

Review

Histone Deacetylase Inhibitors: An Attractive Therapeutic Strategy Against Breast Cancer

CHRISTOS DAMASKOS^{1,2*}, SERENA VALSAMI^{3*}, MICHAEL KONTOS^{4*}, ELEFThERIOS SPARTALIS², THEODOROS KALAMPOKAS⁵, EMMANOUIL KALAMPOKAS⁶, ANTONIOS ATHANASIOU⁴, DEMETRIOS MORIS⁷, AFRODITE DASKALOPOULOU^{2,8}, SPYRIDON DAVAKIS⁴, GERASIMOS TSOUROUFLIS¹, KONSTANTINOS KONTZOGLU¹, DESPINA PERREA², NIKOLAOS NIKITEAS² and DIMITRIOS DIMITROULIS¹

¹Second Department of Propedeutic Surgery, ⁴First Department of Surgery, Laiko General Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece;

²N.S. Christeas Laboratory of Experimental Surgery and Surgical Research, Medical School, National and Kapodistrian University of Athens, Athens, Greece;

³Blood Transfusion Department, Aretaieion Hospital, Medical School, National and Kapodistrian Athens University, Athens, Greece;

⁵Assisted Conception Unit, Second Department of Obstetrics and Gynecology, Aretaieion Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece;

⁶Gynaecological Oncology Department, University of Aberdeen, Aberdeen, U.K.;

⁷Lerner Research Institute, Cleveland Clinic, Cleveland, OH, U.S.A.;

⁸School of Biology, National and Kapodistrian University of Athens, Athens, Greece

Abstract. *With a lifetime risk estimated to be one in eight in industrialized countries, breast cancer is the most frequent type of cancer among women worldwide. Patients are often treated with anti-estrogens, but it is common that some tumors develop resistance to therapy. The causation and progression of cancer is controlled by epigenetic processes, so there is an ongoing interest in research into mechanisms, genes and signaling pathways associating carcinogenesis with epigenetic modulation of gene expression. Given the fact that histone deacetylases (HDACs) have a great impact on chromatin remodeling and epigenetics, their inhibitors have become a very interesting field of research. Aim: This review focused on the use of HDAC inhibitors as anticancer treatment and explains the mechanisms of therapeutic effects on breast cancer. We*

anticipate further clinical benefits of this new class of drugs, both as single agents and in combination therapy. Molecules such as suberoylanilide hydroxamic acid, trichostatin A, suberoylbis-hydroxamic acid, panobinostat, entinostat, valproic acid, sodium butyrate, SK7041, FTY720, N-(2-hydroxyphenyl)-2-propylpentanamide, Scriptaid, YCWI, santacruzamate A and ferrocenyl have shown promising antitumor effects against breast cancer. HDAC inhibitors consists an attractive field for targeted therapy against breast cancer. Future therapeutic strategies will include combination of HDAC inhibitors and chemotherapy or other inhibitors, in order to target multiple oncogenic signaling pathways. More trials are needed.

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer-related death among women worldwide. According to the American Cancer Society about 12% U.S. women will develop breast cancer during their lifetime. Moreover, in 2015, about 2,300 men were diagnosed with breast cancer and 440 died from the disease (1, 2).

In approximately 90% of breast cancer cases, estrogen receptor α (ER α), progesterone receptor (PR), or the human epidermal growth factor receptor2 (HER2/ERBB2) proto-oncogenic receptor are expressed. In many of these patients, treatment with anti-estrogens (e.g. aromatase inhibitors, tamoxifen, fulvestrant) and HER2-targeted agents has improved their survival significantly (3, 4). However, despite

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*These Authors contributed equally to this article.

Correspondence to: Christos Damaskos, MD, M. Sc., Ph.D., 17 Agiou Thoma Street, Athens, 11527, Greece. Mobile: +30 6948467790, Fax: +30 2132061772, e-mail: x_damaskos@yahoo.gr

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these treatments, it is common that some tumors develop *de novo* or acquire resistance to anti-estrogen and HER2-targeted therapies, so these tumors can recur (5).

Regarding the 10% of breast cancer cases that are negative for ER α , PR and HER2, and therefore are called 'triple-negative', there is no clinically-proven type-specific drug target and only genotoxic chemotherapy is used (6).

Molecularly, based on gene expression, breast tumors can be classified into luminal A, luminal B, HER2-enriched and basal-like. Luminal A and B tumors express ER, while basal-like are triple-negative tumors (7-9).

Given the incidence and the severity of the disease, new therapeutic targets need to be identified in order to treat breast cancer. Research in mechanisms that lead to resistance to establish therapies shows that promising targets for breast cancer treatment are histone deacetylases (HDAC) (10).

