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# Histone deacetylase inhibition and estrogen signalling in human breast cancer cells

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**Abstract**

Estrogens are steroid hormones, which act through specific nuclear estrogen receptors (ERα and ERβ) and are important regulators of breast cancer growth. These receptors control gene expression by recruiting transcriptional cofactors that exhibit various enzymatic activities such as histone acetyltransferase or histone deacetylase (HDAC) which target histone as well as non-histone substrates. The ERα itself and some of the transcriptional regulators have been shown to be acetylated proteins. Research performed over the last decade has highlighted the role of HDAC inhibitors (HDACi) as modulators of transcriptional activity and as a new class of therapeutic agents. In human cancer cells, inhibition of HDACs controls the expression of the ERα gene and the transcriptional activity in response to partial antiestrogens such as 4-hydroxytamoxifen. Various HDACi strongly inhibit breast cancer cell proliferation and ERα-negative (ER−) appear less sensitive than ERα-positive (ER+) cell lines. $p21^{WAF1/CIP1}$ gene expression, in relation with ERα levels, could play a role in this differential response of breast cancer cells to hyperacetylating agents.


*Keywords:* Histone deacetylase; Estrogen receptor; Antiestrogen; Breast cancer; Transcription; Cell proliferation

## 1. Introduction

Estrogens are key regulators of cell growth and differentiation and therefore implicated in the control of growth and maintenance of target tissues including the genital tracts, mammary gland, cardiovascular and skeletal systems. A large number of clinical and experimental data have highlighted the crucial role of these hormones in the development of female gynaecological cancers (breast, endometrium, ovary) [1].

The estrogen dependency of these tumours has been successfully exploited to develop an antihormonal chemotherapy [2]. Unfortunately, treatments with antiestrogens are not effective in all tumours and acquired resistance results in patient relapse [3].

The effects of estrogens and antiestrogens are primarily mediated through the interaction with specific nuclear estrogen receptors (ERα and ERβ) acting as ligand-dependent transcription factors [4]. These two receptors share common structural features exhibiting variable degrees of homology. The COOH-terminal part is a multifunctional ligand binding domain which also allows dimerisation and transcription activation in response to hormone. Ligand binding creates a new interface, which allows the interaction of a huge variety of transcription regulators.

These coactivator proteins act as multimolecular complexes either by stabilising the formation of a preinitiation complex or by disrupting the chromatin structure and facilitating the access to DNA for other transcriptional regulators. Transcription activation by ER implicates the recruitment of several coactivators such as CBP/p300, p/CAF and members of the SRC1 family. These coactivators have been shown to possess acetyltransferase activity, which, by acetylating lysines at the carboxy-terminus of histones, destabilises nucleosomes and facilitates transcription. Histone acetylation appears in fact to be one component of a more global histone code involving other

*Abbreviations:* HAT, histone acetyltransferase; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; ER, estrogen receptor; OHTam, 4-hydroxytamoxifen; TSA, trichostatin A; SAHA, suberoylanilide hydroxamic acid

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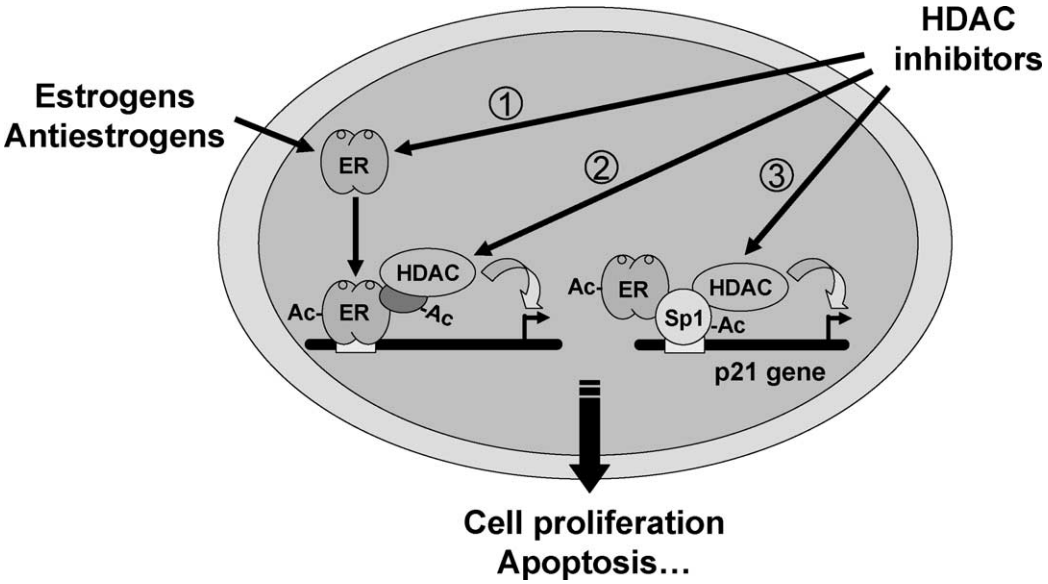


