Oestrogen receptor α increases p21^{WAF1/CIP1} gene expression and the antiproliferative activity of histone deacetylase inhibitors in human breast cancer cells

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Abstract

We analysed the antiproliferative activity of various histone deacetylase (HDAC) inhibitors such as trichostatin A (TSA) on human breast cancer cells. We observed a lower sensitivity to HDAC inhibition for oestrogen receptor negative (ER[−]) versus positive (ER⁺) cell lines. This differential response was associated neither with a modification of drug efflux via the multidrug resistance system nor with a global modification of histone acetyltransferase (HAT)/HDAC activities. In contrast, we demonstrated that in ER⁺ breast cancer cells the p21^{WAF1/CIP1} gene was more sensitive to TSA regulation and was expressed at higher levels. These differences were observed both in transient transfection experiments and on the endogenous

p21^{WAF1/CIP1} gene. The Sp1 transcription factor, which was shown to interact *in vitro* with both class I and class II HDACs, is sufficient to confer the differential sensitivity to TSA and participated in the control of p21^{WAF1/CIP1} basal expression. Finally, re-expression of ER α following adenoviral infection of ER[−] breast cancer cells increased both p21^{WAF1/CIP1} protein accumulation and the growth inhibitory activity of TSA. Altogether, our results highlight the key role of ER α and p21^{WAF1/CIP1} gene expression in the sensitivity of breast cancer cells to hyperacetylating agents.

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Introduction

The implication of ovarian hormones in the development of breast cancer has been widely demonstrated and oestrogen dependency of these tumours has been successfully exploited to develop an antihormonal chemotherapy (Santen *et al.* 1990). Unfortunately, treatments with anti-oestrogens are not effective in all tumours and acquired resistance also results in patient relapse. The effects of oestrogens and anti-oestrogens are primarily mediated through the interaction with specific nuclear oestrogen receptors (ER α and ER β), acting as ligand-dependent transcription factors (Sommer & Fuqua 2001). ER β expression decreases during breast cancer carcinogenesis (Roger *et al.* 2001) and the major form of ER in breast cancers appears to be ER α at both the protein and mRNA levels (Brouillet *et al.* 2001). The absence of ER α expression in so-called ER-negative (ER[−]) breast cancers is the main cause of *de novo* resistance to antihormonal therapies, which is associated with aggressive cancer (Jordan 1998).

Besides endocrine therapies, other types of chemotherapy are available and during the last few years new therapeutic targets have been characterised, in particular those involving chromatin structure. The basic unit of chromatin is the nucleosome, which is formed by the

packaging of eukaryotic DNA with core histones (Woodcock & Dimitrov 2001). Post-translational modifications of histone tails (acetylation, methylation and phosphorylation) alter the nucleosomal structure and play a major role in the regulation of gene transcription (Strahl & Allis 2000). 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 as being associated with the appearance of various cancer types, mainly myeloid leukaemia or lymphoma (Pandolfi 2001).

Several specific HDAC inhibitors (trichostatin A, SAHA, apicidin, FR901228...) have been shown to modify reversibly or irreversibly the balance between HAT and HDAC activities (Marks *et al.* 2000). *In vitro*, in various cell models, these compounds 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 efficacy of some of these inhibitors in blocking tumour growth (Marks *et al.* 2000), mammary tumours in particular (Vigushin *et al.* 2001). 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 six months (Warrell *et al.* 1998). Phase I clinical trials are currently under way for several of these molecules (Kramer *et al.* 2001).

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 (Van Lint *et al.* 1996). 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 HDAC inhibitors (Kramer *et al.* 2001).

In this study, we have analysed the repressive activity of various HDAC inhibitors on proliferation of human breast cancer cells and have observed a difference in sensitivity between ER+ and ER- breast cancer cell lines. The comparative analysis was performed between the two cell types at the level of endogenous histone acetylation, HAT and HDAC activities and gene expression. Our results suggest that ERα, by controlling the expression of the p21^{WAF1/CIP1} gene, could be a key regulator of the differential response of breast cancer cells to HDAC inhibitory drugs.