HDAC Classification and Mechanism of Action

Cell biology is based on the epigenetic modulation of gene expression (11, 12). The nucleosome is an organizing structure of DNA, of which packaging affects gene regulation. It consists of a DNA strand (about 200 bp) wound around an octameric core. This core consists of small, basic proteins which are called histones. Each core has two copies of every core histone (H2A, H2B, H3, and H4) and one copy of H1. In total, the nucleosome looks like a string of beads. Histones form the inside core, whereas DNA is located outside of the core (13). However, the *N*-terminal tails of histones extend outwards from the nucleosome protruding between the DNA coils. Histones are modified by substituents that covalently link to the side chains of certain amino acids in their *N*-terminal tails. They are modified by methylation, acetylation, phosphorylation and the opposite processes (14). These modifications alter the secondary DNA structure in order to induce or prevent access by transcription factors to gene promoter regions.

The reversible process of lysine acetylation at the ϵ -amino group of proteinogenic lysine residues is catalyzed by histone acetyl-transferases (HAT). Acetyl groups are removed by functional antagonists of HAT, histone deacetylases (HDACs) (15). In general, histone acetylation is associated with chromatin relaxation, given that it neutralizes the positive charge of lysine residues (16, 17). On the other hand, deacetylation leads to chromatin condensation, creating a structure called heterochromatin. In heterochromatin areas, transcription is repressed (18) (Figure 1).

Many different human HDACs have been identified. According to functional criteria and their homology with yeast proteins, HDACs are classified into four classes (19). These can be further divided into Zn²⁺-dependent classes (class I, II and IV) and NAD-dependent classes (class III). Class I consists of HDACs 1, 2, 3 and 8, whereas class IV has only one member HDAC11. Class II is divided into class IIa

(HDAC4, 5, 7, and 9) and class IIb (HDAC6 and 10) (20). Class III members are commonly known as sirtuins given the fact that they are homologous to silent information regulator 2 (SIR2) of *Saccharomyces cerevisiae*, and consist of SIRT1-SIRT7 (Table I).

HDAC Inhibitors as Anti-breast Cancer Agents

For the above mentioned reason, histone acetylation and deacetylation have a great impact on chromatin remodeling and epigenetics (Figure 1). HDAC inhibitors have a potent role in cancer pathogenesis and progression. A number of reports indicate that in certain types of cancer, HDAC levels are increased (21-24).

Indicatively, we mention the activity of HDAC1. HDAC1 is a prototypical deacetylase, homolog of RPD3, which is yeast and fly HDAC1 ortholog (25) and was observed for the first time using a trapoxin affinity matrix (26). HDAC1 is expressed in many tumor types, such as in pre-malignant and malignant prostate lesions (27), primary human gastric cancer (28) and esophageal carcinomas (29). It was found that if small interfering double-strand RNA (siRNA) molecules suppressed HDAC1, the proliferation and the survival of carcinoma cells were inhibited in a concentration-dependent way (30). Moreover, overexpression of HDAC1 in breast cancer cell line model systems leads to silencing of ER α gene and suppression of ER α protein (31). On the contrary, another study determined the levels of expression of the HDAC1 gene in malignant human breast tissue, using LightCycler-based quantitative real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) analysis in 162 cases of invasive breast carcinoma. It seems that in patients over 50 years of age, with HER2-negative and ER α /PR-positive, tumors without axillary lymph node involvement, *HDAC1* mRNA was highly expressed. There was a correlation between high levels of *HDAC1* mRNA expression and a better prognosis in terms of both disease-free and overall survival (24). It is possible that there are other factors, for example multiprotein complexes, that are involved in the relation between HDAC1 and ER α expression (32, 33).

The aforementioned data are just an example of using HDACs as an anticancer target. HDAC inhibitors, enhance the acetylation of cellular proteins by blocking HDAC activity, (34). According to their chemical structure, HDAC inhibitors are classified into four classes: hydroxamates [*e.g.* suberoylanilide hydroxamic acid (SAHA)], benzamides (*e.g.* MS-275), cyclic peptides (*e.g.* romidepsin) and aliphatic acids (*e.g.* valproic acid). Alternatively, HDAC inhibitors can be classified by their specificity for HDAC subtypes or classes. For instance, SAHA and trichostatin A are pan-HDAC inhibitors, while MS-275 and romidepsin inhibit class I and valproic acid inhibits class I and IIa HDACs (35).