Fig. 1. Cross-talks between HDACi and ER ligands. The interferences between ERα and HDACs described in this review are schematically represented. The cartoon shows the effects of HDACi on ERα expression and acetylation (1), on ERα transcriptional activity in response to partial antiestrogens (2), and on $p21^{WAF1/CIP1}$ expression, which is differentially regulated in ER+ and ER− breast cancer cells (3).

types of post-translational modifications such as methylation and phosphorylation [5].

The steady state level of histone acetylation is regulated by the activities of two types of enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC). Alterations of these enzymes (mutation, translocation, amplification) have been described to be associated with the apparition of various cancer types mainly myeloid leukaemia or lymphoma [6]. Several specific HDAC inhibitors (HDACi) such as trichostatin A (TSA), SAHA, apicidin or FR901228 have been shown to modify reversibly or irreversibly the balance between HAT and HDAC activities [7]. These compounds, which dramatically inhibit cell growth and induce differentiation and apoptosis are currently evaluated in clinical trials [8].

In this review, we will discuss recent results obtained on the role of acetylation in estrogen signalling (Fig. 1) and describe the effects of HDACi on ERα levels, on its transcriptional response to antiestrogens and on the proliferation of breast cancer cells.

## 2. Regulation of ERα expression by HDACi

Upon HDAC inhibition, and despite the strong acetylation of bulk histones, only a small subset of genes has been shown to be significantly regulated either positively or negatively [9,10]. Some of these genes, such as $p21^{WAF1/CIP1}$, are involved in the control of cell growth and could therefore mediate the cellular effects of HDACi [11].

In breast cancer cells, TSA, a potent and reversible HDACi produced a strong decrease in ERα accumulation independently of the presence or absence of ER ligands [12]. The effect was dose-dependent and was not restricted to TSA since a similar regulation was obtained with different HDACi, structurally related or not to TSA, such as SAHA (Fig. 2A), FR901228 or HC-toxin. This observation thus confirmed previous data obtained using sodium butyrate, another HDACi with lower efficiency and specificity [13]. The negative regulation was observed by Western blot in different human breast cancer cell lines such as MCF-7, T47-D, ZR75-1 and CAMA-1 and the same inhibition occurred in ERα-positive ovarian (PEO4) and endometrial (Ishikawa) cells.

The levels of this regulation together with the exact underlying molecular mechanisms remain to be determined. Results showing that the MG132 compound

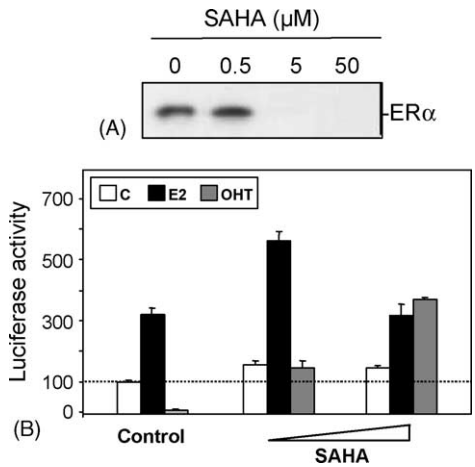


Fig. 2. Effect of HDACi on ERα expression and transcriptional activity. (A) MCF-7 cells were treated for 24 h with increasing concentrations of SAHA and ERα protein expression was detected by Western blot; (B) MELN cells were treated for 24 h with increasing concentrations of SAHA in the absence of ligand (C, white boxes) or in the presence of 10 nM 17-β-estradiol (E2, black boxes) or OHTam (OHT, grey boxes). Results are expressed as relative luciferase units (percentage of values obtained in the absence of ligand and HDACi).

