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Review Article

Manipulating Protein Acetylation in Breast Cancer: A Promising Approach in Combination with Hormonal Therapies?

Aurélien Linares,[1] Florence Dalenc,[2] Patrick Balaguer,[1] Nathalie Boulle,[3] and Vincent Cavailles[1]

[1] *IRCM, Institut de Recherche en Cancérologie de Montpellier, INSERM, U896, Université Montpellier1, CRLC Val d'Aurelle Paul Lamarque, Montpellier, 34298, France*
[2] *CRLC Claudius Regaud, 31052 Toulouse Cedex, France*
[3] *Laboratoire de Biologie Cellulaire et Hormonale, CHRU Arnaud de Villeneuve, 34090 Montpellier, France*

Correspondence should be addressed to Vincent Cavailles, vincent.cavailles@inserm.fr

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Estrogens play an essential role in the normal physiology of the breast as well as in mammary tumorigenesis. Their effects are mediated by two nuclear estrogen receptors, ERα and β, which regulate transcription of specific genes by interacting with multiprotein complexes, including histone deacetylases (HDACs). During the past few years, HDACs have raised great interest as therapeutic targets in the field of cancer therapy. In breast cancer, several experimental arguments suggest that HDACs are involved at multiple levels in mammary tumorigenesis: their expression is deregulated in breast tumors; they interfere with ER signaling in intricate ways, restoring hormone sensitivity in models of estrogen resistance, and they clinically represent new potential targets for HDACs inhibitors (HDIs) in combination with hormonal therapies. In this paper, we will describe these different aspects and underline the clinical interest of HDIs in the context of breast cancer resistance to hormone therapies (HTs).

1. Introduction

1.1. Breast Cancer and Hormonal Therapies. Breast cancer is the most common malignancy and the second most common cause of cancer-related death amongst women in France, Western Europe and North America. About 70% to 80% of infiltrating breast carcinoma are estrogen receptor alpha (ERα) positive, thus offering clinicians the opportunity of hormonal therapies (HTs) in adjuvant and/or metastatic situation. Modulation of estrogen signaling pathways using antiestrogens (such as Tamoxifen or Fulvestrant) or more recently aromatase inhibitors (such as Exemestane, Letrozole, or Anastrozole) was indeed one of the first recognized targeted therapies and is currently the first-line treatment for ERα positive tumors [1]. The effectiveness of HTs is directly linked to the expression and functionality of ERα. Several retrospective studies and clinical trials have demonstrated that tumors expressing both ERα and progesterone receptor (PR) respond significantly better to HTs than those with low receptor expression [2, 3]. Among patients who have a tumor expressing both ERα and PR, a benefit from HTs is seen in about 60% of cases, but the initial response is often not durable, since tumors become resistant to hormonal manipulation, leading to an "endocrine-resistant disease". Moreover, patients with breast carcinoma lacking ERα (ERα negative) will not benefit from these therapies, as the expected efficiency of HTs in this situation is less than 10%.

Definition of the specific genetic lesions and molecular processes that determine clinical endocrine resistance is still incomplete. Candidate molecular pathways of intrinsic and acquired resistance to HTs emphasize the importance of signaling networks which control cell proliferation (e.g., acting *via* epidermal growth factor receptor type 2 (HER2) or insulin-like growth factor-1 receptor (IGF-1R)) or survival (through molecules such as Bad or Bcl-2) [4, 5]. In addition, polymorphisms in metabolizing enzymes such as the hepatic drug-metabolizing cytochrome P450 2D6 (CYP2D6) may

reduce the therapeutic benefit from tamoxifen (for a review, see [6]). Today, the main challenges in mammary cancer research are thus the development of more specific biomarkers to predict response or resistance to hormonal therapy and the development of new combined targeted therapies of hormone therapy-insensitive or therapy-resistant tumors.

1.2. Nuclear ER Signaling. Estrogens, like many other hormones, elicit numerous biological responses. They play a major role in the development and maintenance of the female reproductive tract (including the mammary glands) and are also involved in breast tumorigenesis. They act on target tissues through binding to two ER isoforms (ERα and ERβ), which are members of the nuclear hormone receptor (NR) superfamily [7]. Upon interaction with ERs, estrogens induce a conformational change, which favors receptor dimerization and recruitment to promoter elements either directly through their DNA-binding domain or indirectly through interaction with other transcription factors. ER complexes then recruit transcriptional coregulators (coactivators and corepressors) to increase or inhibit target gene transcription [7]. In most cases, transcriptional cofactors are recruited as multiprotein complexes that could act either sequentially or simultaneously, depending on the considered gene. Many transcriptional coregulators of NRs exhibit enzymatic activities that participate in their mechanism of action. For example, several coactivators—CBP/p300, pCAF, SRC-1, and SRC-3—are acetyltransferases that are able to modify various lysine residues located in the amino terminal tails of histones. Conversely, inhibitory complexes associated with corepressors, contain histone deacetylases (HDACs) whose activity counteracts that of acetyltransferases (HATs). Some other enzymatic activities—including kinases or methyltransferases—displayed by coregulators are also able to modify histone lysines, arginines, or serines. All these posttranslational modifications interfere with each other and represent signals that enable binding of proteins involved in the transcriptional control of gene expression. From a clinical point of view, transcription therapies targeting pathological epigenetic modifications are very promising approaches to improve cancer treatment (see below).

2. Histone Deacetylases and Inhibitors

2.1. Acetylation of Chromatin and Nonchromatin Proteins. Acetylation and deacetylation of the ε-amino group of Lys residue (N^{ε}) is a reversible reaction catalysed by the opposing actions of Lys acetyltransferases and Lys deacetylases. This modification, also described in bacteria, has been first extensively studied in the context of chromatin and histone modifications. As noted above, acetylation and deacetylation of the N-terminal tails of histones contribute to the "histone code" which defines part of the epigenetic landscape involved in the regulation of gene expression. It is now known that in addition to histones and transcription factors, N^{ε}-acetylation target numerous other proteins, such as proteins involved in cell signaling, DNA repair, metabolism, apoptosis, cytoskeleton, and protein folding (see also Section 5 of this paper). N acetyl Lysine may serve as a docking structure for bromodomain, a protein domain that has the ability to recognize acetyl-lysine motifs. N^{ε}-acetylation may either enhance or decrease the function of the protein targeted, depending on the presence of other posttranslational modifications on the protein (such as phosphorylation and methylation) and the effects of N^{ε}-acetylation on protein/protein interactions [8].