Materials and Methods

Plasmids and reagents

Plasmids containing p21^{WAF1/CIP1} promoter sequences in front of the luciferase gene (pWWP and pWP101 constructs) (Sowa *et al.* 1997) were kindly provided by Dr Sowa (Kyoto, Japan). The glutathione S-transferase (GST)-Sp1 and HDAC1 expressing plasmids, together with the Sp1-luc reporter (Doetzlhofer *et al.* 1999), were obtained from Dr C Seiser (VBC, Vienna, Austria) and the HDAC5 and 6 expressing vectors from Dr S Khochbin (INSERM U309, La Tronche, France). The MCK-Luc reporter vector contains the enhancer of muscle creatine kinase gene in front of the TATA box of the major late adenovirus promoter (Zambetti *et al.* 1992). The ERα expression vector (HEGO) was a gift of P Chambon (IGBMC, Strasbourg, France). Trichostatin A (TSA) and doxorubicin were from Sigma (Saint-Quentin, France).

Cell culture

MCF7 (ER+/p53wt), T-47D (ER+/p53 mt), MDA-MB-231 (ER-/p53 mt) and MDA-MB-435 (ER-/p53 mt) breast cancer cells were derived from stocks routinely maintained in the laboratory. MCF7/DOX cells (Julia *et al.* 1994) were obtained from Dr F Pinguet (CRLC, Montpellier, France). Monolayer cell cultures were grown in Ham's F-12/Dulbecco's modified Eagle's medium (1:1) (F12/DMEM) supplemented with 10% foetal calf serum (FCS) (Life Technologies, Cergy-Pontoise, France) and antibiotics. The propagation of the parental Ad5 and recombinant Ad-hERα adenovirus, together with the infection of MDA-MB-231 cells was carried out as described previously (Lazennec *et al.* 2001). Cell prolifer-

ation was evaluated on replicate wells fixed *in situ* with methanol by total DNA measurement using the DABA fluorimetric assay (excitation at 405 nm and emission at 495 nm) on a fluorimeter (Molecular Device, Saint-Germain, France). When appropriate, statistical analyses were performed using the independent samples *t*-test.

Transfection and luciferase assays

For transient transfection experiments, cells were plated at about 80% confluence (10⁶ cells/35-mm diameter well). Plasmids (reporter, expression vector for ERα and CMV-βGal as an internal control) were transfected using the calcium phosphate method as previously described (Philips *et al.* 1993). Cell extract preparation was carried out as recommended by Promega Corporation. Cells were lysed at 4 °C for 10 min in 0.4 ml lysis buffer (25 mM Tris pH 7.8, 2 mM EDTA, 10% glycerol, 1% Triton X-100). Luciferase activity was measured on 100 µl supernatant aliquots during 1 s after injection of 100 µl luciferase detection solution (20 mM Tricine pH 7.08, 1.07 mM (MgCO₃)₄Mg(OH)₂·5H₂O, 2.67 mM MgSO₄, 0.2 mM EDTA, 0.53 mM ATP, 0.27 mM coenzyme A, 0.48 mM luciferin) using a luminometer (Labsystem, Les Ulis, France). When comparing basal levels between different cell lines, transfection data were normalised by the β-galactosidase activities determined as described (Philips *et al.* 1993) and expressed as relative luciferase activities.

Extraction and analysis of histone acetylation

Histones were extracted according to the procedure of Yoshida *et al.* (1990). Briefly, after homogenisation, H₂SO₄-soluble proteins were precipitated by acetone and analysed on acid/urea/Triton (AUT) gels (1 M acetic acid, 8 M urea, 0.5% Triton X-100, 15% acrylamide) which were then stained with Coomassie Brilliant Blue R-250, dried and scanned.

HAT assays

Whole cell extracts were prepared in HAT buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% NP40). Assays were performed using 25 µg histones (Sigma) and 1 µl of 10-fold diluted ¹⁴C acetyl CoA (63 mCi/mM; ICN, Orsay, France) in 30 µl final volume of HAT-buffer and incubating for 1 h at 30 °C. Acetylated histones were then retained on Whatman P-81 phosphocellulose filter paper. The filter papers were air-dried, washed three times in 0.2 M sodium carbonate buffer (pH 9.2) and radioactivity was determined by liquid scintillation counting.

