Specific Activity of Class II Histone Deacetylases in Human Breast Cancer Cells

Vanessa Duong,[1,2,3,4] Caroline Bret,[5] Lucia Altucci,[7,8] Antonello Mai,[6] Céline Duraffourd,[5] Julie Loubersac,[5] Pierre-Olivier Harmand,[1,2,3,4] Sandrine Bonnet,[1,2,3,4] Sergio Valente,[6] Thierry Maudelonde,[5] Vincent Cavailles,[1,2,3,4] and Nathalie Boulle[2,5]

[1]Institut de Recherche en Cancérologie de Montpellier; [2]Institut National de la Santé et de la Recherche Médicale U896; [3]Université Montpellier1; [4]Centre Régional de Lutte contre le Cancer Val d'Aurelle-Paul Lamarque; [5]Laboratoire de Biologie Cellulaire, Centre Hospitalier Universitaire Arnaud de Villeneuve, Montpellier, France; [6]Institute Pasteur-Fondazione Cenci-Bolognetti, Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza University of Rome; [7]Istituto Nazionale di Biostrutture e Biosistemi, Rome, Italy; and [8]Dipartimento di Patologia generale, Seconda Università di Napoli, Naples, Italy

Abstract

Although numerous studies have underlined the role of histone deacetylases (HDAC) in breast physiology and tumorigenesis, little is known on the particular contribution of the various classes of HDACs in these processes. Using estrogen receptor-α (ERα)–positive MCF-7 breast cancer cells, the effects of MC1575 and MC1568, two novel class II–specific HDAC inhibitors, were analyzed on cell proliferation, apoptosis, and estrogen signaling. The specificity of these HDAC inhibitors was validated by measuring histone and α-tubulin acetylation and by the specific *in vitro* inhibition of recombinant HDAC4 using histone and nonhistone substrates, contrasting with the lack of inhibition of class I HDACs. In addition, MC1575 did not inhibit class I HDAC gene expression, thus confirming the specific targeting of class II enzymes. Similar to trichostatin A (TSA), MC1575 displayed a dose-dependent antiproliferative effect and induced cell cycle arrest although this blockade occurred at a different level than TSA. Moreover, and in contrast to TSA, MC1575 had no effect on MCF-7 cells apoptosis. Interestingly, MC1575 was able to increase *p21$^{waf1/CIP1}$* mRNA levels but did not regulate the expression of other genes such as *cyclin D1, p27, p14^{ARF}, Bcl2, Baxα, Trail-R1*, and *Trail-R2*. Finally, MC1575 strongly induced *ERβ* gene expression but did not decrease *ERα* expression, nor did it switch hydroxytamoxifen to an agonist activity. Altogether, these data suggest that the class II HDAC subfamily may exert specific roles in breast cancer progression and estrogen dependence. (Mol Cancer Res 2008;6(12):1908–19)

Introduction

Human histone deacetylases (HDAC) form a large family of 18 members classified into four groups (I to IV) based on sequence homologies (1, 2). Class I enzymes, including HDAC1, HDAC2, HDAC3, and HDAC8, are nuclear proteins with ubiquitous expression. Class II HDACs are divided in two classes: class IIa includes HDAC4, HDAC5, HDAC7, and HDAC9, whereas class IIb is composed of HDAC6 and HDAC10. Class II HDACs have a tissue-specific pattern of expression and can shuttle between the nucleus and the cytoplasm depending on their phosphorylation status. HDAC6 and HDAC10 form a particular group because they both contain two deacetylase domains and because HDAC6 can specifically deacetylate the cytoskeletal protein α-tubulin (3). Indeed, in addition to histones, HDACs have been shown to deacetylate various substrates including transcription factors, chaperones, as well as many regulators involved in DNA repair, cell signaling, or metabolism (2). The diversity of HDACs also suggests differential roles for the various classes of enzymes depending on tissues or cell lines. For instance, recent studies have shown an essential role for HDAC6 in the clearance of ubiquitinated cellular protein aggregates (4), and class IIa HDACs have been involved in cardiac and vascular development, chondrocyte hypertrophy during skeletogenesis, or thymocytes selection (5-8).

HDAC inhibitors (HDI) have shown *in vitro* and *in vivo* activities against various cancer types affecting cell cycle, programmed cell death, differentiation, and angiogenesis (1, 9).

Received 6/24/08; revised 8/22/08; accepted 8/29/08.
Grant support: "Institut National de la Santé et de la Recherche Médicale"; the University of Montpellier I, the "Ligue Nationale contre le Cancer"; the "Association pour la Recherche sur le Cancer"; and the Centre Hospitalo-Universitaire de Montpellier, HEALTH-F4-2007-200767, LSHC-CT2005-518417, and PRIN 2006; and Associazione Italiana per la Ricerca sul Cancro 2007. V.D. was a recipient of fellowships from the French Minister of Research and the "Association pour la Recherche sur le Cancer." P.O.H was supported by the Ligue Nationale Contre le Cancer.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Note: Current address for V. Duong: Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch F-67404, France. V. Duong and C. Bret should be considered as first co-authors. V. Cavailles and N. Boulle should be considered as last coauthors.
Requests for reprints: Vincent Cavailles, Institut National de la Santé et de la Recherche Médicale U896, Institut de Recherche en Cancérologie de Montpellier, Parc Euromédecine, Montpellier, F-34298 France. Phone: 33-4-67-61-24-05; Fax: 33-4-67-61-37-87. E-mail: v.cavailles@valdorel.fnclcc.fr

doi:10.1158/1541-7786.MCR-08-0299

HDIs are thus considered as a new class of anticancer agents and are currently evaluated in several phase I and II clinical trials in patients with hematologic and solid malignancies (10). Recently, one of them, vorinostat, has been approved for the treatment of cutaneous T-cell lymphoma (11, 12).

In breast tumor models, HDIs have potent antiproliferative effects *in vitro* and *in vivo* and interfere with estrogen signaling (13-17). Estrogens effects are mediated by two distinct estrogen receptors, ERα and ERβ, acting as transcriptional factors that belong to the nuclear receptor superfamily (18). We and others have shown that, in ERα-expressing breast cancer cells, HDIs such as trichostatin A (TSA) strongly down-regulate ERα both at the mRNA and protein levels while increasing *ERβ* gene expression (14, 19, 20). By contrast, in ERα-negative breast cancer cells, HDIs and DNA methyltransferase inhibitors synergistically reactivate *ERα* gene expression by releasing various repressors from its promoter, including the class I enzyme HDAC1 (21-23). HDIs such as TSA also increase ERα and ERβ transcriptional activity (14, 19) and, in MCF-7 cells, strongly stimulate the agonist activity of partial antiestrogens such as hydroxytamoxifen (14).

At present, little is known on the specific contribution of the various classes of HDACs in breast tumorigenesis or estrogen responsiveness. Studies using HDIs in breast cancer models have indeed used broad-range, nonselective inhibitors such as TSA or suberoylanilide hydroxamic acid (SAHA) so that no information about the contribution of specific HDACs in biological pathways could be available. Recently, new HDIs displaying specificity against class I, II, or III HDACs have been described and used to identify the roles of these classes of HDACs in various cell responses (24-26). Using specific inhibitors of class II HDACs, the aim of this study was to define the particular contribution of this class of enzymes on cell proliferation, apoptosis, gene expression, and ER signaling in ERα-expressing MCF-7 human breast cancer cells. Altogether, our data show that the class II HDAC subfamily specifically regulates these parameters and may thus exert a particular role in breast tumor progression and estrogen dependence.

Results

Specificity of MC1575 and MC1568 for Class II HDACs

The role of class II HDACs was investigated in ERα-expressing MCF-7 human breast cancer cells using MC1575 and MC1568, two inhibitory compounds displaying class II HDACs specificity (27, 28). The HDAC specificity of MC1575 and MC1568 was first validated by measuring their effects on the levels of acetylated forms of H3 and H4 histones and tubulin (Fig. 1). HDAC blockade induced by MC1575 and MC1568 was confirmed by the accumulation of acetylated H3 and H4 histones in MCF-7 cells, although these compounds were found to be less potent than TSA, in agreement with their pharmacologic properties (28). Moreover, MC1575, MC1568, and TSA, but not MS275, a specific inhibitor for class I HDACs, increased the levels of acetyl-tubulin, indicating their ability to inhibit HDAC6, a member of class II HDAC known to deacetylate α-tubulin (3).