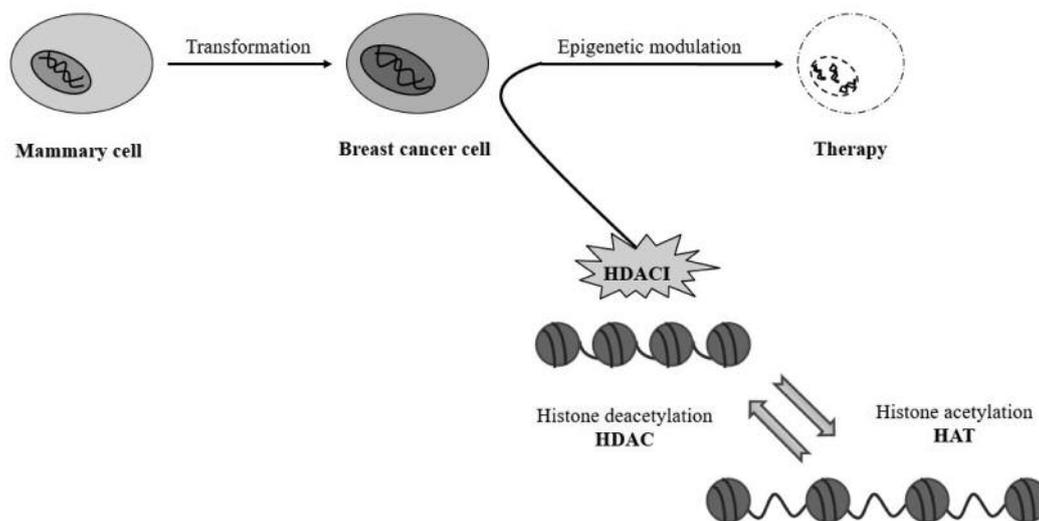


Figure 1. Therapeutic strategy using histone deacetylase inhibitors (HDACI). Lysine acetylation is catalyzed by histone acetyl transferase (HAT); acetyl groups are removed by histone deacetylases (HDAC).

In general, HDAC inhibition leads to inhibition of tumor growth, and apoptosis of cancer cells, whereas normal tissue is not particularly affected. HDAC inhibitors have the ability to decompose and condense not only the histone–DNA complex, but also the acetylation status of non-histone proteins.

Clinical trials using HDAC inhibitors have been performed and their results indicate that HDAC inhibitors have antitumor activity (14, 36-40) and may be clinically beneficial. For example, SAHA/vorinostat and romidepsin were approved by the US Food and Drug Administration for the treatment of cutaneous T-cell lymphoma (36, 37) and peripheral T-cell lymphoma (38) respectively. In addition, panobinostat has demonstrated clinical success for the treatment of multiple myeloma (39). Today many inhibitors are being studied in advanced stages of clinical trials. With regard to breast cancer research, it has been shown that HDAC inhibitors exhibit potent activity in when combined with cytotoxic drugs, aromatase inhibitors, pro-drugs and ionizing radiation (41-44) (Table II).

SAHA. SAHA (vorinostat) is one of the most advanced small-molecule pan-HDAC inhibitors. Its main mechanisms of action are changes in acetylating motif and downstream effects on apoptotic pathways (45). Preclinical data on MCF-7, MDA-MB 231, MDA-MB-435 and SKBr-3 breast cancer cell lines have shown growth inhibition caused by G₁ and G₂-M cycle phase arrest and apoptosis (46). In HER2-overexpressing breast cancer cell lines, SAHA not only facilitates apoptosis in a dose-dependent pattern, but also leads to heat-shock protein 90 (HSP90) acetylation. This acetylation causes dissociation of HSP90 from HER2 and as a result, HER2 is polyubiquitinated and degraded (47).

Table I. Classification of histone deacetylases.

Zn ²⁺ -dependent		NAD-dependent	
Class I	Class II	Class III	Class IV
HDAC1, 2, 3, 8	a: HDAC4, 5, 7, 9 b: HDAC6, 10	HDAC11	SIRT1-7

SAHA was approved by the US Food and Drug Administration in October 2006 for the treatment of cutaneous T-cell lymphoma (36, 37). It has been used in clinical trials for treatment of a variety of solid and hematological tumors such as head and neck cancer (48), breast cancer (49) and Hodgkin lymphoma (50). However, the results of the phase II trial by the California Cancer Consortium against metastatic breast cancer are rather inconclusive. SAHA treatment appeared to have only modest clinical benefits. SAHA as monotherapy for patients with metastatic breast cancer with different molecular subtypes failed to meet the Response Evaluation Criteria In Solid Tumors response criteria (51) for adequate single-agent activity. However, due to its favorable toxicity and ease of administration it might be used as part of a combination therapy (for example co-treatment with paclitaxel and bevacizumab) (49).