HDAC assay

Acetyl-lysine-labelled histones were extracted from K562 cells treated by sodium butyrate as previously described (Rundlett *et al.* 1996). For standard assay, 10 µg ³H-labelled histones were mixed with 20 µg nuclear extract in

50 μ l buffer (50 mM Tris pH 7.5, 100 mM NaCl, 0.1 mM NaEDTA, 0.1 mM AEBSF). The mixture was incubated at 37 °C for 90 min and stopped by the addition of 36 μ l 1 M HCl–0.4 M acetate followed by 800 μ l ethyl acetate. After centrifugation, 600 μ l of the solvent layer was taken into 5 ml toluene scintillation solution for determination of radioactivity.

Protein and RNA analysis

Whole cell extracts were prepared in 0.4 M KCl, 20 mM HEPES (pH 7.4), 20% glycerol, 1 mM dithiothreitol (DTT) and proteases inhibitors. Proteins were quantified using the Bradford assay (Bio-Rad Laboratories, Marnes, France) and 60 μ g were usually analysed. The blots were saturated in TBST buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween 20 (v/v), 5% dehydrated milk (w/v)), incubated with specific primary antibodies for p21^{WAF1/CIP1} (Merck Eurolab, Strasbourg, France), gelsolin or actin (Sigma-Aldrich, Saint Quentin, France), ER α or c-myc (Tebu, Le Perray, France) and with the appropriate second antibody (Sigma-Aldrich). Detection was carried out using the Chemiluminescence Reagent Plus kit (PerkinElmer Life Science, Courtaboeuf, France).

Total RNA was isolated from MDA-MB-231 cells using the TRIzol reagent (Life Technologies) as described by the manufacturer. A specific p21^{WAF1/CIP1} probe was generated and hybridised as previously described (Lazennec *et al.* 2001).

Band shift assay

Nuclear extracts were prepared with the NE-PER kit (Perbio, Bezons, France) and gel retardation assays with the consensus Sp1 DNA-binding probe were subsequently performed as previously described (Thenot *et al.* 1999). Gels were dried and exposed to photo stimulatable plates, scanned and quantified with a Fujix-Bas 1000 phosphorimager (RAYTEST, Courbevoie, France).

GST pull-down assay

In vitro translation and GST pull-down assays were performed as previously described (Thenot *et al.* 1999) except that the bacterial pellet was sonicated in TST buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.05% Tween 20). Binding and washes were carried out in 20 mM HEPES pH 7.5, 100 mM KCl, 10 mM MgCl₂, 10% glycerol, 1 mM DTT, 0.5 mM EDTA and 0.1% Nonidet P-40.

Results

ER α – breast cancer cells are less sensitive to HDAC inhibition

The antiproliferative effect of TSA, a potent but reversible HDAC inhibitor, was analysed by total DNA quantifi-

cation in human breast cancer cell lines expressing (MCF7, T-47D) or not expressing (MDA-MB-231 and MDA-MB-435) functional ER α . As shown in Fig. 1A, TSA exhibited a growth-inhibitory activity in all cell lines, but ER α breast cancer cells were clearly more sensitive to low concentrations of TSA than ER α – cells. For instance, under the conditions of our assay, TSA at a concentration of 12.5 ng/ml was totally ineffective on ER α – cell proliferation, whereas it decreased cell number by 62.8% and 52% in MCF7 and T-47D cells respectively. Similar results were obtained in MCF7 and MDA-MB-231 cells with other known HDAC inhibitors such as the short-chain fatty acid *n*-butyrate or HC-toxin, a naturally occurring cyclic tetrapeptide (Fig. 1B). Together, these results indicated that ER α breast cancer cells exhibit an increased sensitivity to structurally unrelated HDAC inhibitors.

Overexpression of membrane pumps that extrude a wide range of anticancer drugs (Filipits *et al.* 1999) could explain the different efficacy of HDAC inhibitors in ER α and ER α – breast cancer cells. We therefore wanted to eliminate the possibility that the resistance of ER α – breast cancer cells to HDAC inhibitors could be caused by an increase of the general multidrug resistance (MDR) phenotype. We first used an MCF7 subtype selected for its resistance to doxorubicin (Julia *et al.* 1994) and showed that in these cells, the efficacy of TSA was not decreased at all but rather it was slightly increased (Fig. 1C). The use of two MDR inhibitors, namely verapamil (a calcium channel blocker, inhibitor of P-gp) and probenecid (inhibitor of MDR-associated protein) revealed that these compounds did not increase the sensitivity of MDA-MB-231 cells to TSA (not shown). Altogether, these results suggested that the different sensitivity of ER α – breast cancer cells was not due to a modification of the MDR phenotype.