We next measured the effects of MC1575 on purified class I HDAC1, HDAC2, HDAC3, and HDAC8 and class II HDAC4 (Fig. 2). MC1575, unlike SAHA, had a weak inhibitory effect on the enzymatic activity of class I HDAC1, HDAC2, HDAC3, and HDAC8 even at the highest dose tested (20 μmol/L, 10-30% inhibition; Fig. 2A). By contrast, at 20 μmol/L, MC1575 displayed a 65% inhibitory effect on the activity of HDAC4, a representative member of class IIa HDAC (Fig. 2B). Recently, Lahm et al. described trifluoroacetyl-lysine as a new nonhistone acetylated lysine substrate specific for class IIa HDACs (29, 30). Using this substrate, MC1575 at 5 and 20 μmol/L was found to efficiently inhibit HDAC4 enzymatic activity, at levels similar to SAHA (65% inhibition at 20 μmol/L; Fig. 2B). Similar results were obtained using MC1568 (data not shown).

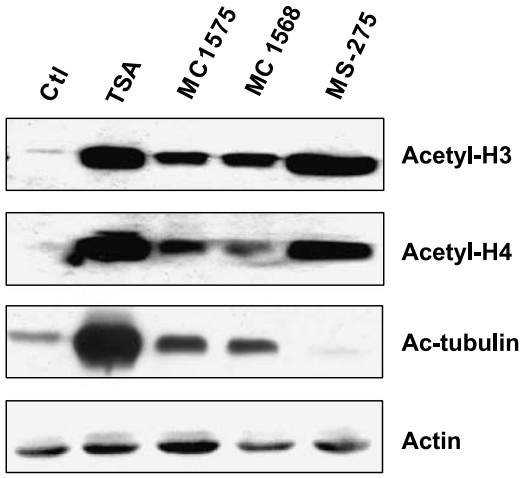

FIGURE 1. Effects of MC1575 and MC1568 on deacetylase activity in MCF-7 cells. MCF-7 cells were treated for 6 h with TSA (1.7 μmol/L), MC1575 (20 μmol/L), MC1568 (20 μmol/L), or MS-275 (1 μmol/L). Cell lysates were analyzed by Western immunoblotting with anti–acetylated histone H3 (*Acetyl-H3*), anti–acetylated histone H4 (*Acetyl-H4*), or anti–acetylated tubulin (*Ac-tubulin*) antibodies. Actin was used as a loading control.

Regulation of HDAC Gene Expression by HDIs

The cellular effects of HDIs are thought to rely on their ability to inhibit HDAC enzymatic activity. However, regulation of HDAC expression by HDIs may also play a role in the alterations of cell behavior, as recently suggested by Dokmanovic et al. (31), showing a strong down-regulation of HDAC7 expression by SAHA in various normal and tumor cell lines. We thus analyzed the effects of TSA and MC1575 on the expression of class I, II, and IV HDACs (HDAC1-HDAC11) by treating MCF-7 cells with either HDI and measuring the corresponding mRNA levels.

Of the 11 HDACs analyzed, HDAC2, HDAC3, and HDAC8 (class I) and HDAC7 and HDAC10 (class II) were found to be significantly regulated by TSA (Fig. 3). Similar to the results obtained by Dokmanovic et al., TSA strongly inhibited HDAC7 gene expression ($P = 0.0006$, versus control cells). Although, to a lesser extent, TSA was also found to significantly down-regulate mRNA levels of HDAC2, HDAC8, and HDAC10 ($P = 0.0175$, $P = 0.0003$, and $P = 0.0017$ versus control cells, respectively) and to increase that of HDAC3 ($P < 0.0001$ versus control cells). These regulations by TSA were specific because

no variations of HDAC1 mRNA levels were noticed in the same conditions (Fig. 3).

Interestingly, and in contrast to TSA, the class II–specific inhibitor MC1575 did not modulate the mRNA levels of any of these HDACs but instead induced the expression of class II HDAC6 (P = 0.0095, versus control cells; Fig. 3). We also observed a trend toward an increase in HDAC9 mRNA levels on TSA and MC1575 treatment. However, because of the high

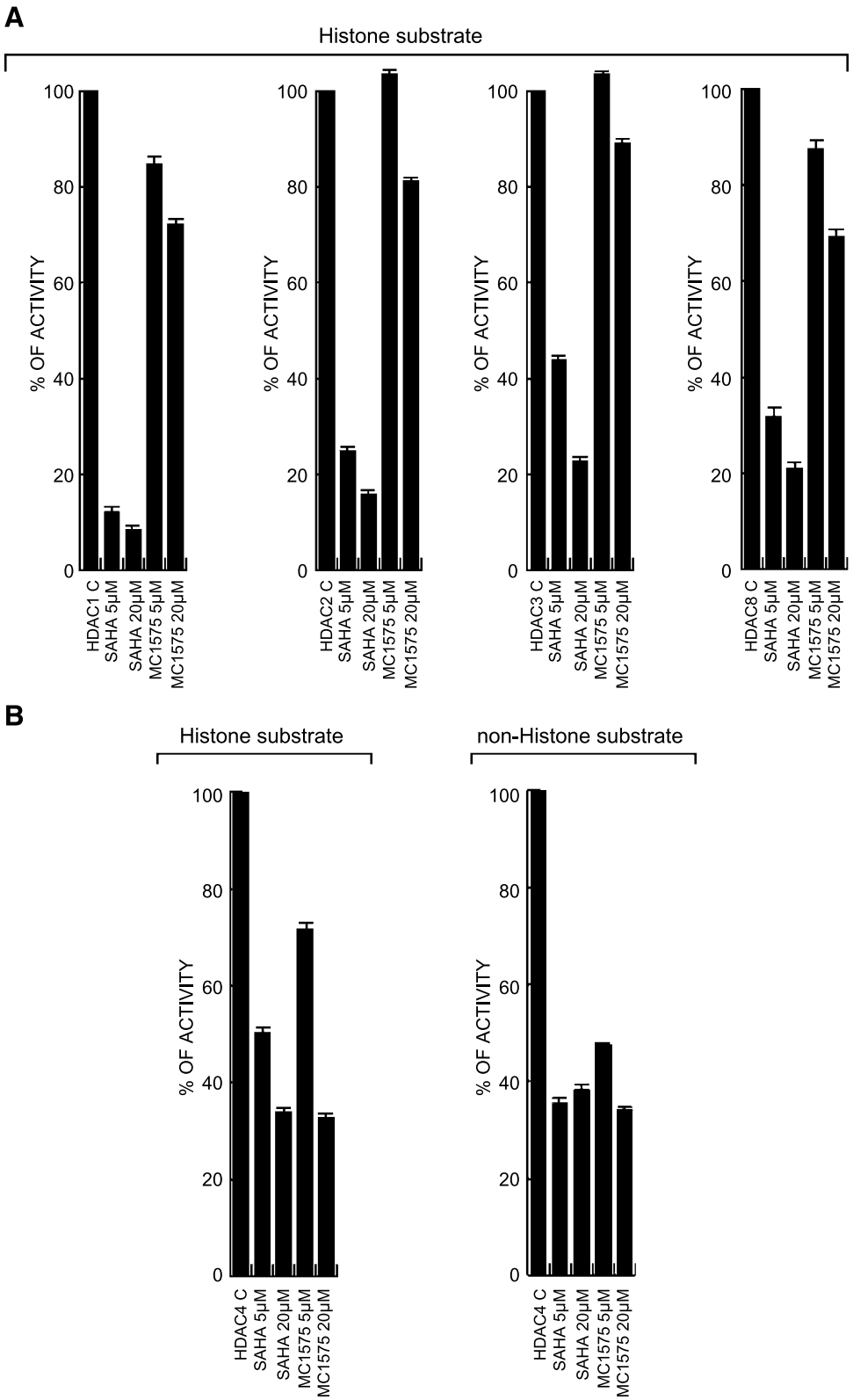

FIGURE 2. HDAC specificity of MC1575. **A.** Histone deacetylase activity of class I enzymes (HDAC1, HDAC2, HDAC3, and HDAC8). Recombinant HDAC1, HDAC2, HDAC3, and HDAC8 were expressed and the deacetylase activity of purified HDAC was measured, using fluorescent substrates, in the absence [control (*C*)] or presence of SAHA or MC1575 (5 and 20 μmol/L). The deacetylase activity of the various HDACs in presence of HDI is expressed as percentage of the control (HDAC alone). Columns, mean of triplicates; bars, SD. **B.** Histone and nonhistone deacetylase activity of class IIa HDAC4. The histone deacetylase activity of HDAC4 was quantified as in **A**. The nonhistone deacetylase activity was measured using trifluoroacetyllysine as substrate.