Although both enzymes are involved in the modulation of protein acetylation, HDACs have been extensively studied as therapeutic targets, in particular in the context of cancer, while few studies have been performed on the clinical benefits of regulating HATs.

2.2. The HDAC Family. Up to now, eighteen human HDACs have been identified. They are divided into 4 families according to sequence homologies: class I (HDAC1, -2, -3, and -8) and class II (HDAC4, -5, -6, -7, -9, and -10) are homologous to the yeast histone deacetylases Rpd3 and Hda1, respectively, and share some degree of sequence homology. Class IV HDAC11 has been discovered more recently and shows similarities to both yeast Rpd3 and Hda1. Class I, II, and IV enzymes present a zinc ion-dependent catalytic domain. By contrast, class III enzymes (called sirtuins) are homologous to the yeast protein Sir2 and use NAD as a cofactor [9].

In the past few years, the crystal structure of the catalytic domain of human class I HDAC8 and class II HDAC4 and HDAC7 has been elucidated and several knockout mice targeting various HDACs have been generated, thus providing insights into their structure and physiological functions [10–13]. The diversity of HDACs suggests differential roles for the various classes of enzymes depending on tissues or cell lines. Accordingly, HDACs have been linked to cell cycle and proliferation and to the differentiation of various tissues. In addition to these physiological roles, the HDAC family has been involved in the physiopathology of human diseases including cancer. Fusion proteins containing HDACs complexes as well as deregulation of protein acetylation and/or HDACs expression have indeed been shown for various hematopoietic or solid tumors [14, 15]. Such findings have long encouraged the development of HDAC inhibitors as anticancer agents.

2.3. HDAC Inhibitors. Sodium butyrate (NaBu) was the first HDAC inhibitor (HDI) to be discovered in the late seventies, being initially found to have antitumor activity by inducing cell differentiation. Since then, various HDIs with different structures and potencies have been synthesized or purified from natural sources, and their effects as anticancer drugs are now widely documented. In 2006, suberoylanilide hydroxamic acid (SAHA or Vorinostat) was the first HDI approved by the FDA for the treatment of cutaneous T-cell lymphoma [16]. Today, the development of HDIs for the treatment of cancer is still ongoing and 80 phases I and II clinical trials are currently underway to validate these drugs alone or in association with other therapies in patients with hematological or solid tumors (see Section 6) [17, 18].

Different studies using cDNA array approaches have shown that around 10% of genes are modulated by HDIs,

with differences in the genes altered linked to the cell model, the time of culture, the concentration, and the HDIs used [19, 20]. Nevertheless, HDIs have been shown to have potent antitumor effects *in vitro* and *in vivo* on various cancer types affecting tumor cells at multiple levels: induction of cell cycle arrest, apoptosis and differentiation, inhibition of angiogenesis, inhibition of cell migration and invasion, and increase in antitumor immunity, response to radio- and chemotherapies (for reviews see [14, 21, 22]).

One of the challenges for the next years will be the development of more selective HDIs that would target specific HDAC isoforms to offer the patients the best therapeutic responses with the lowest toxicity. Specific HDIs have thus been described targeting class I HDACs and class II HDACs or HDAC8, some of them being tested in clinical trials, such as class I-specific MGCD0103 (Mocetinostat) in Hodgkin lymphoma [23]. Another challenge will be to search for biomarkers of clinical response to HDIs [24]. Some biomarkers have already been proposed such as histone H3 and H4 acetylation in tissues or peripheral blood mononuclear cells, HDAC2 tissue expression [25], gene expression profiles [26], or more recently expression of HR23B, a protein involved in the targeting of ubiquitinylated proteins to the proteasome [27]. Despite encouraging results, the identification of potential biomarkers of response to HDIs is critically needed for future trials that will combine these drugs with endocrine therapy.

3. HDACs and Breast Cancer

3.1. HDAC Expression in Breast Cancers. HDAC expression in breast tumors has not been described for all members of the HDAC family, but mostly concerns class I HDAC1, -2, and -3 and class IIb HDAC6 at the protein and/or mRNA levels. Analysis of their prognostic significance in breast carcinoma has been performed in some studies (see below and [15] for a review).

Regarding mammary tumor progression, Suzuki et al. [28] reported a marked reduction in histone acetylation from normal mammary epithelium to ductal carcinoma *in situ* (DCIS) whereas most cases showed similar levels of acetylation in DCIS as compared to invasive ductal carcinoma. This suggests that alterations of histone acetylation are an early event in breast tumor progression. The authors also described a significant but smaller decrease in HDAC1, HDAC2, and HDAC6 protein levels during tumor progression. Greater reductions in HDAC1 protein levels were observed from normal to DCIS in estrogen-receptor negative and high-grade breast tumors (Table 1). According to the authors, such discrepancy (i.e., concomitant decrease in HDAC expression and histone acetylation) could be linked to the relative activities of both HATs and HDACs, as altered expression of HATs has been described in various cancers. It is also possible that the expression of other HDACs, not analyzed in this study, is increased during breast cancer progression, thus encountering for the global reduction in histone acetylation.

Analyzing invasive breast carcinoma, Krusche et al. detected HDAC1 protein expression in the nucleus of mammary luminal epithelial cells, but not in basal cells, and observed the presence of nuclear HDAC1 and HDAC3 proteins in 40% and 44% of breast tumors, respectively. They also found that HDAC1 and 3 protein levels correlated significantly with estrogen and progesterone receptors expression and that HDAC1 was an independent prognostic marker of better disease-free survival (DFS), but not overall survival (OS) in patients with invasive breast carcinoma

Table 1: Expression of HDACs in relation with ER.