Another trial in patients with advanced ER-positive breast cancer investigated co-treatment of SAHA and tamoxifen, an anti-estrogen, and showed that this combination was beneficial and reversed hormone resistance (41). In 40% of patients who had tried hormonal therapy and chemotherapy

Table II. *Histone deacetylase inhibitors and their action against breast cancer.*

Substance	Action
SAHA	Promotion of EMT of triple-negative breast cancer cells <i>via</i> HDAC8/FOXA1 signals
Trichostatin A	Inhibition of tumor growth, repression of cyclin D1 up-regulation
Syberoylbis-hydroxamic acid	p53-dependent way of apoptosis, regulation of p21, p27, BAX
Panobinostat	Restoration of ER α expression, inhibition of metastasis (co-treatment with letrozole)
Entinostat	Reversion of EMT, prevention of metastasis
Valproic acid	Anti-proliferative action, regulation of p21, cell-cycle arrest, apoptosis
Sodium butyrate	Apoptosis
SK7041	Cytotoxicity, G2-M phase arrest, apoptosis
FTY720	Reactivation of ER α expression, enhancement of hormonal therapy
Compound 2	Anti-proliferative action, target HDAC8
Scriptaid	Inhibition of tumor growth
YCW1	Autophagic cell death, inhibition of BNIP3, cytotoxicity (co-treatment with ionizing radiation)
SCA	Anti-proliferative action, degranulation of cytotoxic T-cells
Ferrocenyl	Inhibition of proliferation of ER α + and ER α - cells

EMT: Epithelial–mesenchymal transition; FOXA1: forkhead box protein A1; BAX: BCL2-associated X; ER α : estrogen receptor α ; FTY720: fingolimod; YCW1: octanedioic acid (3-(2-(5-methoxy-1*H*-indol-1-yl)ethoxy)phenyl)-amide N-hydroxyamide; BNIP3: BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; SCA: santacruzamate A.

before, this combination led to tumor regression or stabilization of the disease. Given that the sample size was limited (43 patients), a randomized trial is needed in order to study the effects of this co-treatment over the efficacy of tamoxifen alone. Moreover, in presence of SAHA, tamoxifen induces apoptosis rather than antiproliferative effect, probably because of HDAC2 inhibition (52). Researchers suggested that new studies associated with acetylation bioassays and HDAC2 expression in tumors is required in order to validate the antitumor efficacy of hormonal therapy and HDAC2 inhibitors (41).

A research studied the activity of SAHA on triple-negative breast cancer, using MDA-MB-231 and BT-549 cells (53). The results revealed that SAHA promotes the *in vitro* trafficking of triple-negative breast cancer cells *via* induction of epithelial–mesenchymal transition (EMT) phenotype. This phenotype is known to contribute to cancer recurrence and metastasis (54). It should be noted that during SAHA treatment, expression and subcellular translocation of EMT-related transcription factors (55), zinc finger protein SNAIL (SNAIL), zinc finger protein SNAI2 (SLUG), basic helix-loop-helix transcription factor twist-related protein (TWIST) and zinc finger E-box-binding homeobox (ZEB) did not change. This indicates that EMT-related transcription factors are not involved in SAHA-induced EMT.

Forkhead box A1 (FOXA1) mediates SAHA-induced EMT in triple-negative breast cancer cells (53). FOXA1 is a growth inhibitor that can be used as a prognostic factor in human breast cancer (56). At the beginning of SAHA treatment, FOXA1 expression decreased and its nuclear translocation was down-regulated, in a time-dependent manner. Subsequently, SAHA-induced EMT was weakened by overexpression of

SNAIL. Moreover, in human triple-negative cell lines MDA-MB-231 and BT-549, SAHA-induced down-regulation of *FOXA1* transcription was attenuated by silencing of HDAC8, but not HDAC6 (53). Studies claimed that SAHA activity is mediated by HDAC6 and HDAC8 (57, 58). HDAC8 is a very unique class I HDAC given the fact that it lacks the conserved C-terminal domain (59) and its expression profile is different from those of HDACs 1-3 (60). *HDAC8* knockdown does not influence histone acetylation (61), but it has been claimed that it could act at certain promoter sites of *FOXA* (53).

Trichostatin A. Trichostatin A (TSA) is an antifungal antibiotic and is found in cultured mammalian cells and in fractionated cell nuclear extracts at low nanomolar concentrations. In general, as an HDAC inhibitor, TSA not only represses HDAC activity, but also arrests the cell cycle in G₁ and G₂ phase, induces cell differentiation and the reversion of transformed cells in culture (62). An early study revealed TSA to be a potent inhibitor of tumor growth of human breast cancer cells. Furthermore, ER α -positive breast cancer cell lines were up to 10 times more sensitive to growth inhibition than ER α -negative cell lines (63).

In another study, ER-negative human breast cancer cells were treated with TSA and RT-PCR results showed that ER mRNA was re-expressed in a dose- and time-dependent manner, without changes in ER α CpG island methylation. In MDA-MB-231 cells, these results correlated with sensitivity of the ER locus to DNase I. Treatment in mouse models with TSA in combination with the hypomethylating agent or 5-aza-2'-deoxycytidine (AZA), inhibited the progress of the tumor and indicated better results compared with TSA, and AZA alone (64).