relieves the TSA-mediated decrease of ERα accumulation ([14] and R. Margueron, unpublished observations) provide evidence for a direct or indirect involvement of the proteasome system in this regulation. Moreover, our unpublished data suggest that the regulation by TSA takes place, at least partly, at the transcriptional level thus supporting previous observation with sodium butyrate [13]. Transcription of the *ERα* gene is very complex since it involves at least seven different promoters spanning over 300 kb (for a review, see [15]). Our data indicate that, after transient or stable transfection, a reporter construct encompassing 4.48 kb of the proximal 5′ region containing the two first promoters [16] does not significantly respond to TSA. Further experiments are in progress to delineate the region of the *ERα* gene that could support the negative regulation by HDACi and to identify the transcription factor(s) whose acetylation and transactivation might be regulated upon HDAC inhibition.

Another interesting aspect concerning HDAC inhibition and ERα expression deals with data obtained in ERα-negative human breast cancer cells. The group of N.E. Davidson has initially reported that treatment of such cells by TSA could lead to a dose- and time-dependent re-expression of ERα mRNA [17]. This result could be related to the loss of ERα expression, which is observed in ERα-positive breast cancer cells when HDAC1 is stably over-expressed [18]. However, when assessed at the level of the ERα protein, it seems that HDAC inhibition has no effect by itself [19,20]. By contrast, TSA [19] or Scriptaid [20] could potentiate the effect of DNA methyltransferase inhibitors such as 5-aza-2′-deoxycytidine on the re-expression of the ERα protein.

## 3. Acetylation of ERα and transcription cofactors

ERα activity is controlled at the post-translational level by different types of modifications such as phosphorylation, ubiquitination or glycosylation. It has recently been shown that ERα as other nuclear receptors could also be acetylated [21–23]. The group of R. Pestell initially reported that ERα was modified in vitro by p300 on two lysine residues located in the hinge region (between the DNA- and ligand binding domains) [21]. Mutation of the two sites enhanced hormone sensitivity suggesting that acetylation normally decrease ligand sensitivity. Interestingly, the frequent mutation of the lysine at position 303 has recently been demonstrated in premalignant lesions of the breast [24]. Moreover, this acetylation site appears conserved among several members of the nuclear receptor superfamily and acetylation of the androgen receptor has been also evidenced on the same residues [22]. However, in this case, mutation of the acetylation sites abolishes dihydrotestosterone-dependent transactivation of the receptor. Concerning ERα, it remains to determine which parameters are affected upon acetylation, such as for instance receptor stability, intracellular localisation or its ability to interact with coactivators or corepressors.

In addition to nuclear receptors themselves, several other factors involved in hormone signalling are substrates of acetylases. Various components of the basal transcription machinery such as TFIIE and TFIIF [25] have been described as acetylated proteins. Some nuclear receptor transcription cofactors are also modified by acetylation. The first example has been reported by Evans and coworkers for ACTR/SRC3 [26]. This cofactor possesses an intrinsic acetylase activity and is also acetylated by CBP/p300 on three lysine residues located near the first LxxLL motif involved in the interaction with agonist-liganded nuclear receptors. Acetylation of ACTR, which is increased in vivo upon estrogen stimulation, seems to play an important role in transactivation. Upon acetylation, there is a modification of the charge distribution on the receptor binding interface resulting in a decrease in the association of ACTR/SRC3 with ERα. The control of receptor/coactivator association by acetylation could be an important regulatory mechanism in hormone action at the transcriptional level.