HDAC	HDAC expression	References
HDAC1	Reduced expression from normal to DCIS (ER- tumors)	[28]
	Correlation with ER expression	[29]
	High level of mRNA in ER+ breast cancers	[30]
HDAC2	Locus deletion in ER+ PR+ breast cancers	[31]
	Underexpressed in ER+ breast cancers	[32–55]*
HDAC3	Overexpressed in ER+ breast cancers	[44]*
	Correlation with ER expression	[29]
	Underexpressed in ER+ breast cancer	[56]*
HDAC4	Overexpressed in ER+ breast cancers	[45, 47]*
	Underexpressed in ER+ breast cancers	[38, 43, 53, 54, 57]*
HDAC5	Overexpressed in ER+ breast cancers	[34, 37, 39, 51, 52, 54, 57, 58]*
HDAC6	Overexpressed in ER+ breast cancers	[45, 47, 51, 53]*
	High level of mRNA in ER+ breast cancer	[59]
	Increased expression in ER+ breast cancer	[60]
	Underexpressed in ER+ breast cancers	[32, 34, 37, 50–52]*
HDAC7	Overexpressed in ER+ breast cancers	[36–38, 40, 44, 45, 47, 53, 58, 61]*
HDAC8	Underexpressed in ER+ breast cancers	[45, 47, 56, 62]*
HDAC9	Underexpressed in ER+ breast cancers	[32, 34, 35, 37–40, 45, 47, 52–54, 58]*
HDAC10	Overexpressed in ER+ ductal breast cancer	[48]*
HDAC11	Overexpressed in ER+ breast cancers	[35, 37–40, 43, 45, 47, 51–53, 56, 58]*
SIRT1	Overexpressed in ER+ breast cancers	[38, 40, 46, 52, 53]*

References with * were obtained from the Oncomine database. Increased or decreased expression was considered statistically significant at $P < .05$.

[29]. Similarly, Zhang et al. analyzed HDAC1 mRNA levels in invasive breast tumors and showed that HDAC1 mRNA levels were elevated in ER and PR positive tumors. They also found that patients with breast tumors displaying high levels of HDAC1 mRNA levels tended to have a better prognosis; however, in this study, HDAC1 was not found to be an independent prognostic marker of either DFS or OS [59].

Several studies have focused on HDAC6 expression in breast carcinoma. The rational for such studies relies on initial results showing that HDAC6 was as an estrogen-responsive gene identified by a microarray approach and that it could modulate mammary tumor cell motility *in vitro* [60, 63]. More recently, Lee et al. also showed that HDAC6 was required for anchorage-independent growth of breast tumor cells [64]. HDAC6 protein was detected in 65% [59] and 77% [60] of breast carcinoma, with a cytoplasmic localization of the protein in both studies. Higher levels of HDAC6 mRNA were found in small, low-grade and ER+, PR+ breast tumors, that is, tumors of better prognosis, but this result was not confirmed at the protein level [59]. When analyzing the different studies, the prognosis significance of HDAC6 expression in invasive breast carcinoma remains controversial [15]. For instance, Yoshida et al. found that high levels of HDAC6 correlated with a negative prognosis survival whereas Zhang et al. showed that high levels of HDAC6 mRNA and protein was linked to improved DFS but not OS [59, 65]. On the other hand, Saji et al. did not link HDAC6 expression to DFS or OS, but found increased expression of HDAC6 in a subgroup of ER-positive, tamoxifen-responsive breast carcinoma.

Fewer studies have been performed on HDAC2 in breast carcinoma although its expression is frequently altered in cancer [14, 15]. In a recent analysis of genetic alterations associated with breast cancer subtypes, Hu et al. found deletions/loss of the HDAC2 locus in ER-positive and PR-positive breast tumors, but no data on HDAC2 expression were presented in this study [31]. HDAC2 mutations resulting in loss of HDAC2 protein and resistance to apoptosis induced by HDIs have been described in colon cancer [66]. However, to our knowledge, no mutations in HDAC2 or any other HDACs have been described in breast cancer.

In addition to these published data, we have performed data mining on HDAC expression in breast cancer using the Oncomine database (Compendia Bioscience, Ann Arbor, MI, USA-www.oncomine.org/). As shown in Table 2, the expression of some HDACs appears to be deregulated in breast cancers as compared to normal breast tissues. This is particularly true for HDAC2 and HDAC11 (overexpressed in cancer) or HDAC4–6 and the class III enzyme SIRT1 (underexpressed in cancer). In addition, the same data mining approach reveals that the expression of HDAC3–7, 10, 11, and SIRT1 at the mRNA level is higher in ER-positive breast cancers (Table 1).

In conclusion, although careful analysis of their expression and consequences in breast cancer have not already been performed for all members of the HDAC family, several studies and Oncomine data analysis underline the potential role of HDAC deregulation in breast tumor progression.

Table 2: Expression of HDACs in breast cancers.

Enzyme (locus)	Total studies	Increased expression in BC	Decreased expression in BC
HDAC1 (1p34)	5	1	1
HDAC2 (6q21)	7	**5**	2
HDAC3 (5q31)	5	1	2
HDAC4 (2q37.3)	6	1	**5**
HDAC5 (17q21)	5	1	**4**
HDAC6 (Xp11.23)	8	0	**3**
HDAC7 (12q13.1)	5	0	1
HDAC8 (Xq13)	5	2	1
HDAC9 (7p21.1)	6	4	2
HDAC10 (22q13.31)	8	0	1
HDAC11 (3p25.1)	6	**3**	1
SIRT1 (10q21.3)	9	0	**3**

From Oncomine database (Compendia Bioscience, Ann Arbor, MI, USA-www.oncomine.org/). Differential expression in breast cancer (BC) versus normal breast tissue was considered significant at $P < .05$. Bold numbers correspond to the strongest deregulations.

3.2. Effects of HDI on Breast Cancers—Experimental Data. In breast tumor models, HDIs have potent antiproliferative effects *in vitro* and *in vivo* and interfere with estrogen signaling regulating ERα and ERβ expression and function (see Section 4 and 5).

