the combination of vorinostat with weekly capcitabine is currently recruiting patients with metastatic breast cancer who have received no more than two prior therapies (NCT00719875) [101]. A Phase I study with vorinostat and ixabepilone (belonging to the class of epithilones) is being conducted to identify the maximum tolerated dose and schedule. This study also allows patients who have Her2 neu-positive disease who have failed trastuzumab and any number of prior therapies (NCT01084057) [102]. A Phase II study of neoadjuvant chemotherapy with carboptatin and nab-paclitaxel in locally advanced breast cancer with or without vorinostat is currently ongoing [42]. This study will be crucial to confirm if addition of vorinostat to standard chemotherapy improves tumor response (NCT00616967).

In summary, various clinical trials of a combination of HDACi with chemotherapy are currently ongoing. Most studies thus far have shown that this approach is feasible with limited toxicity. Several studies have demonstrated that at the doses administered, HDACi do result in acetylation of histones in primary tumors and PBMCs, demonstrating that they achieve adequate therapeutic levels. What is still unclear is whether such a molecular response can be linked to clinical response. In addition, several studies have linked expression of HDAC2 in the primary tumor to response, but further work is needed to confirm if this could be a biomarker that could help target HDAC therapy to specific tumors. It is also unclear if presence of HDAC2 is only indicative of response to certain classes of HDACi. Furthermore, results from randomized studies are needed to confirm that addition of HDACi therapy to chemotherapy truly improves outcomes. So, although the current data are encouraging, more work is needed to confirm the role of HDACi in combination with chemotherapy in breast cancer.
With hormone therapy
A clinical Phase II study of vorinostat in combination with tamoxifen was conducted by Munster et al. to attempt to reverse acquired hormone resistance in breast cancer patients who had progressed on previous hormone therapy [43]. Patients with ER-positive metastatic breast cancer, who progressed on up to two prior hormonal therapies and up to three prior chemotherapy regimens for metastatic cancer, were enrolled. Patients were treated with a combination of vorinostat 400 mg/day for 21 of 28 days and tamoxifen 20 mg/day. Patients who had received tamoxifen as adjuvant therapy were included, but those who had received it in the metastatic setting were excluded. Bone-only disease was included if at least one bone lesion measured 1 cm by MRI. The study enrolled 43 patients with a median age of 56 years (range 34 to 71 years) and 42 patients completed at least one cycle of therapy, with a median number of four cycles. Interestingly, 41 patients (98%) patients had received at least one AI and 22 (52%) of the patients had received adjuvant tamoxifen. Out of the 34 patients that were evaluable for response at the time of presentation, seven (21%) had a confirmed partial response, and one patient with bone-only disease had a response by PET/CT. Another four patients (12%) had stable disease for more than 6 months. The median duration of response was 8 months. Major grade 3/4 toxicities noted were fatigue (12%), anorexia, weight loss, nausea and vomiting (5%) and hematologic toxicities in the form of thrombocytopenia (7%), lymphopenia (16%) and neutropenia (14%). Pulmonary emboli were observed in two patients (5%). Correlative studies revealed histone H3 and H4 acetylation at day 8, suggesting adequate vorinostat plasma levels in the majority of the patients. Final data are eagerly awaited, but preliminary data are very promising and highlight the potential for HDACi to reverse acquired hormone resistance [43]. Confirmaotry results from final analysis of the data could provide a new alternative therapy for women who have failed hormone therapy.

Preliminary data from Phase II study of entinostat (SNDX 275) was presented in December 2009, and suggested that entinostat may sensitize breast cancer patients to AIs, in patients who have progressed on AI therapy. This is an interesting study design in which eligible postmenopausal women who have progressive disease after 3 or more months on AI therapy were continued on the same AI along with entinostat. The primary objective was to determine the clinical benefit rate (CR = PR + SD for over 6 months) during the first six cycles. Thus far, 24 patients have been enrolled, with a median age of 69 years. A total of 15 patients (80%) had received tamoxifen. Patients had both visceral (45%) and skeletal metastases (40%). Among the ten patients who had completed at least two cycles, preliminary analysis indicated that the longest duration of stable disease was more than 6 months in one patient and more than 5 months in two patients. Preliminary analysis of biomarkers in paired samples from six patients indicated that HDAC inhibition correlated with changes in cellular molecular targets. Entinostat was reported to be well tolerated and the majority of adverse events were reported to be mild-to-moderate in severity, although more data are awaited [44]. Preliminary data support the hypothesis that HDAC inhibition is sensitizing the resistant tumor to endocrine therapy, but further correlative data are eagerly awaited to understand the mechanism of this sensitization. A more innovative design to answer this question would have been to randomize the patients who progressed on the AI therapy to entinostat alone or entinostat and AI.