Effect of HDAC inhibitors on histone acetylation

To further analyse the mechanism of this differential sensitivity of ER α and ER α – breast cancer cells to HDAC inhibitors, we then compared the effect of TSA at the level of histone hyperacetylation, which is likely to be one of the first consequences of HDAC blockade. Using AUT gel electrophoresis, both the non-acetylated and the mono-acetylated forms of histone H4 were detected in the absence of TSA (Fig. 2A). A strong dose-dependent increase in the accumulation of acetylated forms of H4 was observed upon TSA addition in both MCF7 and MDA-MB-231 cells without significant differences between the two cell lines. We noted that in MDA-MB-231 cells at high concentrations of TSA, the fraction of non-acetylated forms of H4 was more important than in MCF7 cells but this difference was not observed when comparing T-47D and MDA-MB-435 cells (data not shown). These results demonstrated that in both cell lines HDAC inhibition

Figure 1 Effect of histone deacetylase inhibitors on proliferation of human breast cancer cell lines. (A) The four cell lines expressing (white bars) or not expressing (black bars) endogenous ER α were treated during 96 h with vehicle alone or increasing amounts of TSA (ng/ml). Cell count was quantified as described in Materials and Methods. Results are the means ($\pm$ S.D.) of four experiments and are expressed as a percentage of controls. (B) The same experiment as in A was realised in MCF7 (white bars) or MDA-MB-231 (black bars) cells treated with increasing concentrations of HC-toxin (ng/ml) or sodium butyrate (mM). (C) MCF-R (doxorubicin (Dox) resistant cell line, black symbols) and MCF7-S (control MCF7 cells, white symbols) cells were treated by increasing concentrations of either doxorubicin (circles) or TSA (triangles). Treatments were performed exactly as in A and the results, expressed as a percentage of control, are the means ($\pm$ S.D.) of three experiments.

Figure 2 Effect of TSA on histone H4 acetylation and HAT/HDAC activities. (A) Histones purified from MCF7 and MDA-MB-231 cells treated during 9 h by vehicle or increasing concentrations of TSA were analysed on an acid-urea-Triton gel. (B) HAT (left panel) or HDAC (right panel) enzymatic activities were measured as described in Materials and Methods using MCF7, T-47D, MDA-MB-231 and MDA-MB-435 whole cell extracts. Results are the means ($\pm$ S.D.) of three independent experiments and are expressed as a percentage of MCF7 levels. White bars, cell lines expressing endogenous ER α ; black bars, cell lines not expressing endogenous ER α .

was effective and that no major difference in terms of sensitivity to TSA was detected at the level of bulk histone acetylation.

HAT and HDAC enzymatic activities were then analysed in the four breast cancer cell lines. No significant quantitative differences concerning the total HAT or HDAC activities were observed between the different cell lines in relation to ER status (Fig. 2B). Moreover, we also showed that the *in vitro* activity of endogenous HDACs isolated from MCF7 and MDA-MB-231 cells was inhibited to the same extent by increasing concentrations of TSA (not shown). This result indicated that global modifications of the HAT/HDAC ratio were probably not directly involved in the differential response of breast cancer cells to hyperacetylating agents.

Effect of TSA on gene expression

We then tested the effect of HDAC inhibition at the gene level and measured by Western blot the TSA regulation of

endogenous gene expression in ER+ and ER- breast cancer cells. As shown in Fig. 3A, the accumulation of gelsolin, an actin binding protein, was increased by TSA in both MCF7 and MDA-MB-231 cells. Other genes such as c-myc (Fig. 3A) or HDAC1 (data not shown) reported as regulated by HDAC inhibitors (for review see Kramer *et al.* 2001), were insensitive in our conditions.

Interestingly, the levels of p21^{WAF1/CIP1}, a cyclin-dependent kinase inhibitor, were transiently increased in MCF7 and MDA-MB-231 cells, and the effects of TSA occurred at lower concentrations of drug in ER+ breast cancer cells. Maximal induction of p21^{WAF1/CIP1} was obtained for both MCF7 and T-47D cells at 100 ng/ml, whereas induction was only slightly detectable at 500 ng/ml in MDA-MB-231 and undetectable in MDA-MB-435 cells at any of the concentrations used (Fig. 3B). Therefore, the increased sensitivity of p21^{WAF1/CIP1} gene expression to TSA in ER+ cell lines appeared to be correlated with the antiproliferative activity mediated by HDAC inhibitors.