Various HDI have been shown to inhibit the proliferation of breast tumor cell lines, as well as normal human breast epithelial cells with IC50 ranging from nM to few mM depending on the HDIs tested [67–69]. This antiproliferative effect was found to be more pronounced in ER positive breast tumor cells than in ER negative ones [70, 71]. In various tumor models, this effect was in part linked to the induction of the cell cycle inhibitor p21 by HDI [70, 72]. Interestingly, p21 gene was found to more sensitive to HDI in ER positive than in ER negative mammary tumor cells, which may explain the observed difference in inhibition of cell proliferation upon HDI treatment according to the ER status [70]. Moreover, HDI were found to decrease Cyclin D1 expression and stability in mammary tumor cells and to inhibit phosphorylation of the retinoblastoma protein [71, 73–76]. Depending on the cell model, modifications of other cell cycle regulators have also been described including p27 and cyclin B1 [76, 77]. Accordingly, HDI induce cell cycle blockade at the G0-G1 and/or the G2/M level [74, 76–79].

Table 3: HDACs and estrogen signaling.

HDAC	Effect on estrogen signaling	References
HDAC1	Recruited to the silenced ERα promoter	[80]
	Present on ER-target gene promoter region	[81]
	Knockdown reduces ERα levels	[82]
	Directly interact with ERα-suppresses ERα activity in 293T cells	[83]
HDAC2	Present on ER-target gene promoter region	[84]
	Knockdown reduces ERα levels	[82]
HDAC3	Present on ER-target gene promoter region	[81]
HDAC4	Present on ER-target gene promoter region	[85]
	Binds the N-terminal A/B domain of ERα	[86]
HDAC5	Repress ERα promoter via MEF2	[87]
	Directly interacts with ERα-Represses ERα activity	
	KO associated with upregulation of ERα signaling	
HDAC6	Knockdown reduces ERα levels	[82]
	Bind the AF2-domain of ERα	[88]
	Regulates ERα degradation via hsp90 acetylation	[89]
HDAC7	Present on the pS2 gene promoter region	[84]
	Represses ERα activity-Required for E2-dependent repression	[90]
HDAC9	Repress ERα promoter via MEF2	[87]
	Directly interacts with ERα-Represses ERα activity	
	KO associated with upregulation of ERα signaling	
SIRT1	Deacetylates ERα *in vitro*	
	Knockdown reduces ERα levels	[91]

Most of these studies have been performed using HDI of broad range specificity. Recently, Duong et al. showed that inhibition of class II HDACs, using specific chemical compounds, also led to inhibition of mammary tumor cells proliferation in a dose-dependent manner, with higher potency in ER-positive than in ER-negative cell lines. In this study, specific inhibition of class II HDACs induced p21 expression, leading a cell-cycle blockade at the G0-G1 level [74]. Thus, although class II HDACs have been linked to cell differentiation, they may also be involved in cell proliferation, at least in this tumor model.

In vitro, HDI were found to induce apoptosis in breast tumor cells expressing or not ERα [74, 77, 92]. Depending on the cell type and/or the HDI used, apoptosis was linked to activation of the intrinsic (mitochondrial) and/or the extrinsic pathway. Some studies have shown upregulation of the proapoptotic Bak and Bim members along with a downregulation of the antiapoptotic survivin, XIAP and Bcl2 proteins in breast tumor cells [79] whereas others have found strong upregulation of the death receptors upon HDI [92, 93]. In addition, HDIs can efficiently sensitize breast cancer cells to TRAIL-mediated death signaling *in vitro* and in preclinical *in vivo* models [77, 79, 94–96] and can significantly increase the apoptotic effects of various drugs targeting breast tumors.

HDI are also involved in cell differentiation. For instance, Davis et al. showed that NaBu induced cell differentiation in normal breast epithelial cell line as well as in breast cancer cells as indicated by accumulation of lipid droplets [67]. Using valproic acid (VPA), Travaglini et al. confirmed this result by measuring milk lipid production in cell cultures and showed that this effect was independent of the mammary cells ER status [76].

The antiproliferative and proapoptotic effects of HDIs observed *in vitro* were confirmed in preclinical mice or rat breast cancer models [68, 95, 97, 98]. HDIs were indeed shown to have anti-tumor activity *in vivo*, alone or in combination with other therapies, by inhibiting tumor growth or inducing tumor regression depending on the models, and this was found for ERα-expressing [68] as well as ERα-negative [98] breast tumor models. Interestingly, Hirokawa et al. further showed that the class I-specific HDI FK228 (depsipeptide or Istodax) was able to inhibit the growth of tamoxifen-resistant MCF-7 xenografts in nude mice (see below clinical studies) [97]. More recently, Palmieri et al. found that Vorinostat prevented the development of brain metastasis using a preclinical model of triple-negative breast cancer [99].

Taken together, these preclinical studies indicate that HDI have anti-tumor effects in breast cancer, targeting ERα-positive and ER-negative cells as well as the most aggressive mammary tumor types (tamoxifen-resistant and triple-negative tumors).

4. Regulation of ER Expression by HDACs

During the last decade, several groups have investigated the mechanisms by which HDACs regulate ER expression in breast cancer cells. These studies, which mostly concern ERα, have highlighted the multiplicity of the regulations involved (see Table 3 and Figure 1).

4.1. Negative Regulation of ERα Expression. In several ERα-expressing human cancer cells from different origin (breast, endometrium, ovary…), treatment with HDAC inhibitors such as trichostatin A (TSA), Vorinostat, FR901228, HC-toxin, VPA, LBH589 (Panobinostat), or NaBu produced a marked decrease in ERα expression at the mRNA and protein levels, which is independent of the presence or absence of ER ligands [82, 100, 101]. The mechanisms of this effect seem to involve different types of regulation which take place both at the transcriptional and posttranscriptional levels.