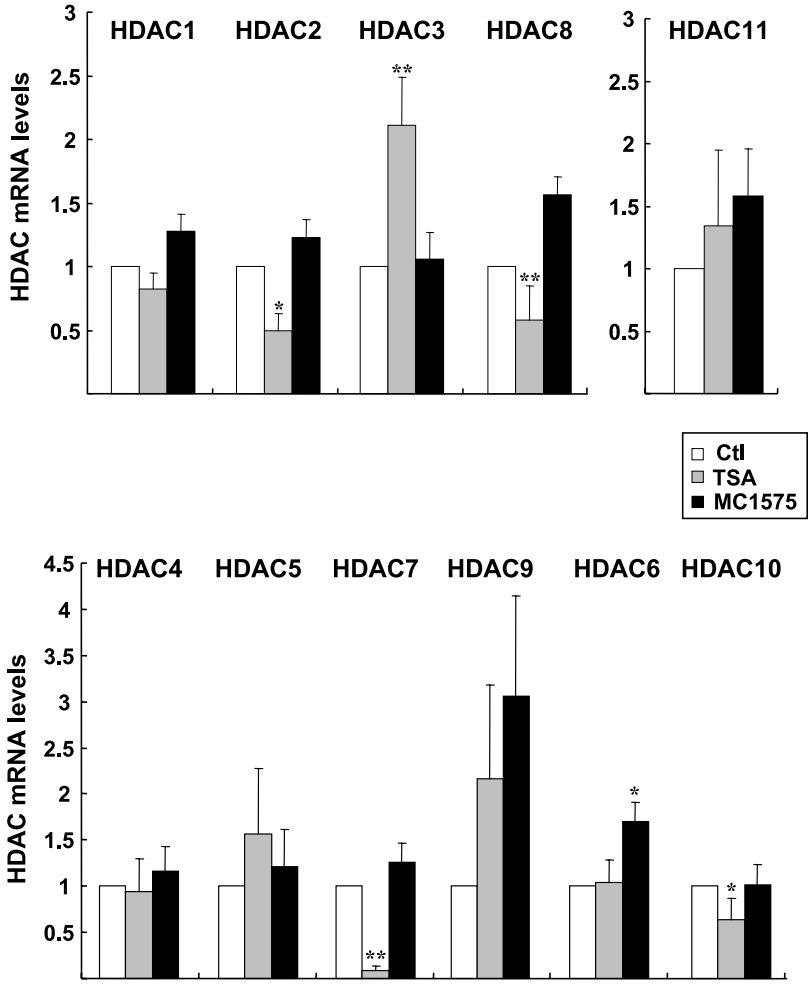

FIGURE 3. Regulation of HDAC gene expression by HDIs. MCF-7 cells were treated with TSA (1.7 μmol/L), MC1575 (20 μmol/L), or vehicle alone [control (*Ctl*)] for 20 h and mRNA levels for the 11 HDACs were measured by real-time quantitative PCR. Results are expressed relative to the *TBP* housekeeping gene and to the mRNA levels measured for the untreated control cells used as reference. Columns, mean of eight (for TSA) or four (for MC1575) independent cell cultures; bars, SD. Raw data were used for statistical analysis. *, $P \leq 0.01$; **, $P \leq 0.001$.

variability in HDAC9 mRNA values due to the weak expression of its gene in MCF-7 cells, these variations on HDI were not found to be statistically significant. Most importantly, none of the class I HDACs was down-regulated by MC1575, strengthening the specific targeting of class II enzymatic activity by this HDI.

Effects of Class II HDIs on Breast Cancer Cell Proliferation, Cell Cycle, and Apoptosis

We then compared the effect of increasing concentrations of TSA and MC1575 on mammary tumor cell proliferation (Fig. 4). As previously shown (15), TSA exhibited a potent antiproliferative activity on MCF-7 cells, which was less pronounced on the ERα-negative MDA-MB 231 breast cancer cell line. MC1575 was also found to have a dose-dependent growth-inhibitory activity on MCF-7 cells, albeit at higher concentrations (micromolar) than TSA (nanomolar), which is consistent with their respective IC_{50} (28). Similar results were obtained using MC1568 (data not shown). Interestingly, ERα-negative MDA-MB 231 cells were also less sensitive to MC1575 treatment compared with ERα-positive MCF-7 cells (Fig. 4).

Flow cytometry analysis further confirmed the dose-dependent antiproliferative effects of TSA and MC1575 on MCF-7 cells (Fig. 5A; Table 1). This effect was observed with the lowest concentration of MC1575 or MC1568 tested (5 μmol/L). However, although both TSA and MC1575/1568 induced cell cycle arrest, a different profile was obtained with either HDI, showing accumulation of cells in the G_2-M phase of the cell cycle for TSA, and in the G_1 phase for MC1575 and MC1568.

HDIs have been shown to induce apoptosis through various pathways in tumor cells, so we investigated whether MC1575 and MC1568 had the same effect in MCF-7 cells (Fig. 5B). TSA was found to markedly induce MCF-7 cell apoptosis, whereas MC1575 and MC1568 had no effects even at the highest dose tested (20 μmol/L). These results were consistent with those obtained using flow cytometry analysis as observed from the fraction of cells in the sub-G_1 phase of the cell cycle, which increased on TSA treatment but remained similar to the control on MC1575 or MC1568 treatment (Fig. 5A; Table 1).

Inhibition of Class II HDACs and Expression of Cell Cycle and Apoptosis Regulators

Because HDIs affect MCF-7 cell proliferation and cell cycle progression, we next analyzed the expression of some cell cycle regulators in response to TSA or MC1575 treatment (Fig. 6A). As previously shown for various HDIs, TSA and MC1575 were found to increase with equal potency the cell cycle inhibitor

$p21^{cip1/waf1}$ at the mRNA and protein levels. However, both HDIs differently affected the expression of other cell cycle regulators: TSA markedly decreased the expression of *cyclin D1* at the mRNA and protein levels and decreased *p27* and $p14^{ARF}$ gene expression, whereas MC1575 had no or a modest effect on these parameters.

When measuring mRNA levels for *Bcl2* and *Baxα*, two members of the Bcl2 family involved in the intrinsic apoptotic pathway, we found that TSA had a strong inhibitory effect on the expression of the antiapoptotic *Bcl2* gene while modestly but significantly decreasing that of the proapoptotic *Baxα* regulator (Fig. 6B). In addition, TSA was found to regulate the expression of members of the death receptors family involved in the extrinsic pathway, decreasing *TRAIL-R1* expression and markedly inducing *TRAIL-R2* mRNA accumulation (~13×). By contrast, MC1575 had no or only a very modest effect on these regulators, in accordance with its lack of regulation of MCF-7 apoptosis. Altogether, these results suggest that, in breast cancer cells, cell cycle and apoptosis regulators are specifically and differentially targeted by the various classes of HDACs.

Inhibition of Class II HDACs and ER Signaling

Several HDIs, including TSA, have been shown to differentially regulate ERα and ERβ expression and transcriptional activity in ERα-expressing mammary tumor cells (19, 20). These studies have been done using nonselective HDIs, and we wanted to assess if a specific inhibition of class II HDACs would have similar effects in MCF-7 cells.

Consistent with previous data, TSA had marked effects on the expression of both ER isoforms in MCF-7 cells, down-regulating ERα both at the mRNA and protein levels while increasing ERβ mRNA levels (Fig. 7A and B). ERα and ERβ isoforms followed different kinetics on TSA treatment, as ERα inhibition was detected at 6 hours and maintained after 20 hours, whereas ERβ induction was strongest at 6 hours and decreased thereafter, suggesting different regulation pathways (Fig. 7A, and data not shown). Interestingly, MC1575 showed a different effect than TSA on these two parameters, as it had only a weak and not significant inhibitory effect on *ERα* expression (mRNA and protein) but a marked and stronger stimulatory effect on *ERβ* gene expression.