A double-blind randomized, placebo-controlled, Phase II study of the steroidal AI exemestane, with or without entinostat, is currently being conducted. The purpose of this trial is to assess whether the addition of entinostat to exemestane can overcome and/or delay the development of acquired resistance to AIs. Postmenopausal women with ER-positive metastatic breast cancer, who had progressed on a nonsteroidal AI, were enrolled in this study. A total of 114 patients are planned to be randomized to either exemestane alone or exemestane with entinostat or exemestane with placebo. Correlative studies are planned to analyze changes in acetylation and gene expression in peripheral blood monocytes, as well as levels of circulating endothelial cells before and after initiation of therapy. The study is designed to evaluate the hypothesis that the addition of entinostat will prolong the PFS by 2.3 months [45]. This study design will help answer the question of whether adding HDAC inhibition to endocrine therapy delays development of resistance and improves outcomes.

In summary, adding HDACi to endocrine therapy shows some early promise in improving responses of tumors that have failed hormone therapy. This is indeed very encouraging as patients who fail multiple endocrine therapies have very few options other than chemotherapy. It is still unclear if there are specific biomarkers in the tumors that could predict sensitivity to this combination approach. Another exciting opportunity is the use of HDACi to induce endocrine sensitivity in ER-negative tumors. Although the preclinical rationale is convincing, efficacy of this approach in the patient population needs to be demonstrated. Completion of the ongoing studies will help in developing the best approach to using these HDACi/endocrine therapy combinations in breast cancer.
With Her2-targeted therapies
A Phase II study of the HDACi vorinostat in combination with trastuzumab in patients with advanced metastatic and/or local chest wall recurrence, in patients with breast cancer resistant to trastuzumab-containing regimens, was conducted by Swaby et al., as part of the Eastern Cooperative Oncology Group [46]. The primary objective was to assess the response rate by RECIST criteria. The first part of the trial was an open-label, dose-escalation schema. A total of 6 patients were treated with vorinostat 200 mg b.i.d. plus trastuzumab 6 mg/kg every 3 weeks, and no dose-limiting toxicities were observed. Of the 16 patients enrolled at the time of presentation, ten patients were centrally confirmed to have Her2-neu positive disease, five were Her2-neu negative, and one had insufficient disease. None of these patients had an objective response. One of five patients who had centrally confirmed Her2-neu positive disease had an objective response. The preliminary data indicate that addition of HDACi to trastuzumab alone in heavily pretreated patients was not associated with a response; however, the data are preliminary and more data are awaited.

A pilot Phase II study of vorinostat and lapatinib in patients with advanced solid tumors in women with recurrent, locally advanced or metastatic breast cancer, designed to evaluate response and biomarkers of epithelial–mesenchymal transition and breast cancer stem cells is currently ongoing (NCT01118975) [101]. Eligible patients who have failed trastuzumab receive 300 mg (if tolerated this can be 400 mg) of vorinostat for 4 days on and 3 days off along with 1250 mg of lapatinib.

A Phase I/II multicenter trial with panobinostat (LBH), a pan-HDACi, with trastuzumab alone, in patients with metastatic breast cancer who had previously progressed on trastuzumab, is currently ongoing (NCT00567879) [47]. Patients receive panobinostat either intravenously (10, 15, and 20 mg/m² on days 1 and 8, every 3 weeks) or orally (15, 20, 30 and 40 mg three-times weekly, continuously) in combination with trastuzumab (2 mg/kg weekly). In this ongoing trial, 33 patients with Her2-positive breast cancer (of which 16 [48%] are ER/PR negative) have received treatment so far. For the intravenous arm, two out of six patients experienced DLTs (grade 4 thrombocytopenia and GI toxicity) in the 20 mg/m² cohort, and this cohort is currently expanded to 12 patients. For the oral arm, two of eight patients experienced DLTs in the 20 mg cohort, and this cohort has been expanded to confirm the MTD. Overall, the most frequent adverse events reported included gastrointestinal events of nausea, vomiting and diarrhea, and hematological events of thrombocytopenia and neutropenia. To date, no clinically significant EKG changes have been reported from the 820 EKGs performed. Two patients with liver metastases experienced tumor reduction of 29% (one patient each in the intravenous 10 mg/m² and oral 20 mg cohorts). Eight of the 25 patients (32%) with measurable disease had stable disease. Five of these patients received more than 18 weeks of treatment with the combination. These preliminary results demonstrate that the combination of panobinostat and trastuzumab is well tolerated. Another study with the addition of paclitaxel to the above regimen is ongoing (NCT00788931) [101].