4.1.1. At the Transcriptional Level. A first level of inhibition of ERα expression takes place at the transcriptional level. Indeed, several studies have reported a decrease in ERα mRNA accumulation upon treatment with various HDAC inhibitors [100–102]. Concomitant treatment by TSA and cycloheximide, a protein synthesis inhibitor, did not affect the observed repression of ERα mRNA accumulation, suggesting a direct role for HDAC activity in the maintenance of

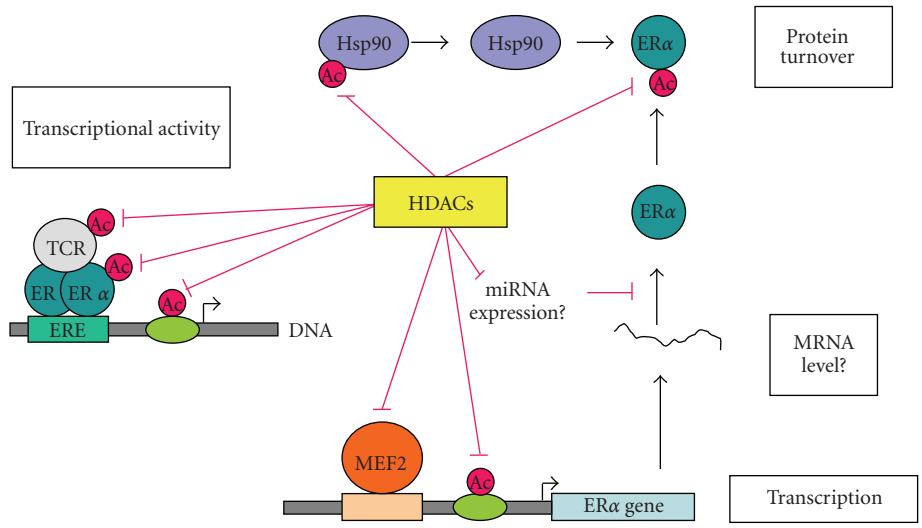

Figure 1: HDAC and estrogen signaling. HDACs are involved in estrogen-genomic mechanisms mediated in part through estrogen response element (ERE) targeting. ERα and numerous transcriptional coregulators (TCR) are acetylated proteins (acetyl mark is represented by a red circle) which are substrates for HDAC. By removing acetyl marks, HDAC regulate the transcriptional activity of ERα. HDACs also regulate the expression of ERα at the transcriptional level, in part through the control of MEF2 activity. They also modulate the level of ERα mRNA by a mechanism which might involve miRNA expression. Finally, HDACs also regulate ERα stability, and one mechanism appears to involve Hsp90 acetylation.

ERα transcription [73, 103]. Our unpublished data indicated that the stability of ERα mRNA was not significantly modified in ERα-expressing MCF7 cells treated with TSA.

Transcription of the ERα gene is driven by several different promoters which span over 300 kb (for a review, see [104]). In MCF7 and Ishikawa cells, levels of transcripts originating from promoters A, B, and C were all decreased upon TSA treatment. In endometrial cells, this effect was associated with a reduction of the amount of acetylated H3 and H4 on the three promoters confirming the inhibition of their activity [103].

Interestingly, both HDAC5 and HDAC9 (class II enzymes) have been shown to participate in the regulation of the ERα promoter by repressing the activity of MEF2 [87]. A recent study also reported that inhibition of SIRT1 by sirtinol or invalidation of the *SIRT1* gene was associated with a decrease of ERα expression in mammary cells which was the consequence of a transcriptional regulation [91]. Finally, several other HDACs could be involved in this negative regulation since a reduction in ERα expression was observed upon depletion of either HDAC1, HDAC2, or HDAC6 by siRNA in T47D breast cancer cells [82]. In these cases, the levels of regulation remain to be defined.

4.1.2. Regulation of mRNA Stability. A regulation of ERα mRNA expression could also take place at the posttranscriptional level since it has been reported that TSA when administered in combination with 5-Aza2′-deoxycytidine (5-azadC or Dacogen) could decrease ERα mRNA stability through altered subcellular localization of the RNA-binding protein, HuR [105]. In addition, several miRNAs (miR-206 for instance) have recently been reported to target ERα mRNA (for a review, see [106]), and some of these miRNAs could be HDI induced and involved in the decreased expression of ERα.

4.1.3. Regulation of Protein Stability. Several data support a regulation at the posttranslational level. Results showing that the MG132 inhibitor relieves the TSA-mediated decrease of ERα accumulation ([102] and R. Margueron, unpublished observations) provide evidence for a direct or indirect involvement of the proteasome system in this regulation.

At the molecular level, one mechanism could involve the Hsp90 chaperone complex which binds to and maintains ERα in a ligand-binding conformation [107] and whose inhibition results in ubiquitin-mediated degradation of ERα by the proteasome [108]. Indeed, the chaperone function of Hsp90 has been shown to depend on HDAC activity, and HDAC6-specific inhibition leads to hyperacetylation of Hsp90, decreases its association with ERα, and results in ERα ubiquitination and depletion [89]. Data reported by Yi et al. confirmed that inactivation of the heat shock protein-90 (Hsp90) is involved in Vorinostat-induced ERα degradation and that the ubiquitin ligase CHIP (C-terminal Hsc70 interacting protein) enhances Vorinostat-induced ERα degradation [109].

By contrast, a recent paper indicated that TSA-induced acetylation of ERα in T47D cells was accompanied by an increased stability of the ERα protein [110]. Interestingly, in this study, overexpression of p300 also induced acetylation and stability of ERα by blocking ubiquitination.

4.2. Reexpression of ERα in ER-Negative Cells. An interesting aspect concerning ERα expression and HDAC inhibition

deals with data obtained in ERα-negative human breast cancer cells. The group of N. E. Davidson initially reported that treatment of such cells by TSA [111] or VPA [112] could lead to a dose- and time-dependent reexpression of ERα mRNA. In addition, TSA [113] or Scriptaid (another hydroxamic acid with HDI activity) [114] could potentiate the effect of DNA methyltransferase inhibitors such as 5-azadC on the reexpression of the ERα protein.

In ERα-negative MDA-MB-231 cells, the silenced ERα promoter has a repressive chromatin structure associated with DNA-methyltransferase 1 (DNMT1), DNMT3b, HDAC1, and H3-K9 methylation [80, 115]. The molecular mechanisms by which HDI reactivated silenced ERα gene in MDA-MB-231 cells include chromatin structure reorganization: for example, TSA induces acetylated histone H3 and H4 but reduces HDAC1 and H3-K9 methylation at the ERα promoter [80]. Chromatin immunoprecipitation analysis showed that binding of TFAP2C to the ERα promoter was blocked in ERα-negative cells, but that treatment with 5-azadC/TSA enabled TFAP2C and polymerase II binding [116].