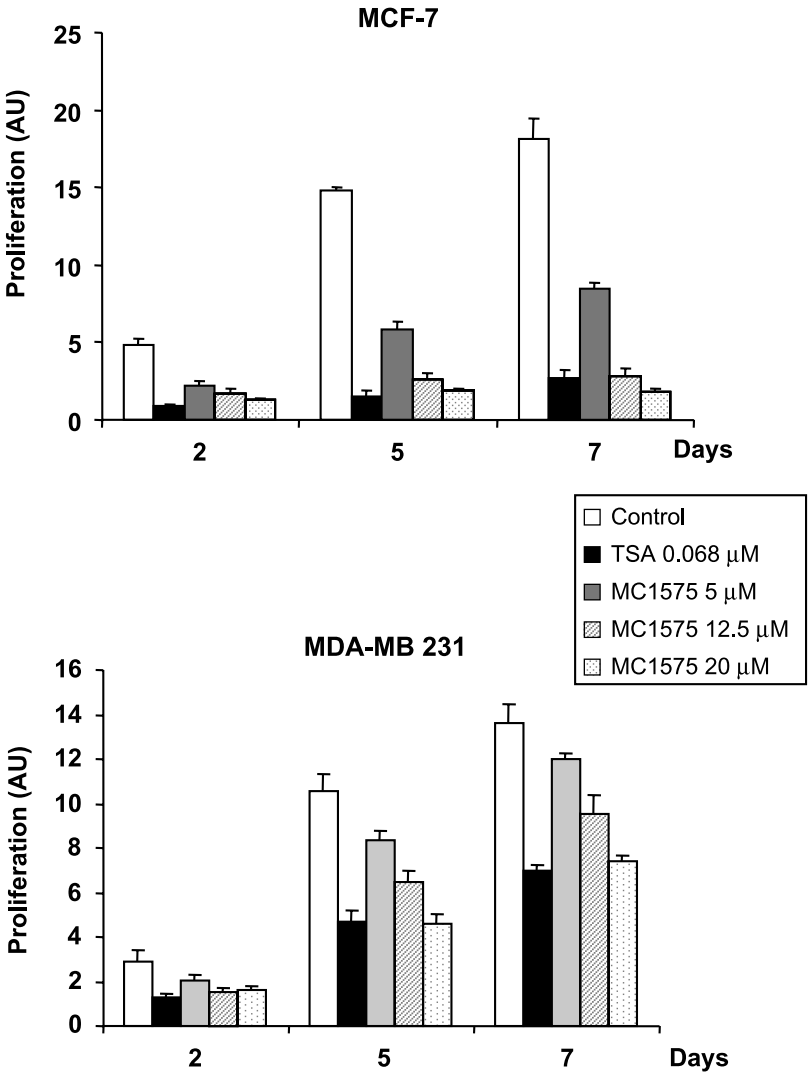

FIGURE 4. Effects of class II HDI on breast tumor cell proliferation. ERα-positive MCF-7 cells or ERα-negative MDA-MB 231 cells were treated with TSA (0.068 μmol/L), MC1575 (5, 12.5, and 20 μmol/L), or solvent alone (control) and cell proliferation was measured by diaminobenzoic acid assay at days 2, 5, and 7. Columns, mean arbitrary units (*AU*) from triplicate wells; bars, SD. Representative of four independent experiments.

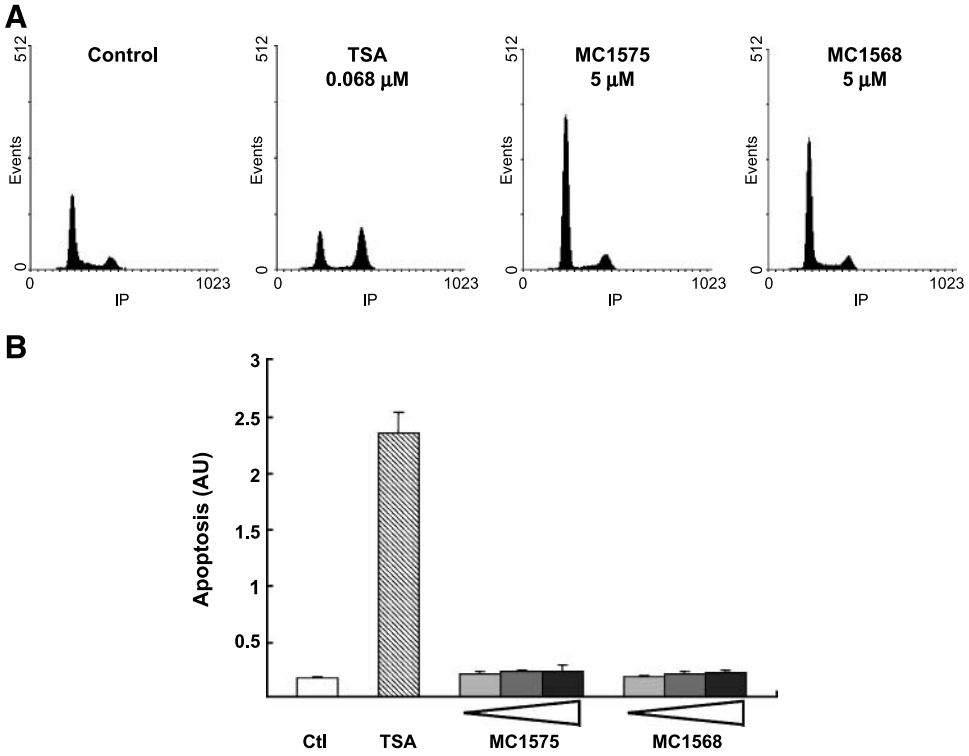

FIGURE 5. Effects of class II HDI on cell cycle and apoptosis in MCF-7 cells. **A.** MCF-7 cells were treated for 20 h with increasing concentrations of TSA, MC1575, MC1568, or solvent alone (control). Cell phase distribution was determined by propidium iodide staining and fluorescence-activated cell sorting analysis. Representative results obtained for control cells or cells treated with TSA (0.068 μmol/L), MC1575 (5 μmol/L), or MC1568 (5 μmol/L). **B.** MCF-7 cells were treated for 40 h with TSA (0.068 μmol/L), increasing concentrations of MC1575 and MC1568 (5, 12.5, or 20 μmol/L), or vehicle alone (control), and apoptosis was measured using the Cell Death Detection ELISA kit. Columns, mean arbitrary units from four wells; bars, SD.

We next studied the effects of TSA and MC1575 on ERα transcriptional activity (Fig. 7C). Recent reports from our laboratory have shown that HDI, including TSA, enhanced the ligand-dependent activity of ERα and ERβ and that partial antiestrogens such as hydroxytamoxifen switch their antagonist activity to an agonist one on HDAC inhibition, this latter effect being related to ERα down-regulation (14, 19). In stably transfected bioluminescent MCF-7 cells (MELN clone), TSA strongly enhanced the transcriptional activity of ERα in the presence or absence of ER ligands, and switched hydroxytamoxifen to an agonist, without modifying the behavior of the pure antagonist ICI 182,780. Inhibition of class II HDACs using MC1575 weakly enhanced ERα transcriptional activity as compared with TSA, but, in contrast to TSA, did not switch the partial antiestrogen hydroxytamoxifen to an agonist, in accordance with its lack of effect on ERα expression in MCF-7 cells.

Table 1. Effects of Class II HDI on MCF-7 Cell Cycle

% of Cells	Ctl	TSA (μmol/L)			MC1575 (μmol/L)			MC1568 (μmol/L)		
		0.068	0.34	1.7	5	12.5	20	5	12.5	20
Sub-G_1	3.2	3.8	10.4	9.5	3.3	1.7	3.5	2.3	2.9	3.5
G_1	59.2	35.8	25.2	28.1	73.5	76.1	72.1	69.6	70	73.3
S	19	10.5	19.8	13.1	9.6	7.1	8.9	14.6	13.1	14.3
G_2-M	18.6	49.9	44.7	49.3	13.6	15.2	15.5	13.5	14	8.9

NOTE: Percentages of MCF-7 cells in the various phases of the cell cycle in response to HDI are presented.
Abbreviation: Ctl, control.

Discussion

Several studies have underlined the role of HDACs in breast physiology and tumorigenesis (13, 15-17, 32, 33). Some studies have focused on particular HDACs (HDAC1, HDAC3, and HDAC6) and their roles in breast carcinoma (34-36). However, little is known on the specific contribution of the various classes of HDACs in mammary tumorigenesis, which is an important issue from a cognitive point of view and in the context of HDI development as promising anticancer therapies. The aim of this study was thus to investigate this issue in a model of estrogen-responsive breast tumor cell line using HDIs specifically targeting the class II HDAC subfamily.

MC1575 and MC1568 are newly designed synthetic inhibitors of class II HDACs (27, 28). Although these HDIs are less potent than TSA in inhibiting HDACs, their selectivity against class II HDACs has been shown using various models (24, 25, 27, 28). We confirmed this selectivity in MCF-7 cells and by means of recombinant HDACs, showing that MC1575 and MC1568 had no or a weak effect on the histone deacetylase activity of class I enzymes (Figs. 1 and 2).

Class IIa HDACs expressed in mammalian cells have been shown to recruit class I HDACs, which display high deacetylase activity, and several reports have questioned whether class IIa HDACs by themselves had an intrinsic deacetylase activity. Recently, Lahm et al. (30) showed that the low catalytic activity of mammalian class IIa HDACs was linked to the presence of a unique histidine residue in the catalytic domain of these enzymes in place of the tyrosine residue observed in the conserved active site of class I HDACs. Despite this structural particularity, class IIa HDACs were shown to possess a weak but measurable enzymatic

activity *in vitro* against acetyl-lysine histone substrates. We also found that recombinant HDAC4, which is representative of class IIa HDACs family according to sequence and structure homologies, had histone deacetylase activity *in vitro* and showed that MC1575 efficiently inhibited this activity, being as efficient as SAHA at the highest dose tested. Interestingly, by screening a panel of acetylated lysine-like molecules, Lahm et al. also identified trifluoroacetyl-lysine as a substrate specific for class IIa HDACs, on which these enzymes were highly active, suggesting that mammalian class IIa HDACs may have additional biological substrates and activities, different from canonical histone deacetylation (29, 30). Using this specific substrate, we also showed that MC1575 and MC1568 were potent inhibitors of HDAC4 catalytic activity.