Alao *et al.* found that in breast cancer cells, TSA affects cyclin D1. It seems that TSA represses cyclin D1 up-regulation. This repression was found in both ER α -dependent and -independent cell lines. In particular, in the ER α -positive MCF-7 breast cancer cell line, in addition to the inhibition of ER α and cyclin D1 transcription, TSA led to ubiquitin-dependent proteasomal degradation of cyclin D1 and arrested the cell cycle in G₁-S phase. However, in the ER α -negative MDA-MB-231 breast cancer cell line, while TSA led to degradation of cyclin D1, it did not particularly affect its transcription and led to G₂-M phase arrest. These results indicate that both cyclin D1 degradation and inhibition of ER α transcription determine the sensitivity of ER α -positive breast cancer cells to growth inhibition by TSA (65).

Suberoylbis-hydroxamic acid. Suberoylbis-hydroxamic acid (SBHA) is a HDAC inhibitor that has a similar structure to SAHA and TSA. It has shown anticancer activity against many types of tumors, such as medullary thyroid and lung cancer (66, 67). Studies in both A549 lung cancer cells and MCF-7 breast cancer cells indicated that SBHA treatment induced a significant level of apoptosis (67, 68). Specifically, SBHA seemed to induce p53-dependent apoptosis of MCF-7 breast cancer cells. Another investigation studied the association of SBHA with cancer cell proliferation and apoptosis. The results revealed that treatment with SBHA not only inhibited MCF-7 cell proliferation, but also induced apoptotic cell death in a concentration-dependent manner. The cell cycle was arrested at G₀-G₁ phase. In addition, levels of p21 (cyclin-dependent kinase inhibitor 1 (WAF1)) and p27 (kinesin-like protein (KIP1)) proteins increased, as did apoptosis regulator BCL-2-like protein 4 (BAX) expression, whereas apoptosis regulator B-cell lymphoma 2 (BCL2) expression was reduced (69). p21 and p27 are inhibitors of cyclin-dependent kinases, hence they are involved in cell-cycle control (70) and appear to suppress MCF-7 cell proliferation *in vitro* (71). BAX is a pro-apoptotic member of the BCL2 family and a downstream target gene of p53 that mediates p53-dependent apoptosis (72). Lack of BAX is associated with p53-induced apoptosis in neurons (73). Research on MCF-7 cells treated with SBHA showed shrinkage, chromatin condensation and nuclear fragmentation with Hoechst 33258 staining. However, the antiproliferative potency of SBHA appeared to be less than that of SAHA (69).

Panobinostat. Panobinostat (LBH589) is a pan-HDAC inhibitor recently approved for treatment of multiple myeloma with tumor progression after immunomodulating agents and bortezomib. It expresses its inhibitory activity against solid tumors, such as lung, breast, ovarian cancer and multiple myeloma (74). According to one study, in ER-negative human breast cancer lines MDA-MB-231 and MDA-MB-435 treated with panobinostat for 24 hours, ER α mRNA and protein

expression were restored, whereas CpG island at the ER promoter was not demethylated. After at least 96 hours from termination of panobinostat treatment, the expression of ER α mRNA was maintained. Chromatin immunoprecipitation analysis showed panobinostat treatment released DNA (cytosine-5)-methyltransferase 1 (DNMT1), HDAC1, and the H3 lysine 9 (H3-K9) methyltransferase SUV39H1 from the ER promoter. There was a connection between these alterations and active chromatin formation. This formation led to an increase of acetylated H3 and H4, a decrease in methylated H3-K9 and false binding of heterochromatin protein 1 (HP1 alpha) at the promoter (75).

Kubo *et al.* studied panobinostat in combination to aromatase inhibitors. They used aromatase inhibitor-resistant breast cancer cell lines and tumors and found that by stopping the cell cycle at G₂-M phase and by inducing apoptosis, growth of aromatase inhibitor-resistant cells was prevented *in vitro* and *in vivo* by panobinostat. In addition, while resistance to aromatase inhibitor is reflected by the overexpression of Nuclear factor -kappa-B p105 subunit (NF- κ B1), with treatment with panobinostat, the level of NF- κ B1 in tumors fell (76, 77). Furthermore, a recent phase I clinical trial in patients with metastatic breast cancer tested the safety and efficacy of the combined treatment of panobinostat and the aromatase inhibitor letrozole. This trial tested the safety and efficacy of this co-treatment. Researchers found that this co-treatment was safe and recommended a tolerable dose for the phase II clinical trial (panobinostat at 20 mg orally 3 times weekly and oral letrozole at 2.5 mg daily) (78). These data indicate that panobinostat may soon have a role in the treatment of metastatic breast cancer in the future.