In the ERα-negative human breast cancer cell lines MDA-MB-231 and MDA-MB-435, treatment with Panobinostat at 100 nM for 24 hours restored ERα mRNA and protein expression without a concomitant demethylation of the ERα promoter CpG island [117]. Importantly, the expression of ERα mRNA was sustained at least 96 hours after withdrawal of Panobinostat treatment. The same laboratory reported that reexpression of ERα protein upon treatment with Vorinostat, another pan-HDI, was coupled with loss of EGFR in MDA-MB-231 cells, which overexpress EGFR [118].

4.3. Regulation of ERβ Expression. Fewer studies have described the effects of HDACs or HDAC inhibition on ERβ expression in cancer cells. Recent studies reported the upregulation of ERβ expression in ovarian [119] and prostate cancer cells at the mRNA and protein levels [120].

In breast cancer cells, HDI have been shown to clearly increase ERβ expression at least at the transcriptional levels in both ERα-negative [121] and ERα-positive cells [122]. Moreover, treatment with HDI was found to strongly enhance the transcriptional activity of ERβ [121, 122]. According to Jang et al. [121], ERβ induction upon HDI treatment could be involved in the sensitization of ERα-negative breast cancer cells to hormonal therapy (see below).

5. Roles of HDACs and HDIs in ER Signaling

In addition to their role in the regulation of ER expression, a large set of data also support a major role of HDACs in the control of transcriptional signaling by estrogens (Table 3 and Figure 1).

5.1. Several Components of the ER Signaling Pathway Are Acetylated Proteins. Several types of posttranslational modifications have been described as targeting nuclear receptor (for a review, see [123]) and could modify several parameters such as DNA-binding activity, interactions with positive or negative transcriptional regulators, and stability or subcellular localization of the protein. It has also been shown that ERα as other nuclear receptors could be modified at the posttranslational level by addition of acetylated groups on lysine residues [124, 125]. The group of Pestell initially reported that ERα was acetylated *in vitro* by p300 on two lysine residues located in the hinge region of the protein [126]. Mutation of the two amino acids resulted in an enhancement of hormone sensitivity, suggesting that acetylation normally decreases ligand response. More recently, using a variety of biochemical and cell-based approaches, Kim et al. identified two other lysines within ERα (K266 and K268) as primary targets of NCOA2-dependent p300 acetylation [127]. In this study, acetylation of these residues increased DNA-binding activity of the receptor in gel shift assay and ligand-dependent transactivation in transient transfection experiments. It should be noted that K266/268 are not conserved in ERβ, and until now, acetylation of this nuclear receptor has not been reported. Moreover, the specific deacetylases which remove these marks are still mostly unknown although preliminary data suggested that both the NAD^+-dependent SIRT1 enzyme or TSA sensitive HDAC are able to deacetylate ERα *in vitro*.

In addition to nuclear receptors themselves, several other factors involved in estrogen signaling are acetyltransferase substrates. Indeed, several nuclear receptor coregulators such as ACTR/SRC3, SRC-1 and TIF2 [128], PGC1α [129], RIP140/NRIP1 [130], or HDAC1 [131] are also modified by acetylation, and this highlights the complexity of the effects resulting from the modulation of the acetylation balance in response to HDAC inhibition (see below).

5.2. Direct and Indirect Recruitment of HDACs by ERs. Using the chromatin immunoprecipitation technique (ChIP), the presence of several HDACs has been detected on various ER-target promoters. For instance, both HDAC1 and HDAC7 are present on the *pS2* gene promoter region [84, 90]. In the presence of partial antiestrogens such as tamoxifen or raloxifen, HDAC2 and HDAC4 [85] or HDAC1 and HDAC3 [81] have been evidenced on the pS2 promoter or on other estrogen target promoters such as the *c-myc* or *cathepsin D* genes.

Several studies have reported different modes of HDAC recruitment by ERα. A direct association of HDAC1 with the DNA binding and AF2 domains of ERα has been demonstrated both by GST pull down and coimmunoprecipitation [83]. A more recent study failed to confirm this result, but it described the *in vitro* interaction with class II HDAC5 and 9 [87]. Finally, HDAC4 was shown to bind the N-terminal A/B domain of ERα [86] and, more recently, the physical E2-dependent association of HDAC6 with the AF2-domain of ERα expressed as a fusion with a membrane targeting signal was reported [88].

In addition to direct association with the receptor, HDACs could also be indirectly recruited to target promoters. Indeed, a huge number of ER transcription coregulators which bind the receptor in the presence of agonists (for instance RIP140 [132–134], SHP [135], and REA [136]) or in the presence of antagonists (such as NCoR or SMRT

[137, 138]) are able to recruit different HDACs. This indirect recruitment of HDACs belonging to the different classes of enzymes also increases the complexity of the relationship between acetylation and estrogen signaling.

5.3. Role of HDACs in the Control of ER Transcriptional Activity. As already mentioned, HDACs not only regulate ER expression *via* intricate mechanisms but also participate in the formation of ER transcriptional complexes. Different approaches, based on the modulation of their enzymatic activity or their expression levels, have indeed investigated whether or not they actively participate in the regulation of estrogen transcriptional signaling.

5.3.1. Effect of HDAC Inhibitors on ER Transcriptional Activity. Using MCF-7 or HeLa cells transfected with an ERE-containing luciferase reporter plasmid, we showed that inhibition of HDAC activity increased transactivation of both ERα and ERβ in the presence of agonist ligands [122]. In ERα-expressing cells, HDAC inhibitors also abolished the transrepression ability of partial antiestrogens and increased their agonist activity through a mechanism which requires the reduction of ERα expression [100]. This effect was not obtained with class II selective inhibitors [74].

5.3.2. Class I HDAC. Very few data are available concerning the role of class I HDACs in the control of ER activity. Kawai et al. suggested that HDAC1 overexpression in 293T cells suppresses the E2-dependent transcriptional activity of ERα [83]. However, it is difficult to ascertain that this effect was indeed due to a modulation of receptor transactivation and not a simple reflect of a strong decrease in receptor levels. Using a Knockdown strategy, it has been reported more recently that selective depletion of HDAC2 in T47D cells resulted in a decrease in PR levels but it is unclear whether this is due to a modulation of ERα activity [82].