In addition to the well-documented *in vitro* and *in vivo* inhibition of HDAC enzymatic activity, HDI may also affect global acetylation levels by controlling HDAC expression. Using the nonselective HDI TSA, we observed that class II HDAC7 was markedly down-regulated at the mRNA level in various breast cancer cell lines (Fig. 3, and data not shown), thus confirming previously published data (31). We also found that TSA could significantly modulate the expression of other class I and class II HDACs (down-regulation of HDAC2, HDAC8, and HDAC10 and increase of HDAC3). Interestingly, none of these negative regulations was observed using the class II–specific HDI MC1575. Altogether, these results, along with previous studies (25, 28), confirm that MC1575 and MC1568 are specific class II HDI, as they do not inhibit class I HDAC activity or expression.

Using these specific inhibitors, we first addressed the effect of class II HDAC inhibition on human breast cancer cell proliferation. Nonselective HDIs have been shown to display antiproliferative effects in various tumor models, including

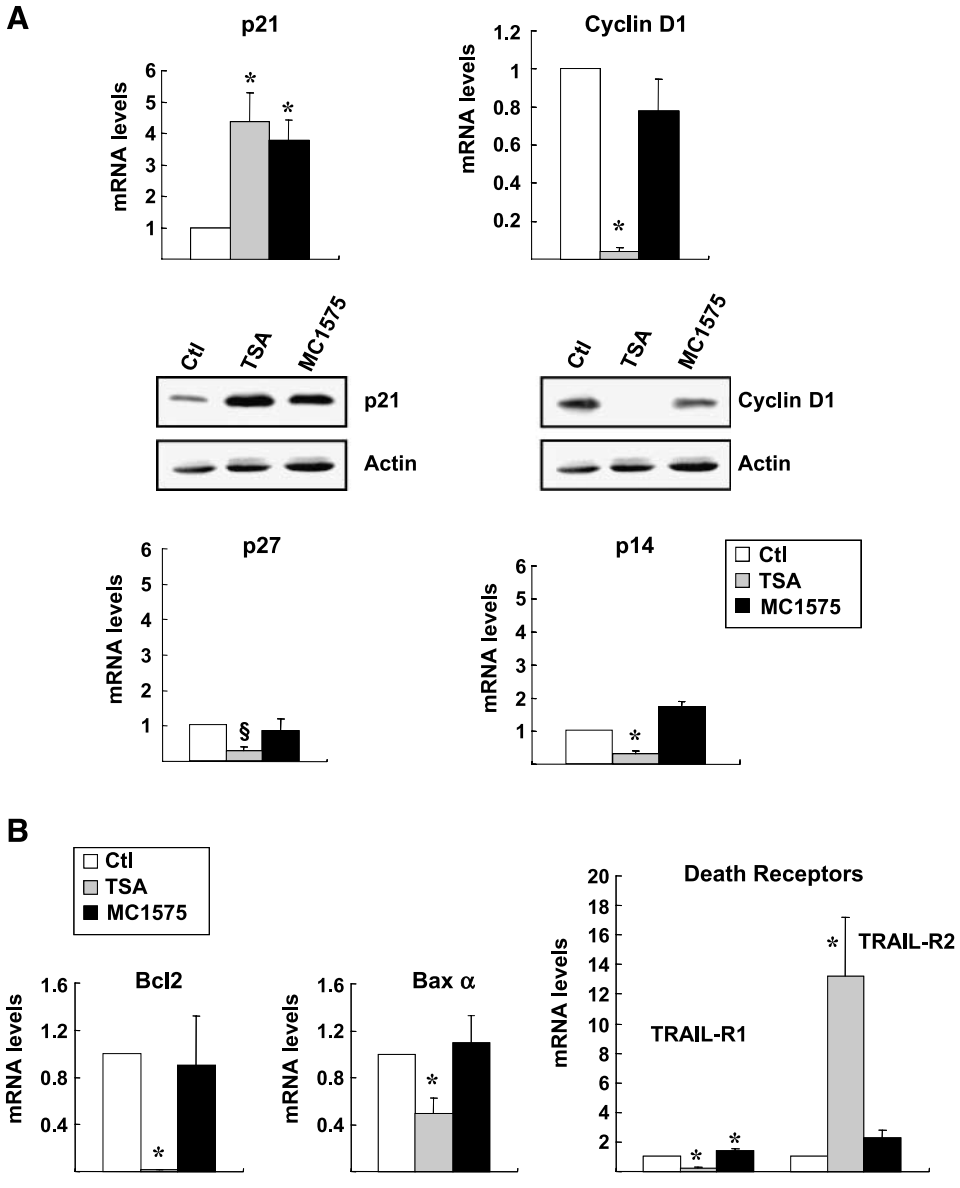

FIGURE 6. Effects of class II HDI on the expression of cell cycle and apoptosis regulators. **A.** MCF-7 cells were treated with TSA (1.7 μmol/L), MC1575 (20 μmol/L), or vehicle alone (control) for 6 h (p27 and $p14^{ARF}$) or 20 h ($p21^{cip1/waf1}$ and cyclin D1) and mRNA levels for *p21, cyclin D1, p27*, and *p14* genes were measured by real-time quantitative PCR. Results are expressed relative to the *TBP* housekeeping gene and to the mRNA levels measured for the untreated control cells used as reference. Columns, mean of four independent cell cultures; bars, SD. For p21 and cyclin D1, Western blot analysis was done in the same conditions, with actin as a loading control. **B.** MCF-7 cells were treated for 20 h with TSA (1.7 μmol/L), MC1575 (20 μmol/L), or vehicle alone (control), and *Bcl2*, *Baxα*, *TRAIL-R1*, and *TRAIL-R2* mRNA levels were quantified by real-time quantitative PCR. Columns, mean of four independent cell cultures; bars, SD. Raw data were used for statistical analysis. §, $P = 0.05$; *, $P < 0.05$, compared with control cells.