Entinostat. Tumor cells undergo EMT which enables spread from the primary site. This transition makes a cancer cell's phenotype invasive (79), allowing establishment of metastases which leads to mortality (80). Recent data show that escape from the primary site may be achieved by a subset of cells within a breast tumor. These cells are termed as tumor-initiating cells (TICs). TICs produce progenitor cells, which cannot self-renew, but may comprise the greater part of the tumor (54). Entinostat (MS-275), a selective inhibitor of class I HDACs, reverses the EMT phenotype. This reversion is a result of epigenetic repression of E-cadherin (81, 82). A new study showed that treatment with entinostat reduced the level of TICs in triple-negative breast cancer cells. These results may indicate that entinostat can prevent development of metastasis (43).

HDACs may in general also influence non-histone proteins, such as Hypoxia-inducible factor 1-alpha (HIF1 α), which participate in drug resistance, EMT and development of TICs (83). Given the fact that HDAC inhibitors are related to EMT and TICs, a number of studies have been performed. In particular, valproic acid and SAHA (vorinostat) appeared to

reduce the population of TICs in breast cancer cell lines (84). TSA seemed to induce a TIC phenotype in head and neck cancer cells and increased EMT (85). However, as mentioned, valproic acid, SAHA and TSA block class I and II HDACs, whereas entinostat specifically blocks class I HDACs. It is understandable that further research is needed on the regulation of EMT and TICs by specific HDACs in order to identify potential clinical benefits.

Yardley *et al.* conducted a phase II, double-blind, placebo-controlled study of the aromatase inhibitor exemestane with and without entinostat in postmenopausal women with metastatic, ER-positive breast cancer. In this trial, 134 patients with advanced ER-positive breast cancer resistant to aromatase inhibition received entinostat combined with exemestane. Co-treatment enhanced the progression-free survival from 2.3 to 4.3 months and the median overall survival from 19.8 to 28.1 months compared with exemestane alone. Further research is needed to validate this result (86).

Valproic Acid. Valproic acid causes hyperacetylation of the *N*-terminal tails of H3 and H4 *in vitro* and *in vivo* and inhibits HDAC activity, probably by binding to the catalytic center and blocking the access of the substrate (87). It has been used for treatment of epilepsy and bipolar disorder (88, 89). Valproic acid is a potent inhibitor of class I HDACs, but it also inhibits class II HDACs (90). Additionally, by inducing gene targeting of cyclin-dependent kinase inhibitor p21 (WAF1), valproic acid influences differentiation and has antiproliferative effects (91). p21 regulates cell-cycle progression, given the fact that is involved in both G₁-S and the G₂-M transition. Valproic acid is found to suppress the growth of ER-positive breast cancer cells at a lower dose than that required for ER-negative cells (92). Moreover, the extent of cell growth suppression by valproic acid is not related to estrogen sensitivity (93).

Studies in HER2-negative breast cancer cell lines indicated that valproic acid has antiproliferative activity (92-94). In studies of HER2-overexpressing breast cancer cells, at a clinically achievable dose of 1 mM, valproic acid reduced proliferation by cell-cycle arrest and induction of apoptosis; moreover HER2-overexpressing breast cancer cells were more sensitive to valproic acid treatment than HER2-negative ones (95). This antiproliferative activity of valproic acid seems to be a result of HSP90 dysfunction, which hyperacetylates HSP70. The hyperacetylation of HSP70 affects HER2 protein, which is a client protein of HSP90. HSPs are highly conserved molecular chaperones. Specifically HSP90 controls the intracellular trafficking and folding of some cellular proteins. Dysfunction of HSP90 destabilizes and degrades HSP90 client proteins and leads to apoptosis (96).

Furthermore, Mawatari *et al.* used immunohistochemical techniques and terminal deoxynucleotidyl transferase dUTP nick end labeling assay and found that valproic acid induced apoptosis of SKBR3 cells by activating caspase-3, which

induces apoptosis in the form of cleaved caspase-3 (95). Recently, co-treatment of valproic acid and the prodrug capecitabine, which is commonly used for metastatic breast cancer in different settings, resulted in antiproliferative and pro-apoptotic effects on breast cancer cells both *in vitro* and *in vivo*, and this might become an innovative antitumor strategy against metastatic breast cancer (97).

Sodium Butyrate. Sodium butyrate, as member of the HDAC inhibitor family, was shown to exhibit an effective anticancer ability (98). It leads to relaxation of chromatin structure, allowing easier access to transcription-related proteins. These abilities made sodium butyrate a widespread treatment against many types of solid tumor (99, 100). In addition, studies in MCF-7 cells demonstrated that sodium butyrate has a dose- and time-dependent anti-proliferative effect (101).