5.3.3. Class II HDAC. Class II HDACs have also been demonstrated to act as important modulators of ERα activity. A recent paper from the Olson's laboratory has demonstrated a role for HDAC5 and 9 in cardioprotection mediated by ERα [87]. Upregulation of ERα signaling in female mice deleted for either HDAC5 or -9 dramatically diminishes cardiac dysfunction following myocardial infarction. This cardiac protection appears to be due, at least in part, to the induction of neoangiogenesis in the infarcted region via upregulation of the ER target gene Vascular Endothelial Growth Factor (VEGF). These findings reveal a key role for MEF2 and class II HDACs in the regulation of cardiac ER signaling and the mechanisms underlying the cardioprotective effects of estrogen. Accordingly, van Rooij et al. showed that HDAC5 and HDAC9 repressed estrogen-dependent transcriptional activation by ERα.

Another class II HDAC, HDAC7, seems to play a unique role in E2-dependent repression of gene expression [90]. Indeed, in transient transfection experiments, increasing concentrations of HDAC7 inhibited ERα activity in a dose-dependent manner although the catalytic activity of HDAC7 did not appear to be required. More interestingly, knockdown of HDAC7 using siRNA resulted in complete loss of E2 repression of different target genes such as RPRM, CXCR4, or NEDD9.

HDAC4 has also been shown to regulate transactivation by ERα in the presence of either estradiol or antiestrogens such as tamoxifen or raloxifen [86]. Overexpression or silencing of HDAC4 impacted (negatively or positively) ERα activity in a cell type-specific manner.

Finally, HDAC6 may also participates in rapid action of estrogens (the so-called nongenomic action of ER), since it has been proposed that upon estrogen stimulation, a complex containing ERα and HDAC6 is rapidly translocated at the membrane, where HDAC6 could functionally interact with the microtubule network and cause tubulin deacetylation [88]. However, analysis of E2-induced tubulin deacetylation remains to be analyzed in HDAC6 knockdown or knockout models.

5.3.4. Class III HDAC. A single study has investigated whether class III HDACs play a role in the regulation of ERα activity [91]. This work demonstrated that sirtinol, an inhibitor of the SIRT1 deacetylase activity, inhibited estrogen-dependent gene transcription in different breast cancer cell lines. This observation could be related to previous data showing that the loss of SIRT1 expression in female mice is associated with a defect in mammary gland development [139].

6. Effects of HDIs on HT Response

6.1. In Vitro Experiments

6.1.1. ERα-Positive Breast Tumors. As discussed briefly in Section 3, several HDIs have been shown to reverse acquired hormone resistance in ERα-positive breast cancer cells lines. For example, Hirokawa et al. showed that treatment of tamoxifen sensitive and insensitive MCF-7 cells with depsipeptide not only inhibited tumor cells proliferation *in vitro* and *in vivo* but also abrogated tamoxifen-resistance. These data suggest that HDIs could be useful for the treatment of breast cancers which become resistant to currently used estrogen antagonists such as tamoxifen [97]. Moreover, Hodges-Gallagher et al. suggested that this resensitization upon HDI, was not limited to tamoxifen, but could also be observed with aromatase inhibitors [140]. The mechanisms by which HDIs may reverse acquired hormone resistance in ERα-positive breast tumor cells are probably complex and may involve different mechanisms according to tamoxifen or antiaromatase treatments. For instance, inhibition of HDAC enzymatic activity modulates ERα and ERβ expression and may control the relative agonist activity of partial antiestrogens (see above) [100]. Moreover, HDIs block the activation of PAK1 [97], a growth factor pathway, which may contribute to tamoxifen resistance [141]. In addition, De Los Santos et al. showed that a combination of Vorinostat and fulvestrant (a pure steroidal anti-estrogen also known as ICI 182.780) was more potent than fulvestrant alone

to regulate the expression of cell cycle proteins, to induce downregulation of ERα, and to decrease the transcription of ERα target genes in MCF-7 breast cancer cells [75].

To our knowledge, no preclinical study has been published evaluating the effects of a treatment combining HDI and HTs (tamoxifen or aromatase inhibitor) on the delay of endocrine acquired resistance in ERα-positive breast cancer cells or in xenografts.

6.1.2. ERα-Negative Breast Tumors. As stated in Section 1, HTs are ineffective in ERα-negative breast carcinoma. Preclinical studies have shown that ERα repression in these tumors may be due to epigenetic modifications. The discovery of HDACs recruitment in ERα gene promoter provides a rationale for inhibiting HDACs activity to release ERα transcriptional repression as a potential therapeutic strategy (see Section 4). Several laboratories have reported that HDIs could reverse hormone resistance in human ERα-negative breast cancer cells. The combination of TSA and 5-azadC, a DNMT inhibitor, restored sensitivity to tamoxifen in MDA-MB 235 human breast cell lines and in nude mice. This effect was due to the reexpression of a functional ERα and the level of tamoxifen growth suppression paralleled that of ERα reexpression [98]. Similarly, restoration of ERα expression by the pan HDI Panobinostat in MDA-MB 231 cells enhanced sensitivity to 4-hydroxy-tamoxifen (an active metabolite of tamoxifen) [117]. So, reexpression of ERα might at least in part mediate the antiproliferative effect of tamoxifen, although other mechanisms are likely to be involved. For instance, Jang et al. observed that pretreatment of ERα-negative MDA-MB 231 and Hs578T breast cancer cells with TSA alone could restore response to tamoxifen whereas no apparent ERα could be detected in the treated cells. The mechanism involved might be linked to the upregulation of ERβ expression [121]. Other mechanisms may involve modulation of growth signaling pathways. Zhou et al. indeed showed that Panobinostat allowed a decrease in EGFR expression together with the suppression of EGF-initiated signaling pathways involved in the loss of tamoxifen antiestrogenic effect including phosphorylated PAK1, p38MAPK, and AKT [142]. Treatment of ERα-negative and hormone resistant human breast cancer cells MDA-MB 231 or xenografts with the HDI SNDX275 (MS275 or Entinostat) led to an upregulation of ERα and aromatase expression. Importantly for clinical perspectives, these up regulations resulted in a sensitization of MDA-MB 231 cells and xenografts to a treatment with an aromatase inhibitor (Letrozole). The same authors reported inhibition of growth, cell migration, and formation of micrometastasis by treatment with Entinostat plus letrozole (Sabnis et al., communication at the San Antonio Breast Cancer Symposium 2009). Altogether, these results provide the basis of therapies combining tamoxifen (or aromatase inhibitors) and HDIs for the treatment of hormone refractory ERα-negative breast cancer and open a new perspective for the management of ERα-negative breast cancer.