Figure 3 Effect of TSA on the regulation of gene expression. (A) MCF7 and MDA-MB-231 cell lines were exposed to TSA (500 ng/ml) for the indicated periods and gelsolin, c-myc and p21^{WAF1/CIP1} expressions were analysed by Western blot. (B) p21^{WAF1/CIP1} levels were determined in MCF7, T-47D, MDA-MB-231 and MDA-MB-435 cell lines treated with increasing amounts of TSA for 17 h.

Differential regulation of the p21^{WAF1/CIP1} gene by TSA

To analyse in more detail the regulation of p21^{WAF1/CIP1} gene expression in ER+ and ER- breast cancer cells, we performed transient transfection experiments. We used plasmids containing the luciferase reporter gene under the control of the human p21^{WAF1/CIP1} promoter (pWWP and pWP101 reporter constructs corresponding respectively to regions of 2400 bp and 101 bp upstream of the transcription start site). As shown in Fig. 4, both the large (pWWP in panel A) and the proximal (pWP101 construct in panel B) promoter region of the p21^{WAF1/CIP1} gene conferred a strong induction by TSA both in ER- and ER+ breast cancer cells. However, in accordance with our results on the endogenous gene (Fig. 3), ER+ cells were more sensitive to TSA with either reporter, the EC₅₀ for the induction of pWWP ranging from 50 ng/ml for MCF7 and T-47D to about 320 ng/ml for the two ER- cell lines.

Since one of the proximal Sp1 sites had been identified as the major TSA-responsive sequences in the p21^{WAF1/CIP1} promoter in MG63 osteosarcoma cells (Sowa *et al.* 1997), we analysed the response of the Sp1-luc reporter plasmid in the ER+ and ER- breast cancer cell lines. This construct, which is a luciferase reporter construct bearing only three copies of the GC rich Sp1 binding site in front of a TATA box, also exhibited a strong response to TSA treatment which again was more efficient in MCF7 cells as compared with MDA-MB-231 cells (Fig. 4C). As a

control, the MCK-Luc construct that contains the muscle creatine kinase enhancer in front of a minimal promoter (Zambetti *et al.* 1992) was similarly induced in both cell types (Fig. 4D). This indicated that the differential TSA regulation through Sp1 sites was not a general effect, in accordance with the results shown in Fig. 2.

Next, we sought to determine whether the increase in sensitivity to TSA of ER+ breast cancer cells could be associated with a modification, either in the nature or in the amounts of transcription factors that interact with the Sp1 binding sites. We first carried out gel retardation assays using nuclear extracts from either MCF7 or MDA-MB-231 cells treated or not with TSA for 9 or 24 h. As shown in Fig. 5A, no significant differences were observed between the two cell types in the absence or presence of inhibitor indicating that TSA-induced hyperacetylation in breast cancer cells did not alter the total DNA binding activity on Sp1 binding sites.

We then performed GST-pull-down experiments in order to define which type of HDACs could interact *in vitro* with Sp1 (Fig. 5B). HDAC1, 3, 5 and 6 were all similarly recruited by GST-Sp1, whereas no signal was obtained with GST alone. The strength of these interactions was comparable to that of ERα previously described as binding to Sp1. These results suggest that both class I and class II HDACs could be recruited by Sp1 and play a role in the differential response of p21^{WAF1/CIP1} promoter to TSA treatment.

Figure 4 Differential responsiveness to TSA of p21^{WAF1/CIP1} promoter constructs. Transient transfection experiments using pWWP (A), pWP101 (B), Sp1-Luc (C) or MCK-Luc (D) constructs were performed in the two ER+ (open symbols) or ER- (closed symbols) breast cancer cell lines treated with increasing amounts of TSA for 24 h. Results are expressed as a percentage of the maximum luciferase activity and are the means ($\pm$ S.D.) of three independent experiments.