Preliminary results from the Phase I study of panobinostat (LBH 589) in combination with capecitabine, with or without lapatinib, was recently presented at the ASCO annual meeting 2010 [48]. This study has three main objectives: establishing the MTD and DLT of panobinostat with capecitabine, assessing the safety and tolerability of lapatinib and panobinostat, and evaluating the tolerability and efficacy of the triplet. Accrual to stage I is complete with 15 patients receiving capecitabine 825 (n = 4) and 1000 mg/m² (MTD, n = 11) b.i.d. for 14 out of 21 days along with oral panobinostat 30 mg twice weekly, demonstrating the safety of this regimen with a DLT of grade 4 thrombocytopenia. Other grade 3 toxicities include anemia, thrombocytopenia, hand–foot syndrome, dehydration, fatigue and peripheral edema. One patient had an objective response and 27% had stable disease. The study will be continued, and the panobinostat dose will be changed to 20 mg three-times weekly (NCT00632489) [101].

In summary, preclinical rationale supporting the benefit of HDAC inhibition to improve responses to Her2-based therapy led to design of several clinical trials with this combination. Unfortunately, so far we have not seen any dramatic responses with this approach, although it is a little premature to draw any conclusions regarding efficacy as these studies are not yet completed. The one consistent observation so far is the lack of increased cardiac side-effects, but further data are awaited. A neoadjuvant study with weekly paclitaxel, trastuzumab and vorinostat followed by doxorubicin and cyclophosphamide in Her2-positive breast cancer patients is currently ongoing (NCT00574587) [101]. Although not a randomized study, this may shed further light on the efficacy of adding HDACi therapy to Her2-based therapy.

With radiation therapy
Vorinostat has been demonstrated to radiosensitize tumor cells in vitro, as assessed by both radiation-induced DNA damage and clonogenic cell survival [49]. Lawrence et al., in their recent abstract, showed that vorinostat downregulates key genes involved in double
strand DNA repair (Rad 50, Rad 51, XRCC2, XRCC 3, XRCC 6), as assessed by quantitative PCR, suggesting that vorinostat’s mechanism of radiosensitization could be via epigenetic modulation of genes involved in DNA repair mechanisms [50]. They are currently conducting a Phase I trial of whole brain radiation delivered in daily fractions of 2.5 Gy over 3 weeks (total dose 37.5 Gy) along with vorinostat (200 or 400 mg o.d.) on days of radiation therapy, in patients with advanced solid tumors and newly diagnosed brain metastases. Although this study will only determine the safety of this approach and will not confirm efficacy, future studies may confirm that HDACi given in combination with radiation therapy improves outcomes.

■ HDAC inhibitors in ductal carcinoma in situ

Broccoli sprouts are rich in sulforaphane, which is an isothiocyanate that has been shown to exert anticancer effects through histone acetylation, induction of p21 and Bax, and induction of cell cycle arrest and apoptosis in various cancer cell lines, including breast cancer cell lines. Based on these data, there are currently two trials using broccoli sprout extract open for patients with ductal carcinoma in situ of the breast [51].