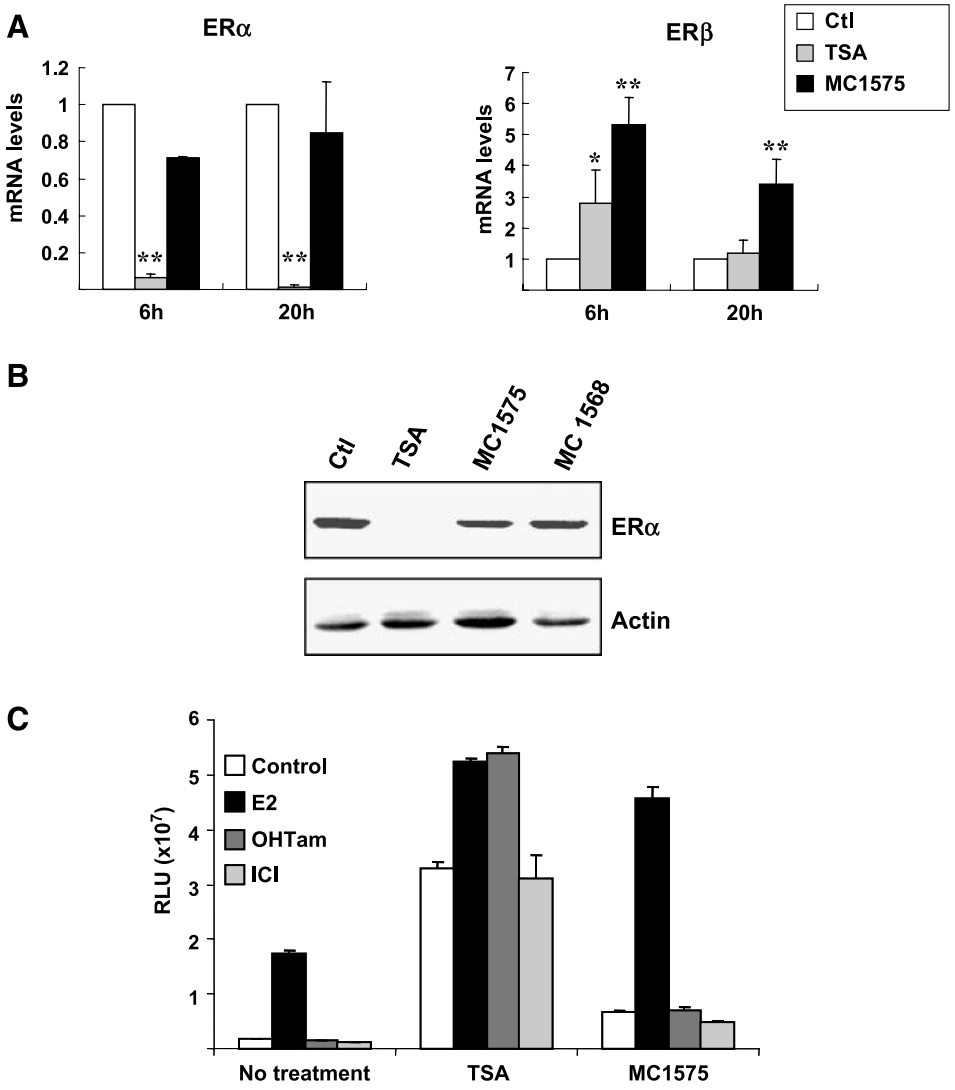

FIGURE 7. Effects of HDI on ERα and ERβ expression and activity in MCF-7 cells. **A.** MCF-7 cells were treated for 6 or 20 h with TSA (1.7 μmol/L), MC1575 (20 μmol/L), or vehicle alone (control), and mRNA levels for ERα and ERβ were quantified by real-time quantitative PCR. Results are expressed relative to the *TBP* housekeeping gene and to the mRNA levels measured for the control cells used as reference. Columns, mean of at least four independent cell cultures; bars, SD. Raw data were used for statistical analysis. *, $P < 0.05$; **, $P \leq 0.01$, compared with control cells. **B.** MCF-7 cells were treated for 20 h with TSA (1.7 μmol/L), MC1575 (20 μmol/L), MC1568 (20 μmol/L), or vehicle alone (control), and ERα protein levels were analyzed by Western immunoblotting. Actin was used as a loading control. **C.** MELN cells were treated for 20 h with control vehicle, 17β-estradiol (*E2*; 10^{-8} mol/L), hydroxytamoxifen (*OHTam*; 10^{-8} mol/L), or ICI 182,780 (*ICI*; 10^{-8} mol/L) in the absence or presence of TSA (1.7 μmol/L) or MC1575 (20 μmol/L), and luciferase activity was quantified. Columns, mean relative luciferase units (*RLU*) from triplicate wells; bars, SD. Representative results of three independent experiments.

breast tumors, and this effect has been linked to cell cycle arrest at the G_1-S and/or G_2-M checkpoints (1, 9). Among the few genes regulated by HDI, the cell cycle inhibitor $p21^{cip1/waf1}$ is consistently up-regulated by these compounds, which may explain, in part, their antiproliferative effects (37). Studies based on HDAC1-deficient cells or siRNA approaches suggest a predominant role of class I HDACs, and more particularly HDAC1 and HDAC3, in the control of cell proliferation and cell cycle (38-40). For instance, Lagger et al. (39) found that HDAC1-deficient embryonic stem cells presented reduced proliferation rates along with an increased expression of the cell cycle inhibitors $p21^{waf1}$ and $p27^{KIP1}$. Similarly, Glaser et al. (38), using a siRNA approach, showed that inhibiting the expression of HDAC1 or HDAC3 in HeLa cells induced morphologic changes and reduced their proliferation, in contrast to inhibition of class II HDAC4 and HDAC7, which had no effects. Finally, analysis of HDAC5, HDAC9, and HDAC4 null mouse phenotypes suggests that class II HDACs are mainly involved in tissue-specific growth and differentiation, rather than in cell proliferation (5, 7).

Our results indicate that, in addition to class I HDACs, class II enzymes may also be involved in cell proliferation and cell cycle control, at least in breast cancer cells. First, we showed that MC1575 and MC1568 were both able to inhibit MCF-7 and MDA-MB 231 cell proliferation in a dose-dependent manner. Moreover, as observed with TSA and other nonselective HDIs, those compounds strongly induced $p21^{waf1}$ gene and protein expression. Interestingly, MC1575 and MC1568 induced different effects on cell cycle and *cyclin D1* expression as compared with TSA, suggesting that class I and class II HDACs may control cell cycle at specific levels.

HDIs have also been shown to induce apoptosis in tumor cell models through various molecular pathways, including regulation of the expression of members of the Bcl2 family, up-regulation of death receptors, and induction of oxidative injury (41, 42). As previously shown, TSA was found to strongly induce apoptosis in MCF-7 cells, to decrease the expression of *Bcl2* gene, and to markedly induce the expression of the death receptor *TRAIL-R2* (43, 44). By contrast, our results clearly showed that inhibition of class II HDACs was not involved in

the proapoptotic effects of HDI in MCF-7 cells. The absence of apoptotic effects on MC1575 and MC1568 treatment was consistent with the weak effects of these compounds on the expression of *Bcl2*, *Baxα*, and death receptor *TRAIL-R1* and *TRAIL-R2* genes. Similarly, Inoue et al. (25) found in another tumor model that inhibition of class I, but not class II, HDACs was critical for sensitization of cells to TRAIL-induced apoptosis, suggesting that our observation in MCF-7 cells may be a more general phenomenon.

Finally, we and others have shown that TSA differentially regulated ERα and ERβ in ERα-positive human breast and ovarian cell lines, leading to a strong decrease in ERα accumulation, contrasting with an increase in ERβ expression (14, 19, 20). In the present study, we confirmed these results and found that the regulation of *ERα* and *ERβ* expression on HDI treatment followed different kinetics, suggesting that various molecular pathways are involved. Moreover, specific inhibition of class II HDACs led to a different profile of regulation for both ER isoforms than TSA, as ERα was weakly altered whereas ERβ was still strongly induced. The use of MS-275, a class I–specific HDI, further suggested that ERα down-regulation was predominantly linked to class I HDAC inhibition (data not shown). In contrast, *ERβ* gene expression was induced by all HDIs, indicating that various classes of HDACs could be involved in its regulation. Whatever the underlying molecular mechanisms, the effects of MC1575 and MC1568 on ERα and ERβ isoforms in MCF-7 cells potentially represent an interesting physiologic condition whereby *ERβ* expression is induced while that of *ERα* remains unchanged.

As previously shown (14), ERα transcriptional activity was strongly induced by TSA. Although MC1575 also increased ERα transcriptional activity, this effect was weaker than that observed with TSA, suggesting that inhibition of class II HDACs was probably not the only factor involved in this regulation. The switch of the partial antiestrogen hydroxytamoxifen to an agonistic transcriptional activity on HDI treatment has been linked to ERα down-regulation (14). Our results are consistent with this hypothesis because on MC1575 treatment, MCF-7 cells displayed high levels of ERα protein together with an ER antagonist activity for hydroxytamoxifen.

From a clinical point of view, it has been suggested that combined therapies associating HDI and antiestrogens could be helpful for the treatment of patients with breast carcinomas expressing or not ERα. HDAC inhibition has indeed been shown not only to enhance the antiproliferative action of antiestrogens on ERα-positive breast cancer cells (45) but also to sensitize ERα-negative breast cancer cells to tamoxifen after reactivation of *ERα* expression (20). In this context, our data highlighting the antiproliferative activity of MC1575 and its effects on ER expression and activity (i.e., higher levels of ERs than in the presence of TSA and persistence of an antagonistic activity for hydroxytamoxifen) suggest that such a drug could be of potential interest for future therapeutic approaches in combination with antiestrogens.

In conclusion, our results evidence major differential effects of class II HDAC inhibition on cell cycle progression, apoptosis, gene expression, and ER signaling in mammary tumor cells, strengthening the notion that the different HDAC subclasses could play specific roles in breast tumorigenesis. It should be stressed that, when considering the different sets of genes analyzed in this study (HDACs, cell cycle, apoptosis regulators, or ERs), MC1575 failed to reproduce the negative regulations of gene expression observed on TSA treatment, while recapitulating most of its positive effects. Further studies using additional HDIs and siRNAs will be needed to identify the role of individual HDAC, especially class II enzymes, on the regulation of gene expression and cellular processes in breast tumor cells. The development of such approaches that specifically target HDAC isotypes will be critically important in the future for a better comprehension of the roles of these proteins in physiologic or pathologic processes and to propose the most ideal therapies with greatest efficacy and least unintended side effects.

Materials and Methods

Reagents

17β-Estradiol and TSA were from Sigma-Aldrich. Hydroxytamoxifen and ICI 182,780 were kind gifts of Sanofi-Aventis and Astrazeneca, respectively. MC1575 and MC1568 were synthesized as previously described (27, 28), dissolved in DMSO, and stored at −20°C before use. MS-275 was obtained from Calbiochem (VWR).

Cell Culture

MCF-7 cells were grown in Ham's F-12/DMEM (1:1) supplemented with 10% FCS (Invitrogen) and antibiotics. Before hormonal treatment, cells were stripped of endogenous steroids by passage in medium without phenol red containing 3% charcoal-stripped FCS (DCC medium). For experiments using estrogen ligands or HDIs, control cells were grown in medium complemented with vehicle alone (ethanol or DMSO). The MELN cell line was derived from MCF-7 cells stably transfected with the ERE-βGlob-Luc-SVneo plasmid (46).