6.2. Clinical Trials. Several HDIs have been used in clinical trials for the treatment of hematological malignancies (with great success in most studies) and for solid tumors although with less impressive clinical efficacy. Concerning breast cancer, the HDIs Vorinostat, Panobinostat, and Entinostat are currently being tested in patients with advanced and/or metastatic disease. The most common adverse events of HDI treatment include fatigue, nausea, diarrhea, thrombocytopenia, and lymphopenia [17, 18]. In metastatic breast cancers, HDIs have limited efficacy as single agents. For example, a phase II study evaluating Vorinostat alone was stopped early due to the absence of objective responses [143]. A phase II study evaluating the efficacy of Panobinostat alone in HER2-negative women with locally recurrence or metastatic breast cancer is still ongoing (NCI clinical trial protocol NCT00777049; see http://www.cancer.gov/).

As stated above, HDIs as single agents have shown limited activity in patients with solid tumor malignancies, thus prompting clinicians to use these compounds in combination with other therapies acting on other targets than HDACs. Such drug combinations interfering with both HDACs and growth factor pathways (HER2, EGFR, BCR-ABL, etc.) have already shown promising anticancer effects *in vitro* [144–146]. Moreover, studies combining an HDI with chemotherapy (Munster et al., communication at the San Antonio Breast Cancer Symposium 2009) or trastuzumab, an HER2 monoclonal antibody, (NCI clinical trial NCT00567879) are ongoing. Preliminary results, in heavily pretreated women who had either relapsed or progressed during trastuzumab combined therapies proved to be promising: Vorinostat or Panobinostat were indeed shown to reverse trastuzumab resistance.

Since there is a good rationale for combining HDI with HTs, several trials involving the combination of a pan- or a selective HDI and an HT (tamoxifen, or aromatase inhibitor) are ongoing (see Table 4). At the SABCS 2009, Munster et al. reported preliminary results of a phase II study, combining Vorinostat (400 mg daily for 21 days of 28 days) and tamoxifen (20 mg daily), in women with ER-positive metastatic breast cancers whose tumor progressed under aromatase inhibitors treatment. Moreover, patients could have received up to 3 chemotherapy regimens for metastatic disease. In the first 42 patients enrolled in this trial, 34 were assessable for efficacy to the date of the report: 7 (21%) had an objective response, and 4 (12%) had stable disease for ≥6 months. These results are encouraging if one considers that the expected response rate for tamoxifen alone at this stage of disease is less than 10% and that the trial of Luu et al., evaluating Vorinostat alone in metastatic breast carcinoma reported no objective responses [143].

Moreover, a preliminary phase II data suggest that Entinostat, a class I selective HDI, may resensitize invasive ER-positive breast cancer patients progressing under aromatase inhibitors although 80% of these patients had already received tamoxifen (Yardley et al., communication at the San Antonio Breast Cancer Symposium 2009). In metastatic breast carcinoma, additional trials involving the combination of an HDI including Vorinostat and Panobinostat with aromatase inhibitors are underway.

Table 4: Clinical trials combining HDI and HT in advanced/metastatic ER-positive breast carcinoma.

HDI	HT	Phase	Patients	Preliminary results	Reference
Vorinostat (SAHA) 200 mg twice daily 14 d/21	Tam	II	AI resistant HR+	34 patients evaluated 21% OR 12% SD	Munster et al. Poster # 6100 SABCS 2009
Entinostat (SNDX275) 5 mg weekly	AI (Exemestane)	II	AI resistant HR+	10 patients with >2 cycles CB > 6 months (1 case) CB > 5 months (2 cases)	NCI clinical trial NCT00676663 Yardley et al. Poster # 6111 SABCS 2009
Vorinostat (SAHA) 200 mg twice daily 14 d/21	AI (anastrozole letrozole, OR exemestane)	II	AI resistant	Ongoing	NCI clinical trial NCT01153672 Linden et al.
Panobinostat (LBH589) once daily on days 1, 3, 5 during 28 d	AI (Letrozole)	I/II	HR−/+ (phase I) triple-negative disease (phase II)	Ongoing	NCI clinical trial NCT01105312 Tan et al.
Vorinostat (SAHA)	Tam	II	Stage I–III (treatment for 2 weeks before surgery)	Ongoing	NCI clinical trial NCT01194427 Stearns et al.

AI: aromatase inhibitor. OR: objective response. SD: stable disease. CB: clinical benefice. HR: hormone receptor. SABCS: San Antonio Breast Cancer Symposium 2009.

7. Perspectives and Conclusions

In conclusion, analysis of the links between ERs and HDACs underline multiple and intricate levels of interactions. Such complexity is reflected in breast tumorigenesis as HDI have opposite effects on ERα expression in ER-positive and ER-negative breast tumor cells. Several important questions remain to be answered in order to further appreciate these transcriptional and cellular crosstalks: what are the roles of the different HDAC isoforms? Do HDAC regulate ER signaling independently of their catalytic activity? Are sirtuins key players in these crosstalks? Do HDACs regulate miRNA which target ER signaling? What is the exact role of HDACs in hormone resistant breast tumors?

Despite these open questions, HDI in combination with chemotherapies or hormonal therapies led to promising results in the context of hormone-resistant breast cancers, and several clinical trials are still ongoing in this field. Further studies are needed to define the best combinations of HDI therapies for the most aggressive breast tumors and to better understand how they impact hormone-resistant breast cancers.

Moreover, as stated in this paper, much work is being done today to define biomarkers that would identify which tumors will better respond to HDI-combined treatments. In the field of breast cancer, it will certainly be important to define biomarkers for the reexpression of ERα in ER-negative tumors along with predictive biomarkers of anti-estrogen sensitivity in hormone resistant tumors in response to HDI treatments. Finally, few data have been performed on triple negative breast tumors, which represent one of the most aggressive groups of breast cancers or in the familial forms of BRCA1 mutated tumors. The role of HDACs and the impact of HDIs in these particular groups could possibly open new therapeutic strategies.

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