More recently, RIP140/NRIP1, another nuclear receptor transcription cofactor, has been identified has an acetylated protein [27]. The p300/CBP-mediated acetylation of RIP140 disrupts its interaction with the corepressor CtBP and could then derepress estrogen-regulated genes.

## 4. Recruitment of HDACs by ERα

Several modes of HDACs recruitment by ERα have been reported. The initial description has been made for the antiestrogen-liganded ERα and involves the N-CoR and SMRT corepressors (see the next section). However, the number of transcription cofactors interacting with ERα and able to recruit HDAC has considerably increased. Interestingly, several modulators of ERα activity, which bind the receptor in the presence of agonists, are now known as HDAC-interacting proteins. We and others have characterised the association of both class I and class II HDACs with RIP140 [28–30]. Other cofactors such as L-CoR [31], MTA1 [32], REA [33] and possibly TIF1α [34] are also able to bind HDACs. Moreover, a direct interaction of HDAC1 with the DNA-binding and AF2 domains of ERα has recently been documented [18], thus increasing the complexity of HDAC recruitment by ERα. The relevance of these interactions has recently been reinforced using the chromatin immunoprecipitation technique (ChIP), demonstrating the presence on the estrogen-activated *pS2* gene promoter, of both HDAC1 and HDAC7 [35].

## 5. Inhibition of HDAC and antiestrogen activity

Antiestrogens such as tamoxifen, which is the most commonly used drug in endocrine therapy to block estro-

gen action [36], exhibit mixed estrogenic and antiestrogenic activities depending on the species, tissue, cell and promoter context [37]. In the presence of 4-hydroxytamoxifen (OHTam), ERα has been shown to interact with the N-CoR corepressor and this binding is decreased by intracellular pathways which switch OHTam from antagonist to an agonist function (in response for instance to forskolin or growth factors) [38]. The relevance of these interactions is supported by transient transfection experiments showing that overexpression of coactivators (SRC1 or L7/SPA) or corepressors (N-CoR or SMRT) controls the activity of antihormones [39–41]. Recent data obtained by ChIP analysis have demonstrated that both SMRT and N-CoR are indeed recruited in the presence of OHTam on ER-regulated target genes such as *pS2* or *cathepsin D* [42]. Moreover, in the presence of partial antiestrogens such as OHTam or raloxifen, HDAC2 and HDAC4 are detected on estrogen target promoters [43], thus indicating that these proteins might play important roles.

Using MCF-7 cells stably transfected with an ERE-containing luciferase reporter plasmid (MELN cells), we have demonstrated that the capacity of partial but not pure antiestrogens to repress ERα activity requires HDAC enzymatic activity [12]. The transrepression of partial antiestrogens (i.e. their ability to decrease ERα activity under the level obtained with unliganded receptor) is abolished by treatment with TSA or other HDACi (Fig. 2B). This effect is independent of the decrease in ERα levels as shown when exogenous receptor is over-expressed upon stable transfection in MELN cells.

By contrast, pure antiestrogens induce a particular conformation of ERα [44] which does not allow the in vitro interaction with the corepressor N-CoR [45]. The inhibitory effect of ICI182780 rather implicates a decrease in ERα stability [46] and/or mobility [47]. Interestingly, in MELN cells where exogenous ERα is constitutively expressed, the transrepression activity of ICI182780 is maintained even in the presence of HDAC inhibition, thus supporting a HDAC-independent mechanism for the inhibitory effect of ICI182780. On the other hand, the transrepression behaviour of ICI182780 is not significantly restored upon exogenous expression of ERβ. This suggests that ERβ stability might not be affected to the same extent in the presence of pure antiestrogens, in support of our recent observation showing a differential effect of ICI182780 on the accumulation of ERα and ERβ proteins [48].

HDACi such as TSA, not only abolish the transrepression ability of partial antiestrogens, but also increase their agonist activity, the latter effect involving a mechanism dependent on the reduction of ERα expression [12]. Previous studies have shown that the relative expression of coactivators versus corepressors is critical for the agonist behaviour of partial antihormones [39,43]. These data altogether suggest that the transcriptional response to partial antiestrogens could depend on the balance between ERs and their transcription cofactors. Hence, modulation of either of these parameters (i.e. expression levels of coactivators, corepressors or receptors) could be of importance in determining the relative agonist/antagonist activity of antiestrogens in breast cancer cells.