Another study treated MCF-7 cells with sodium butyrate in order to correlate it with cell apoptosis and structural alterations. It was found that sodium butyrate suppressed MCF-7 cell viability in a concentration- and duration-dependent manner. Moreover, it indicated that sodium butyrate treatment reduced BCL2 expression in MCF-7 cells. This evidence supported the correlation of sodium butyrate effects with cell apoptosis, which was observed previously in other cell types (102, 103). As apoptotic cells exhibit morphological alterations (104), microscopy revealed cell shrinkage, rounding, large amounts of heterochromatin, chromatin condensation, apoptotic nuclei, minimization of mitochondria and incredibly high cytoplasmic vacuolization (balloon-like vacuoles) in sodium butyrate-treated cells in comparison with control (105).

SK7041. SK7041 belongs to a class of synthetic HDAC inhibitors which are composed of hybridization of TSA and MS-275. In general, the structure of HDAC inhibitors consists of a functional moiety, a cap, and a linker, which connects the cap and functional moiety. The TSA functional moiety is hydroxamic acid. TSA has powerful inhibitory activity at nanomolar concentrations, but is unstable and has low bioavailability (63). MS-275, a synthetic benzamide derivative, consists of a pyridyl ring cap structure and has better physicochemical properties than TSA, but its HDAC-inhibitory activity is low at micromolar concentrations (106). Researchers created a hybrid in order to keep the high inhibitory activity of TSA and enhance its physicochemical properties. For this reason, the cap structure of TSA was replaced with the pyridyl ring of MS-275.

In one study, SK-7041 was evaluated on breast (MDA-MB-231, MCF-7, and SK-BR-3) and lung (A549, NCI-H23, and NCI-H1299) cancer cell lines. It was found that not only was SK-7041 cytotoxic in breast and lung cancer cell lines, but it also showed more potent cytotoxicity than SAHA. It induced histone acetylation and had an antiproliferative effect on these cell lines.

These effects were the result of G₂-M phase arrest and, to some extent, G₁-phase arrest, therefore SK-7041 treatment appears to provoke apoptotic cell death (107). This evidence is supported by other reports on treatment with SK-7041 in gastric and pancreatic cancer cell lines (108, 109). Furthermore, SK-7041 was found to inhibit cell proliferation more selectively in cancer lines than in normal human cell lines (107).

FTY720. FTY720 (fingolimod), a sphingosine analog, is a pro-drug approved by the US Food and Drug Administration for the treatment of multiple sclerosis. A study showed that FTY720 is phosphorylated in the nucleus of both ER-positive and ER-negative breast cancer cells by nuclear sphingosine kinase 2. Nuclear FTY720-phosphate (P) is a class I HDAC inhibitor. FTY720-P, as a powerful inhibitor, is highly involved in histone acetylation and in gene expression.

A recent study which investigated ER α -negative human and mouse breast cancer cells showed that FTY720 induced the re-expression of silenced ER α . In this study, FTY720 was given orally to mice in clinically accepted doses and, as a result, HDAC activity decreased and the evolution and development of breast tumors were diminished (110). They also found that the inhibitor sensitizes cells to tamoxifen and, in comparison with other HDAC inhibitors (*e.g.* SAHA), it enhanced the sensitivity to the treatment (110).

***N*-(2-Hydroxyphenyl)-2-propylpentanamide.** *N*-(2-Hydroxyphenyl)-2-propylpentanamide is an aryl derivative of valproic acid. These derivatives are produced by combining valproic acid and the arylamine core of SAHA with different substituents at its carboxyl group. *N*-(2-Hydroxyphenyl)-2-propylpentanamide was the most promising compound among other derivatives which were submitted to docking simulations. *In vitro* studies showed that it was the best anticancer agent against HeLa, rhabdomyosarcoma and breast cancer cell lines and in particular, triple-negative breast cancer cells. This compound seems to target HDAC8. In addition, *in vitro* studies showed that in comparison with valproic acid, *N*-(2-Hydroxyphenyl)-2-propylpentanamide inhibited cancer cell proliferation at a much lower concentration (111).

Scriptaid. Scriptaid is a member of the hydroxamic class of HDAC inhibitors, so it is similar to TSA (112). Protein expression and ER mRNA function are influenced by the activity of HDAC inhibitor Scriptaid. Specifically, in MDA-231, MDA-MB-435, and Hs578t ER cell lines, not only was ER function restored, but tumor growth was also inhibited. Furthermore, given the fact that dose-dependent re-expression of PR was observed, it seemed that ER functioned. Acetylation of H3 and H4 histone tails was also increased. Co-treatment with Scriptaid and methyltransferase inhibitor AZA gave better results in comparison with Scriptaid, or AZA

alone. We can assume that in regard to ER-negative human breast cancer cells, Scriptaid could have clinical benefits in the dissection of the components of the repressive complex at the methylated ER locus (113).