Basal levels of p21^{WAF1/CIP1} gene expression

As the basal level of p21^{WAF1/CIP1} gene expression was hardly distinguishable in Fig. 3, we modified our Western blot experimental conditions to compare the levels in the four cell lines. We showed that the rates of protein accumulation were significantly higher in the two ER+ cell lines (Fig. 6A upper panel). Moreover, the difference in the basal expression of p21^{WAF1/CIP1} gene between ER+ and ER- breast cancer cells was reproduced in transient transfection experiments using the pWWP plasmid (Fig. 6A lower panel). Low levels of luciferase were measured in the two ER- cell lines, whereas significantly higher amounts were found in the two ER+ cell lines, MCF7 cells exhibiting the highest expression.

Among the four cell lines tested, MCF7 cells are the only ones which express wild-type p53, a strong positive regulator of p21^{WAF1/CIP1} gene expression, which acts through two p53 response elements and also via the Sp1 binding site (Koutsodontis *et al.* 2001). In order to define which regions of the p21^{WAF1/CIP1} promoter were implicated in the differential expression independently of a functional p53, we used T-47D and MDA-MB-231 cell lines which both expressed mutated p53. As shown in Fig. 6B, we noted in both cell lines a weaker expression of the pWP101 reporter as compared with the pWWP

construct, thus indicating the importance of distal sites for the control of the basal expression. Interestingly, both pWP101 and Sp1-Luc reporter plasmids reproduced the difference observed between the two cell lines on the pWWP construct (between 10- and 15-fold).

Together, these results revealed differences in basal expression of the p21^{WAF1/CIP1} gene between ER+ and ER- cell lines, both on the accumulation of the endogenous protein and on the expression of transiently transfected reporters. They also demonstrated that Sp1 binding sites seemed to be key mediators of this differential basal expression of p21^{WAF1/CIP1}.

Effect of ER α expression in ER- breast cancer cells

The increase in p21^{WAF1/CIP1} basal levels in ER+ breast cancer cells could reflect transactivation of the p21^{WAF1/CIP1} gene by oestrogen receptors. In support of this hypothesis, we showed in transient transfection assays that ER α overexpression in MDA-MB-231 cells increased the expression of pWWP and pWP101 constructs by two- to threefold (Fig. 7A). This regulation might result from an Sp1-mediated transactivation as shown by the strong effect of ER α (more than fivefold) on the expression of the Sp1-Luc reporter.

Figure 5 Analysis of the *in vitro* interaction of Sp1 with DNA and HDACs. (A) MCF7 or MDA-MB-231 cells were treated or not treated with 500 ng/ml TSA for the indicated time prior to preparation of nuclear extract for band shift assay using an end-labelled Sp1 probe. The filled arrowheads indicate the specific retarded DNA-protein complexes. (B) Pull-down assays were carried out as described in Materials and Methods using bacterially expressed GST-Sp1 versus GST alone to precipitate ^{35}S -labelled HDACs or ER α . Inputs correspond to 10% of the material used in the assay.

In order to investigate further the role of ER α on the expression of endogenous p21^{WAF1/CIP1} gene, we used an adenoviral-based strategy to reintroduce the ER α protein in receptor-negative breast cancer cells. Infection of MDA-MB-231 cells by the recombinant adenovirus Ad-hER α was previously shown to allow the expression of a functional ER α protein able to activate oestrogen-sensitive reporter genes (Lazennec *et al.* 2001). As shown in Fig. 7B, we confirmed that the levels of endogenous p21^{WAF1/CIP1} mRNA and protein were strongly up-regulated by oestradiol treatment in cells infected with Ad-hER α as compared with control cells infected with the non-recombinant virus Ad5. In addition, we also noticed that, in the absence of hormone, infection with Ad-hER α slightly increased the levels of p21^{WAF1/CIP1} mRNA.