From a clinical point of view, our data are consistent with previous studies dealing with response of patients to endocrine therapy in relation to ERα levels. These papers show that the rate of response to tamoxifen treatment is significantly correlated with ER concentration [49,50]. Almost all patients who progressed on treatment had tumours with a low (less than 20 fmol/mg cytosol protein), but in most cases detectable, ER concentration [50].

## 6. Different sensitivity of breast cancer cells to HDACi

In vitro, in various cell models, HDACi induce histone hyperacetylation and regulate gene expression, leading in general to cell differentiation and inhibition of proliferation. Several studies in animal models have reported the efficiency of some of these inhibitors to block tumour growth [51] and mammary tumours in particular [52]. In addition, the use of such a ‘‘transcriptional therapy’’ has been reported for the treatment of leukaemia using phenylbutyrate in combination with retinoic acid, leading in one case to a complete remission at 6 months [53]. Phases I and II clinical trials are currently under way for several of these molecules [11].

In a recent study [54], we have analysed the antiproliferative effect of various HDACi (such as the short-chain fatty acid *n*-butyrate, TSA or HC-toxin, a naturally occurring cyclic tetrapeptide), by total DNA quantification in human breast cancer cell lines expressing functional ERα (MCF-7, T-47D) or not (MDA-MB-231 and MDA-MB-435). Histone deacetylase inhibition exhibited a growth-inhibitory activity in all cell lines, but ER+ breast cancer cells were clearly more sensitive to low concentrations of TSA than ER− cells (Fig. 3). Previous data, published on MCF-7 and MDA-MB-231 cells using millimolar concentrations of sodium butyrate as an inhibitor of HDACs [55], also support a higher sensitivity of ER+ breast cancer cells. Several lines of evidence indicate that the general multidrug resistance (MDR) phenotype, which extrudes a wide range of anticancer drugs [56], is not involved in the resistance of ER-breast cancer cells to HDACi. This corroborates data obtained with phenylbutyrate [57] or SAHA [58]. In addition, no significant quantitative differences concerning the total HAT or HDAC activities have been observed between the different cell lines in relation with ERα status indicating that global modifications of the HAT/HDAC ratio are probably not directly involved in the differential response of breast cancer cells to hyperacetylating agents [54].

By contrast, we have found that the antiproliferative activity of HDACi is correlated to the expression of

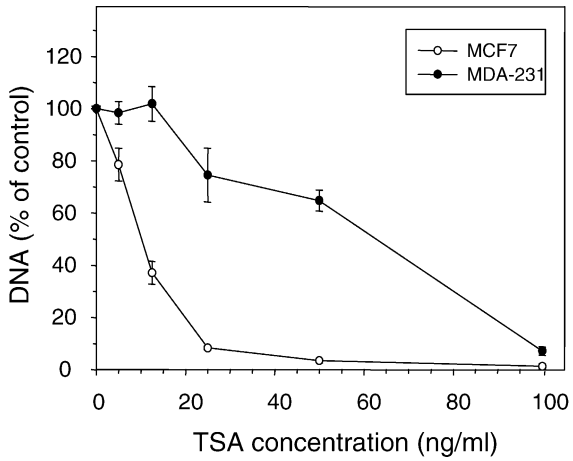


Fig. 3. Effect of HDACi on breast cancer cell proliferation. The MCF-7 (ER+; white symbols) and MDA-MB-231 (ER−; black symbols) cell lines were treated during 96 h with vehicle alone or increasing amounts of TSA (ng/ml). Cell number was quantified by total DNA measurement and results are expressed as percentage of controls. Adapted from [54].