YCW1. Octanedioic acid (3-(2-(5-methoxy-1*H*-indol-1yl)ethoxy)phenyl)-amide *N*-hydroxyamide (YCW1) is a novel HDAC inhibitor which was developed using structure-based analyses (114, 115). YCW1 activity was investigated in the murine triple-negative breast cancer cell line 4 T1 and the human triple-negative breast cancer cell line MDA-MB-231. The authors particularly tested the co-treatment of YCW1 and ionizing radiation (IR) for its antitumor effects. They found that this combination led to an increase of autophagy and endoplasmic reticulum stress, thereby acting as a cytotoxic against triple-negative breast cancer cells (44). Moreover, in comparison with the inhibitory activity of SAHA, YCW1 significantly enhanced toxicity.

It is known that in patients with triple-negative breast cancer, positive BCL2 expression is associated with poor survival and lower sensitivity to chemotherapy (116). BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) is a member of the BCL2 subfamily and is associated with death-inducing mitochondrial proteins (117) and autophagy (118, 119). BNIP3 is not expressed in normal breast tissue but is up-regulated in breast cancer cells (120). In Chiu *et al.*'s study that was mentioned above, BNIP3 levels were significantly decreased in 4 T1 cells that were co-treated with YCW1 and IR in comparison with those treated with YCW1 or IR alone. The authors claimed that this down-regulation of BNIP3 enhanced the antitumor effects of IR and YCW1 by inducing autophagic cell death and cytotoxicity. These results were also confirmed in an orthotopic breast cancer mouse model. Therefore, Chiu *et al.* supported the hypothesis that in triple-negative breast cancer, co-treatment with IR and YCW1 induced autophagic cell death, through the inhibition of BNIP3 (44).

Santacruzamate A. The Panamanian marine cyanobacterium *Symploca* sp. produces a natural bioactive product, santacruzamate A (SCA). SCA seems to act as a HDAC inhibitor and consists of structural domains which also exist in SAHA. In one study, Gromek *et al.* used 40 SCA analogs which were synthesized with alterations in the zinc-binding group, cap terminus, and linker region, three structural motifs that appear in the majority of HDAC inhibitors. Analogs were categorized by their cap. It was found that the proliferation of MCF-7 breast cancer cells was inhibited in two group analogs and other analogs seemed to influence the degranulation of cytotoxic T-cells (121).

Ferrocenyl. Selective ER modulators have many advantages in the treatment of hormone-responsive breast cancer.

However, they are ineffective for ER-negative breast cancer. In recent research, a series of dual-acting ER and HDAC inhibitors were designed with incorporation of a ferrocenyl moiety, leading to novel hybrid ferrocenyl conjugates (FcOBHS-HDAC inhibitors) against breast cancer. These ferrocenyl conjugates can inhibit both the proliferation of ER α -positive (MCF-7) and ER-negative breast cancer cells (MDA-MB-231), and are a very promising strategy against breast cancer (122).

Conclusion

HDAC inhibitors represent the first successful epigenetic therapy against cancer. They seem to be clinically beneficial in subtypes of hematological malignancies. On the contrary, their efficacy against solid tumors is unclear (123). Despite the positive results in some pre-clinical trials, most HDAC inhibitors used as single agents failed to achieve treatment as a single therapy against solid tumors (124). For instance, use of SAHA as monotherapy in 14 patients with metastatic breast cancer failed to demonstrate adequate single-agent activity (49). As such, at the moment, clinical trials tend to combine HDAC inhibitors with cytotoxics, aromatase inhibitors, pro-drugs and chemotherapy (41-44) in order to enhance their effects against tumors.

In a significant proportion of breast cancers, the ER α gene is abnormally methylated at its CpG island. As a result, the expression of ER α gene is inhibited. Consequently, researchers started to study how HDAC inhibitors reverse resistance to anti-estrogen therapies *in vitro*. It was found that HDAC inhibitors can restore sensitivity to ER in endocrine-resistant and triple-negative breast cancer cell lines (125-127).

Except for a phase II trial of SAHA (49), other investigators occurred another phase II trial. Munster *et al.* treated patients with ER-positive endocrine-resistant metastatic disease with SAHA and tamoxifen. This co-treatment induced an objective response rate of 19%, clinical benefit rate of 40% and median response duration of 10.3 months (41). Another trial that studied co-treatment of entinostat and exemestane in patients with advanced ER-positive breast cancer resistant to aromatase inhibition showed that progression-free survival improved compared with exemestane/placebo (86). Moreover, in patients with metastatic breast cancer, researchers investigated the combination of panobinostat and letrozole and we attend phase II trial so panobinostat would be approved for treatment of metastatic breast cancer (78).

It is understandable that the identification of new targets in breast cancer is very promising. Future therapy advances will likely include combination treatment using HDAC inhibitors and chemotherapy or other inhibitors. By combining therapeutics, we can simultaneously target multiple oncogenic signaling pathways and overcome resistance.

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