Finally, we investigated whether ER α expression could modulate the proliferative response of MDA-MB-231 cells to HDAC inhibition. As previously described (Lazennec *et al.* 2001), activated ER α by itself reduced cell proliferation (Fig. 8A). This result correlated with the p21^{WAF1/CIP1} gene up-regulation by ER α in the presence of oestradiol (Fig. 7B). Interestingly, TSA inhibited cell proliferation more efficiently in cells infected with Ad-hER α as compared with cells infected with the non-recombinant Ad5 adenovirus. As shown in Fig. 8A, treatment of cells with 20 ng/ml TSA resulted in growth inhibition of 16, 32 and 46% in the presence of ER α compared with 8, 17 and 19% in the absence of ER α at 2, 4 and 6 days respectively. The difference, although detectable after two days of treatment, was more significant at 6 days. Moreover, Fig. 8B showed that ER α expression markedly increased the sensitivity to HDAC

inhibition at all the concentrations of TSA that were tested. Together, these data support our initial observation on ER+ and ER- breast cancer cells since they (1) demonstrate a link between ER α and p21^{WAF1/CIP1} expression and (2) suggest that the presence of ER α influences the sensitivity of breast cancer cells to growth inhibition by TSA.

Discussion

The use of specific inhibitors to target HDACs has recently emerged as a new potential therapeutic approach in the treatment of cancer (Kramer *et al.* 2001). 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.

In the present study, we have examined the response of human breast cancer cells expressing or not the oestrogen receptor to hyperacetylating agents such as trichostatin A and showed that ER- breast cancer cells are less sensitive to the growth inhibitory activity of these agents. Previous data published on MCF7 and MDA-MB-231 cells using millimolar concentrations of sodium butyrate as an inhibitor of HDACs (Davis *et al.* 2000) also support a higher sensitivity of ER+ breast cancer cells. According to our results, this differential sensitivity is not linked to the multidrug resistance (MDR) phenotype, in agreement with data obtained with phenylbutyrate (Shack *et al.* 1996) or SAHA (Kim *et al.* 1999).

Our work demonstrates that the resistance to hyperacetylating agents observed in ER- breast cancer cell

Figure 6 Analysis of basal expression of p21^{WAF1/CIP1} gene. (A) Basal levels of p21^{WAF1/CIP1} expression in the four human breast cancer cell lines were analysed either by Western blot (upper panel) or in transient transfection experiments using the pWWP reporter vector (lower panel). For Western blot, higher amounts of whole cell extracts were analysed (120 μ g) and exposure time was increased as compared with Fig. 3. Sample loading was verified using actin as an internal control. For transient transfection, luciferase activities were corrected by β -galactosidase. Results are the means ($\pm$ S.D.) of three independent experiments and are expressed as a percentage of MCF7 levels. (B) Basal levels of pWWP, pWP101 and Sp1-Luc in T-47D (white bars) and MDA-MB-231 (black bars) cells. Results, which are expressed in luciferase arbitrary units after correction by the β -galactosidase, are the means ($\pm$ S.D.) of three independent experiments.

lines is associated with low basal levels of p21^{WAF1/CIP1} gene expression and with a decreased sensitivity to TSA of both the endogenous and transfected p21^{WAF1/CIP1} gene. Moreover, we show that ER α itself is able to transactivate the p21^{WAF1/CIP1} promoter and to increase TSA growth inhibitory activity in ER $^-$ cells.

This study highlights the importance of Sp1 sites in the transcriptional regulation by HDAC inhibitor (Sowa *et al.* 1997) 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. We show that different HDACs (from either class I or class II) could be recruited *in vitro* by Sp1, as previously reported for HDAC1 (Doetzlhofer *et al.* 1999). Although the global HDAC activity (Fig. 2B) and the expression of several class I enzymes (HDAC1–3) (data not shown) were not significantly different between ER $^+$ and ER $^-$ breast cancer cells, we cannot rule out the possibility that other HDACs do not present a different expression pattern between the two cell types. Moreover, it has been shown

that the enzymatic activity of HDAC3 is regulated through the interaction with the SMRT and N-CoR corepressors (Guenther *et al.* 2001), and it is therefore possible that the nature of such regulatory partners in HDAC-containing complexes recruited by Sp1 varies from one cell type to another. In addition to HDACs, other factors such as p300 (Xiao *et al.* 2000) participate in the regulation of p21^{WAF1/CIP1} by HDAC inhibitors and could also be differentially expressed or recruited between ER $^+$ and ER $^-$ breast cancer cells.