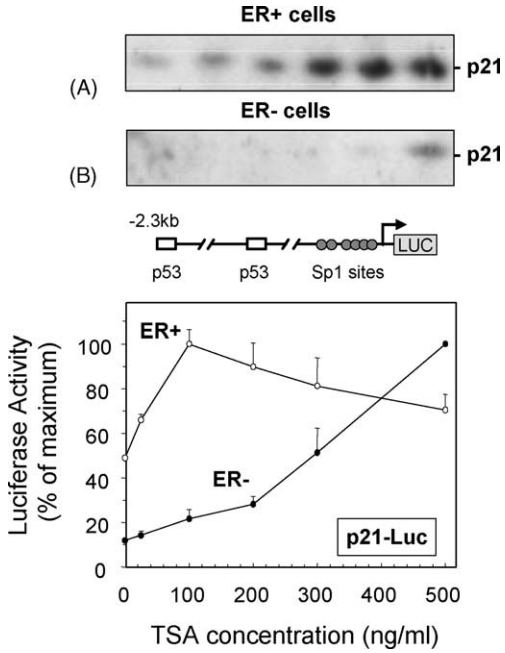


Fig. 4. Effect of TSA on $p21^{WAF1/CIP1}$ expression in breast cancer cells. (A) MCF-7 (ER+) and MDA-MB-231 (ER−) cell lines were exposed to increasing concentrations of TSA for 17 h and $p21^{WAF1/CIP1}$ expression was analysed by Western blot. (B) Transient transfection experiments in MCF-7 (ER+; white symbols) and MDA-MB-231 cells (ER−; black symbols). Cells were transfected with a p21-luciferase reporter construct and treated by increasing amounts of TSA for 24 h. A schematic view of the reporter construct is given showing the position of the p53 and Sp1 response elements. Results are expressed as percentage of the maximum luciferase activity and are the mean (±S.D.) of three independent experiments. Adapted from [54].

$p21^{WAF1/CIP1}$, a cyclin-dependent kinase-inhibitor. In ER+ cell lines, we have observed a higher basal level of $p21^{WAF1/CIP1}$ gene expression, which is more sensitive to TSA regulation. These differences appear both on the endogenous protein levels assessed by Western blot and in transient transfection experiments using plasmids containing the luciferase reporter gene under the control of the human $p21^{WAF1/CIP1}$ gene promoter (Fig. 4). With regard to the association between ERα and $p21^{WAF1/CIP1}$ levels, our data are supported by a clinical analysis in breast tumour samples, which reports a positive correlation between the expression of these two proteins measured by Western blot [59].

Since one of the proximal Sp1 sites has been identified as the major TSA-responsive sequences in the $p21^{WAF1/CIP1}$ gene promoter [60], we have analysed the response of the Sp1-luc reporter plasmid in the ER+ and ER− breast cancer cell lines (Fig. 5). The expression and regulation of the Sp1-luc reporter recapitulate the differences in both the basal expression and differential sensitivity to HDACi [54]. This highlights the importance of Sp1 sites in the transcriptional regulation by HDACi [60] by demonstrating that these elements are sufficient to confer a differential sensitivity to TSA in transient transfection experiments of ER− and ER+ breast cancer cells. The different sensitivity of breast cancer cells to TSA could therefore reflect a role of acetylation in protein–DNA or protein–protein interactions at the Sp1 site, as it has been shown that both Sp1 [61] and Sp3 [62] could be acetylated. However, TSA treatment did not modify quantitatively or qualitatively the binding on Sp1 sites in gel retardation assays using MCF-7 or MDA-MB-231 cell extracts [54], suggesting that the interaction between Sp1 proteins and DNA is probably not altered by acetylation under these conditions.