RNA Extraction and Real-time Quantitative PCR

Total RNA was extracted using RNeasy minikit (Qiagen) according to the manufacturer's conditions. For reverse transcription-PCR, 1.5 μg of total RNA were subjected to reverse transcription using the Omniscript Reverse Transcriptase kit (Qiagen). Real-time PCR quantification was then done using a SYBR Green technology (Light Cycler, Roche). For each sample, mRNA levels of specific genes were corrected for mRNA levels of *TBP*, used as a reference gene, and normalized to a calibrator sample (untreated MCF-7 cells). The primers for *ERα, ERβ, cyclin D1, TBP, p21*, and *p27* genes have been described elsewhere (47-49). Primers for the other genes are depicted in Table 2.

Western Blot Analysis

Whole-cell extracts were prepared in high-salt lysis buffer containing 500 mmol/L NaCl, 50 mmol/L Tris (pH 8), 1% NP40, 1 mmol/L DTT, and proteases inhibitors (Roche Diagnostics). Proteins were quantified using the Bradford assay (Bio-Rad Laboratories), and 60 μg were loaded on SDS-PAGE and transferred onto nitrocellulose membrane. Blots were saturated in TBST buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl,

0.1% Tween 20 (v/v), 5% nonfat dehydrated milk (w/v)]; incubated with specific primary antibodies for cyclin D1 (clone sc-718, Tebu), ERα (clone sc-543, Tebu), $p21^{WAF1/CIP1}$ (Cell Signaling), acetyl-histone 3, acetyl-histone 4 (Upstate Biotechnology), acetyl-tubulin (clone 6-11B-1, Sigma), or actin (Sigma); and probed with the appropriate secondary antibody (Sigma). Detection was done using the Chemiluminescence Reagent Plus Kit (Perkin-Elmer Life Science).

Deacetylase Assays

Full-length HDAC1, HDAC2, HDAC3, HDAC8, and HDAC4 with COOH-terminal His tag were expressed using baculovirus expression systems. For histone deacetylase activity assays, purified HDACs (100 ng for class I HDACs, 250 ng for HDAC4 on histone substrate, 20 ng for HDAC4 on nonhistone substrate) were preincubated or not with HDI for 15 min and were used in the HDAC fluorescent activity assay according to the supplier's instructions (BIOMOL, Palatine House). Fluorescence was quantified using a TECAN Infinite M200 station. For measurement of HDAC4 activity against nonhistone substrate, the trifluoroacetyl-lysine substrate, specific for class IIa HDACs, was synthesized and used as previously described (29, 30). Assays were carried out in triplicates.

Cell Proliferation

For proliferation studies, cells were seeded at 25×10^3 per well in 24-well dishes in Ham's F-12/DMEM supplemented with 10% FCS. After 24 h, cells were treated with either vehicle alone or HDIs at various concentrations, and total cell DNA was quantified by diaminobenzoic acid assay at days 2, 5, and 7 as previously described (19). During cell proliferation assay, treatment with HDI or vehicle alone was renewed every 2 d.

Cell Cycle Analysis

For cell cycle analysis, 2×10^6 cells were seeded in 25-cm^2 flasks. After 24 h, cells were treated for 20 h with either solvent alone or HDI at various concentrations. Cells were then pelleted, washed, and incubated in a staining solution containing 10 μg/mL propidium iodide. The cellular suspension was passed through a FACSVantage flow cytometer (Becton Dickinson). For each sample, at least 2×10^4 events were acquired and analyzed using CellQuest 3.3 (Becton Dickinson) and ModFit LT 3.1 softwares.

Apoptosis Measurement

MCF-7 cells were plated in six-well plates (5×10^4 per well) and treated or not with TSA, MC1575, or MC1568 for 40 h. Apoptosis was quantified 24 h later using the Cell Death Detection ELISA (Roche Molecular Biochemicals) according to the manufacturer's conditions. Values from absorbance measurements at 405 nm were corrected using DNA quantification in separate wells treated in parallel.

Luciferase Assays

For measurement of ERα-dependent transactivation assays, MELN cells were plated in six-well plates (5×10^5 per well).

Table 2. Primer Sequences Used for Quantitative PCR

	Primers	Gene accession no.	PCR size product (nt)
HDAC1	F-5′-CCTGAGGAGAGTGGCGATGA-3′ R-5′-GTTTGTCAGAGGAGCAGATCGA-3′	NM 004964	69
HDAC2	F-5′-GCTCTCAACTGGCGGTTCAG-3′ R-5′-AGCCCAATTAACAGCCATATCAG-3′	NM 001527	75
HDAC3	F-5′-CCCAGACTTCACACTTCATCCA-3′ R-5′-GGTCCAGATACTGGCGTGAGTT-3′	NM 003883	70
HDAC4	F-5′-GACCTGACCGCCATTTGC-3′ R-5′-GGGAGAGGATCAAGCTCGTTT-3′	NM 006037	73
HDAC5	F-5′-CAACGAGTCGGATGGGATGT-3′ R-5′-GGGATGCTGTGCAGAGAAGTC-3′	NM 005474	74
HDAC6	F-5′-TGCCTCTGGGATGACAGCTT-3′ R-5′-CCTGGATCAGTTGCTCCTTGA-3′	NM 006044	69
HDAC7	F-5′-AGCAGCTTTTTGCCTCCTGTT-3′ R-5′-TCTTGCGCAGAGGGAAGTG-3′	NM 015401	66
HDAC8	F-5′-CGGCCAGACCGCAATG-3′ R-5′-CACATGCTTCAGATTCCCTTT-3′	NM 018486	56
HDAC 9	F-5′-AGGCTCTCCTGCAGCATTTATT-3′ R-5′-AAGGGAACTCCACCAGCTACAA-3′	NM 014707	75
HDAC10	F-5′-ATGACCCCAGCGTCCTTTACT-3′ R-5′-CGCAGGAAAGGCCAGAAG-3′	NM 032019	66
HDAC11	F-5′-CCCCTTGGTCATGGGATTT-3′ R-5′-CATCCACACCAGTGCCTATAGC-3′	NM 024827	68
Bcl2	F-5′-GGTGCCACCTGTGGTCCACCTG-3′ R-5′-CTTCACTTGTGGCCCAGATAGG-3′	NM 633.2	459
Baxα	F-5′-ATGGACGGGTCCGGGGAGGAGC-3′ R-5′-CCCCAGTTGAAGTTGCCGTCAG-3′	NM 138761.2	323
$p14^{ARF}$	F-5′-GGTTTTCGTGGTTCACATCC-3′ R-5′-CCTCAGTAGCATCAGCACGA-3′	NM 058195	91
TRAIL-R1 (TNFRS10A)	F-5′-TGCTTCCAACAATTTGTTTGCT-3′ R-5′-CGTGGTGCAGGGACTTCTCT-3′	NM 003844.2	79
TRAIL-R2 (TNFRS10B)	F-5-GGTTCCAGCAAATGAAGGTGAT-3′ R-5′-AAGGGCACCAAGTCTGCAAA-3′	NM 003842.3	75

Abbreviation: nt, nucleotides.

Cells were lysed at 4°C for 10 min in 400 μL of lysis buffer [25 mmol/L Tris (pH 7.8), 2 mmol/L EDTA, 10% glycerol, 1% Triton X-100] and luciferase activity was measured as previously described (50).

Statistical Analysis

Results are expressed as mean ± SD. Statistical analysis was done on raw data using Student's *t* test or Mann-Whitney *U* test for comparison of two groups. A probability level of 0.05 was chosen for statistical significance. Statistical analysis was done using GraphPad InStat version 3.06 (GraphPad Software).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Stephan Jalaguier for critical reading of the manuscript.

References

1. Minucci S, Pelicci PG. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer 2006;6:38–51.

2. Yang X-J, Seto E. HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. Oncogene 2007;26:5310–8.

3. Hubbert C, Guardiola A, Shao R, et al. HDAC6 is a microtubule-associated deacetylase. Nat Rev Cancer 2002;417:455–8.

4. Boyault C, Zhang Y, Fritah S, et al. HDAC6 controls major cell response pathways to cytotoxic accumulation of protein aggregates. Genes Dev 2007;21:2172–81.

5. Chang S, McKinsey TA, Zhang CL, Richardson JA, Hill JA, Olson AN. Histone deacetylase 5 and 9 govern responsiveness of the heart to a subset of stress signals ans play redundant roles in the heart development. Mol Cell Biol 2004;24:8467–76.

6. Dequiedt F, Kasler H, Fischle W, et al. HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis. Immunity 2003;18:687–98.

7. Martin M, Kettmann R, Dequiedt F. Class IIa histone deacetylases: regulating the regulators. Oncogene 2007;26:5450–67.

8. Vega RB, Matsuda K, Oh J, et al. Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. Cell 2004;119:555–66.

9. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov 2006;5:769–84.

10. Liu T, Kuljaca S, Tee A, Marshall GM. Histone deacetylase inhibitors: multifunctional anticancer agents. Cancer Treat Rev 2006;32:157–65.

11. Duvic M, Vu J. Vorinostat: a new oral histone deacetylase inhibitor approved for cutaneous T-cell lymphoma. Expert Opin Investig Drugs 2007;16:1111–20.