A Phase II randomized study of broccoli sprout extract in women with newly diagnosed ductal carcinoma in situ and/or atypical hyperplasia is currently open (NCT00843167) [101]. The objectives are to determine the correlation between supplemental sulforaphane dose and concentrations of sulforaphane and its metabolites in blood, urine and nipple aspiration fluid samples, from women with ductal carcinoma in situ (DCIS) and/or atypical ductal hyperplasia. Additional end points include determining the effect of sulforaphane in the above specimens, such as acetylation of histones and biomarkers of cell proliferation (Ki-67 by IHC), apoptosis (TUNEL assay) and sulforaphane metabolism (isothiocyanate levels). It will also include evaluating the effect of this supplement on HDAC inhibition in peripheral blood mononuclear cells and in normal and cancerous breast tissue. A Phase II trial to examine the effect of a broccoli sprout preparation on specific factors in the breast tissue that are related to breast cancer risk, as well as its effect on protective enzymes such as NADH quinine oxidoreductase (NQ01) and glutathione reductase in the breast, is ongoing (NCT00982319) [101].

Another trial evaluating the role of neoadjuvant vorinostat in patients with DCIS is currently active (NCT00788112). The primary objective is to evaluate the in vivo molecular and biological effects of vorinostat by analyzing changes in proliferation and apoptosis, histone acetylation and HDAC protein expression in the tumor.

In summary, exploring the role of HDAC inhibition as a dietary supplement in a chemopreventive role is very exciting.

Conclusion

Our understanding of the mechanism of epigenetic modifications in breast cancer has evolved significantly over the last decade. This has led to a wealth of translational work demonstrating the role of HDACi as a novel anticancer strategy, aimed at enhancing response to conventional therapies in breast cancer. Clinical studies based on this translational work demonstrate that HDACi may not be very effective as a single-agent therapy, but may improve outcome when combined with endocrine therapy, chemotherapy and other targeted therapies. Perhaps the most innovative use of HDACi is to induce endocrine sensitivity in ER-negative breast cancer. Another exciting new avenue is the role of HDAC inhibition in the prevention of breast cancer. Our ability to monitor the effects of these agents using peripheral blood mononuclear cells is also very appealing. Several questions remain to be answered, such as tumor characteristics or biomarkers that will help to identify tumors that are sensitive to these agents, the most effective combination(s) and whether epigenetic changes in the breast could serve as a high-risk marker. Many key clinical trials are ongoing and results are eagerly awaited to confirm the promise of HDACi therapy in breast cancer. These will serve as a platform to design further larger studies to establish HDACi therapy as a strategy to overcome drug-resistance in breast cancer.

Future perspective

Epigenetic therapy has fulfilled the promise of being an effective therapy in certain hematological malignancies. In solid tumors, much of its role in clinical management is yet to be established. It is unlikely that HDACi will have activity in breast cancer as a single agent but we believe that this group of agents has the potential to be a modulator of cellular response to conventional therapy. Researchers need to incorporate valid tumor biomarkers, including biopsies, while designing clinical trials so that we can identify tumors that are ideally suited for epigenetic therapy.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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**Executive summary**

**Preclinical data**
- Histone deacetylase inhibitors (HDACi) are a novel group of anticancer agents that are showing promising activity in breast cancer.
- In ER-positive cells, HDACi augment endocrine therapy through transcriptional regulation of ER and aromatase and through its effects on nonhistone proteins such as Hsp90.
- In ER-negative cells, HDACi are able to induce endocrine sensitivity by restoring ER expression and function.
- Although the exact mechanisms are unclear, in preclinical models HDACi increase radiosensitivity of the breast cancer cells, perhaps through altering gene-expression leading to cell-cycle arrest.

**Clinical data**
- Completed and ongoing clinical trials of HDACi in breast cancer demonstrate that these agents are generally well-tolerated.
- Clinical trials thus far have not demonstrated significant single-agent activity of HDACi in breast cancer.
- Key role of HDACi in breast cancer appears to be in enhancing sensitivity to endocrine therapy in both ER-positive and ER-negative patients.
- Combining HDAC inhibitors with cytotoxic therapy appears to be promising, but it is unclear if there is an optimal regimen for this approach.
- Histone acetylation in peripheral blood mononuclear cells may be used as surrogate tissue to monitor molecular effects of HDACi.
- Presence of HDAC2 in primary tissue may be predictive of response to HDACi therapy. Further studies are needed to confirm this and identify other biomarkers.
- Early studies suggest that HDACi may play a role in chemoprevention.

**Bibliography**

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- of interest

Therapeutic potential of histone deacetylase inhibitors for breast cancer

Therapeutic Perspective


Viswanathan, Carothers & Ramaswamy


Website

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