## 7. ERα expression and response to HDACi

The increase in $p21^{WAF1/CIP1}$ basal levels in ER+ breast cancer cells could reflect transactivation of the $p21^{WAF1/CIP1}$ gene by estrogen receptors. Such a regulation occurs in transient transfection assays where ERα overexpression in

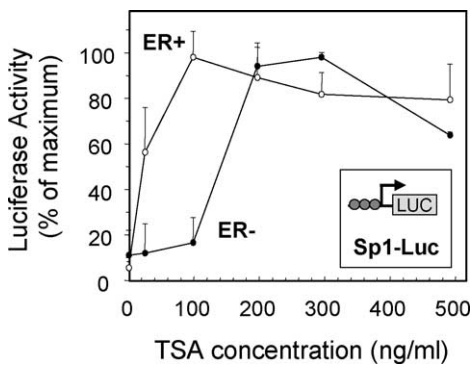


Fig. 5. Effect of TSA on the Sp1-luc reporter plasmid. The Sp1-luciferase reporter construct was transiently transfected in MCF-7 (ER+; white symbols) and MDA-MB-231 cells (ER−; black symbols). Cells were treated with the indicated amounts of TSA for 24 h. Results are expressed as percentage of the maximum luciferase activity and are the mean (±S.D.) of three independent experiments. Adapted from [54].

MDA-MB-231 cells results in a three-fold increase of the expression of $p21^{WAF1/CIP1}$ constructs [54].

An adenoviral-based strategy was used to reintroduce the ERα protein in receptor-negative breast cancer cells in order to investigate further the role of ERα on the expression of endogenous *p21*$^{WAF1/CIP1}$ gene. Infection of MDA-MB-231 cells by the recombinant adenovirus resulted in a strong increase in the levels of endogenous $p21^{WAF1/CIP1}$ mRNA and protein [54]. ERα expression modulates the proliferative response of MDA-MB-231 cells to HDAC inhibition since TSA inhibited cell proliferation more efficiently in cells infected with Ad-hERα as compared to cells infected with the non-recombinant Ad5 adenovirus. These data support our initial observation on ER+ and ER− breast cancer cells since they (1) demonstrate a link between ERα and $p21^{WAF1/CIP1}$ expression and (2) suggest that the presence of ERα influences the sensitivity of breast cancer cells to growth inhibition by TSA. It is, however, clear that the *p21*$^{WAF1/CIP1}$ gene is not the unique mediator of the antiproliferative properties of HDACi, but more likely a key protein acting in combination with other regulators.

From a clinical point of view, the in vivo antitumour activity of hyperacetylating agents (in particular on breast cancer) is becoming more and more documented [52,63–65] and appears to be associated with low toxicity, thus supporting their potential use in anticancer therapy. The need for markers of responsiveness to these drugs could therefore be important, and ERα is a good candidate. Other genes that are linked to the increase of programmed cell death induced upon hyperacetylation could also be of interest and are currently under investigation in our laboratory.

## 8. Concluding remarks

Understanding the molecular mechanisms through which estrogens act is an important challenge in order to improve hormonal therapies used to treat these cancers and to compensate the decrease of estrogens after menopause. Recent advances have improved our knowledge of the biology of steroid hormones and highlighted the cellular and molecular aspects of estrogen signalling.

On the other hand, the use of specific inhibitors to target HDACs has recently emerged as a new potential therapeutic approach in the treatment of cancer [11]. A better understanding of the mechanisms responsible for the sensitivity of a given tumour cell to this class of drugs is required in order to define diagnostic tools allowing the identification of responsive cancers. As already suggested for the treatment of leukaemia [53], combinatory approaches involving other cytotoxic drugs will probably be necessary for the treatment of breast cancer. Future research will be necessary to establish the expression profiles of the eleven HDACs in breast cancer samples. This step is a prerequisite in order to propose the use of more specific HDACi targeting only a subset of enzymes.

It is now clear that the two types of pathways responding to ER and HDAC modulators, cross-talk at various levels (expression and activity of ERα, regulation of $p21^{WAF1/CIP1}$ expression, cell proliferation and apoptosis, etc.). However, the exact mechanisms by which HDACi inhibit ERα expression and cell proliferation remain to be elucidated (levels of control, identification of the HDAC(s) implicated, etc.). Finally, in addition to ERα, ERβ is obviously the other key actor involved in estrogen signalling (for a review, see [66]), but recent data have indicated that ER-related receptors (ERRα, β and γ) [67] also actively influence the estrogen pathway. Future studies will be required to define the effects of HDACi on the expression and activity of these nuclear receptors.

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