12. Marks PA. Discovery and development of SAHA as an anticancer agent. Oncogene 2007;26:1351–6.

13. Davis T, Kennedy C, Chiew Y-E, Clarke CL, deFazio A. Histone deacetylase inhibitors decrease proliferation and modulate cell cycle gene expression in normal mammary epithelial cells. Clin Cancer Res 2000;6:4334–42.

14. Margueron R, Duong V, Bonnet S, et al. Histone deacetylase inhibition and estrogen receptor α levels modulate the transcriptional activity of partial antiestrogens. J Mol Endocrinol 2004;32:583–94.

15. Margueron R, Licznar A, Lazennec G, Vignon F, Cavailles V. Oestrogen receptor α increases p21 waf1/Cip1 gene expression and the antiproliferative activity of histone deacetylase inhibitors in human breast cancer cells. J Endocrinol 2003;179:41–53.

16. Vigushin DM, Ali S, Pace PE, et al. Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer *in vivo*. Clin Cancer Res 2001;7:971–6.

17. Yang X, Ferguson AT, Nass SJ, et al. Transcriptional activation of estrogen receptor α in human breast cancer cells by histone deacetylase inhibition. Cancer Res 2000;60:6890–4.

18. Aranda A, Pascual A. Nuclear hormone receptors and gene expression. Physiol Rev 2001;81:1269–304.

19. Duong V, Licznar A, Margueron R, et al. ERα and ERβ expression and transcriptional activity are differentially regulated by HDAC inhibitors. Oncogene 2006;25:1799–806.

20. Jang ER, Lim S-J, Lee ES, et al. The histone deacetylase inhibitor trichostatin A sensitizes estrogen receptor α-negative breast cancer cells to tamoxifen. Oncogene 2004;23:1724–36.

21. Kenn JC, Yan L, Mack KM, et al. A novel histone deacetylase inhibitor, Scriptaid, enhances expression of functional estrogen receptor α (ER) in ER negative human breast cancer cells in combination with 5-aza 2′-deoxycytidine. Breast Cancer Res Treat 2003;81:177–86.

22. Sharma D, Blum J, Yang X, Beaulieu N, Macleod AR, Davidson NE. Release of methyl CpG binding proteins and histone deacetylase 1 from the estrogen receptor α (ER) promoter upon reactivation in ER-negative human breast cancer cells. Mol Endocrinol 2005;19:1740–51.

23. Zhou Q, Ataja P, Davidson NE. Histone deacetylase inhibitor LBH589 reactivates silenced estrogen receptor α (ER) gene expression without loss of DNA hypermethylation. Cancer Biol Ther 2007;6:64–9.

24. Illi B, Dello Russo C, Colussi C, et al. Nitric Oxide modulates chromatin folding in human endothelial cells via PP2A activation and class II HDACs nuclear shuttling. Circ Res 2008;102:51–8.

25. Inoue S, Mai A, Dyer MJS, Cohen GM. Inhibition of histone deacetylase class I but not class II is critical for the sensitization of leukemic cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. Cancer Res 2006;66:6785–92.

26. Kim IA, Shin JH, Kim IH, et al. Histone deacetylase inhibitor-mediated radiosensitization of human cancer cells: class differences and the potential influence of p53. Clin Cancer Res 2006;12:940–9.

27. Mai A, Massa S, Pezzi R, Rotili D, Loidl P, Brosch G. Discovery of (aryloxopropenyl)pyrrolyl hydroxyamides as selective inhibitors of class IIa histone deacetylase homologue HD1-A. J Med Chem 2003;46:4826–9.

28. Mai A, Massa S, Pezzi R, et al. Class II (IIa)-selective histone deacetylase inhibitors. 1. Synthesis and biological evaluation of novel (Aryloxopropenyl)-pyrrolyl hydroxyamides. J Med Chem 2005;48:3344–53.

29. Jones P, Altamura S, De Francesco R, et al. Probing the elusive catalytic activity of vertebrate class IIa histone deacetylases. Bioorg Med Chem Lett 2008;18:1814–9.

30. Lahm A, Paolini C, Pallaoro M, et al. Unraveling the hidden catalytic activity of vertebrate class IIa histone deacetylases. Proc Natl Acad Sci U S A 2007;104:17335–40.

31. Dokmanovic M, Perez G, Xu W, et al. Histone deacetylase inhibitors selectively suppress expression of HDAC7. Mol Cancer Ther 2007;6:2525–34.

32. Kawai H, Li H, Avraham S, Jiang S, Avraham HK. Overexpression of histone deacetylase HDAC1 modulates breast cancer progression by negative regulation of estrogen receptor α. Int J Cancer 2003;107:353–8.

33. Varshochi R, Halim F, Sunters A, et al. ICI182,780 induces p21Waf1 gene transcription through releasing histone deacetylase 1 and estrogen receptor α from Sp1 sites to induce cell cycle arrest in MCF-7 breast cancer cell line. J Biol Chem 2005;280:3185–96.

34. Krusche CA, Wülfing P, Kersting C, et al. Histone deacetylase-1 and -3 protein expression in human breast cancer: a tissue microarray analysis. Breast Cancer Res Treat 2005;90:15–23.

35. Saji S, Kawakami M, Hayashi S, et al. Significance of HDAC6 regulation via estrogen signaling for cell motility and prognosis in estrogen receptor-positive breast cancer. Oncogene 2005;24:4531–9.

36. Zhang Z, Yamashita H, Toyama T, et al. HDAC6 expression is correlated with better survival in breast cancer. Clin Cancer Res 2004;10:6962–8.

37. Ocker M, Schneider-Stock R. Histone deacetylase inhibitors: signalling towards p21cip1/waf1. Int J Biochem Cell Biol 2007;39:1367–74.

38. Glaser KB, Li J, Staver MJ, Wei RQ, Albert DH, Davidsen SK. Role of class I and class II histone deacetylase in carcinoma cells using siRNA. Biochem Biophys Res Commun 2003;310:529–36.

39. Lagger G, O'Carroll D, Rembold M, et al. Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. EMBO J 2002;21:2672–81.

40. Senese S, Zaragoza K, Minardi S, et al. Role for histone deacetylase 1 in human cell proliferation. Mol Cell Biol 2007;27:4784–95.

41. Nakata S, Yoshida T, Horinaka M, Shiraishi T, Wakada M, Sakai T. Histone deacetylase inhibitors up-regulate death receptor 5/TRAIL-R2 and sensitize apoptosis induced by TRAIL/APO2-L in human malignant tumor cells. Oncogene 2004;23:6261–71.

42. Rosato RR, Grant S. Histone deacetylase inhibitors: insight into mechanisms of lethality. Expert Opin Ther Targets 2005;9:809–24.

43. Chopin V, Slomianny C, Hondermarck H, Le Bourhis X. Synergistic

induction of apotosis in breast cancer cells by cotreatment with butyrate and TNF-α, TRAIL, or anti-Fas agonist antibody involves enhancement of death receptors' signaling and requires p21waf1. Exp Cell Res 2004;298:560–73.

44. Chopin V, Toillon RA, Jouy N, Le Bourhis X. p21waf1/cip1 is dispensable for G_1 arrest, but indispensable for apoptosis induced by sodium butyrate in breast cancer cells. Oncogene 2004;23:21–9.

45. Hodges-Gallagher L,Valentine CD, El Bader S, et al. Inhibition of histone deacetylase enhances the anti-proliferative action of antiestrogens on breast cancer cells and blocks tamoxifen-induced proliferation of uterine cells. Breast Cancer Res Treat 2007;105:297–309.

46. Balaguer P, François F, Comunale F, et al. Reporter cell lines to study the estrogenic effects of xenoestrogens. Sci Total Environ 1999;233:47–56.

47. Anicotte J-S, Iankova I, Miard S, et al. Peroxisome proliferator-activated receptor γ regulates E-cadherin expression and inhibits growth and invasion of prostate cancer. Mol Cell Biol 2006;26:7561–74.

48. Chen Y-X, Fang J-Y, Zhu H-Y, Lu R, Cheng Z-H, Qiu D-K. Histone acetylation regulates $p21^{WAF1}$ expression in human colon cancer cell lines. World J Gastroenterol 2004;10:2643–6.

49. Delfour C, Roger P, Bret C, et al. RCL2, a new fixative, preserves morphology and nucleic acid integrity in paraffin-embedded breast carcinoma and microdissected breast tumor cells. J Mol Diagn 2006;8:157–69.

50. Duong V, Boulle N, Daujat S, et al. Differential regulation of estrogen receptor α turnover and transactivation by Mdm2 and stress-inducing agents. Cancer Res 2007;67:5513–21.

Molecular Cancer Research

Specific Activity of Class II Histone Deacetylases in Human Breast Cancer Cells

Vanessa Duong, Caroline Bret, Lucia Altucci, et al.

Mol Cancer Res 2008;6:1908-1919.