Sp1 family factors have been shown to mediate activation of the p21^{WAF1/CIP1} promoter by a wide variety of transcription factors including p53 (Koutsodontis *et al.* 2001) and some nuclear receptors (Lu *et al.* 2000). In support of the direct interaction of Sp1 with ERs (Fig. 6B and Porter *et al.* 1997), we show that ER α , in addition to p53, participates in the difference in p21^{WAF1/CIP1} expression. Some of these transcription factors could compete for the binding of HDACs on Sp1, as recently shown for p53 (Lagger *et al.* 2003), or form ternary complexes

Figure 7 Exogenous ER α regulates p21^{WAF1/CIP1} expression in MDA-MB-231 cells. (A) The ER α expressing vector (+, black bars) or the corresponding empty vector (–, grey bars) was cotransfected with the pWWP, pWP101 and Sp1-Luc reporter plasmids in MDA-MB-231 human breast cancer cells. Results were normalised for transfection efficiency using β -galactosidase activities and expressed as a percentage of control levels and are the means ($\pm$ S.D.) of three independent experiments. (B) MDA-MB-231 cells were infected with recombinant (Ad-hER α) or non-recombinant (Ad5) adenovirus (multiplicity of infection 100) and treated during 14 h with vehicle alone or with 17 β -oestradiol (E2) (10 nM). Total RNA was extracted and Northern blot was performed using a p21^{WAF1/CIP1} specific probe (upper panel). The same analysis was performed at the protein level (lower panel) as described in Fig. 3.

with Sp1 and HDACs, thereby modifying the response to HDAC inhibitors.

Another possibility is that the different sensitivity of breast cancer cells to TSA reflects a role of acetylation in protein–DNA or protein–protein interactions at the Sp1 site, as it has been shown that both Sp1 (Ryu *et al.* 2003) and Sp3 (Braun *et al.* 2001) could be acetylated. However, TSA treatment did not modify quantitatively or qualitatively the binding on Sp1 sites in gel retardation assays using MCF7 or MDA-MB-231 cell extracts, suggesting that the interaction between Sp1 proteins and DNA is probably not altered by acetylation under these conditions. More interestingly, ER α has also been described as being modified by acetylation, and mutation of the two acetylation sites enhanced hormone sensitivity (Wang *et al.* 2001). This suggested that the antiproliferative activity of TSA in ER+ breast cancer cells might be linked to a decrease in their responsiveness to oestrogens. However, our unpublished observations do not support this hypothesis. First, we failed to reproduce the increase in ligand sensitivity when we mutated both acetylation sites of ER α . Secondly, we checked that TSA treatment did not significantly decrease hormone-dependent transactivation of ER α in transient transfection. Finally, we verified that the sensitivity of MCF7 cell proliferation to TSA was not significantly different in the presence or absence of oestrogens.

Altogether, our results demonstrate that the effects of HDAC inhibitors on breast cancer cell proliferation correlate with the regulation of p21^{WAF1/CIP1} expression (both on the endogenous or transfected p21^{WAF1/CIP1}

gene). Concerning bulk histone acetylation, it should be noted that this regulation required higher concentrations of TSA than those able to decrease cell proliferation and was not significantly different between ER+ and ER– breast cancer cells. This could be due to the fact that, when we analyse global acetylation of total histones, we only assess quantitative changes at the cellular level, whereas the regulation of cell proliferation depends on highly restricted acetylation affecting specific regions of the chromatin, and in some cases, specific nucleosomes in a given promoter. It has been previously described that the acetylation of a limited region of p21^{WAF1/CIP1} promoter was transiently induced in response to HDAC inhibitors (Richon *et al.* 2000), indicating that at least for small amounts, HDAC inhibitors do not affect uniformly the acetylation status of the nucleosomes along the DNA. Moreover, transcriptional activity is not proportional to the acetylation level but depends on the specific arrangement of different post-translational modifications described as the histone code (Jenuwein & Allis 2001). Hence, even if there is no detectable change in the global pool of acetylated histone, local and specific modifications could account for the regulation of genes implicated in the control of cell proliferation.

Concerning the regulation of the p21^{WAF1/CIP1} gene, the difference with the proliferation is less pronounced since, for instance at 25 ng/ml, the accumulation of the p21^{WAF1/CIP1} protein is increased twofold (quantification of three independent experiments, representation not shown). The implication of this protein in the cell

Figure 8 Effect of ER α expression on MDA-MB-231 cell proliferation. MDA-MB-231 cells were infected with recombinant (Ad-hER α) or non-recombinant (Ad5) adenovirus (multiplicity of infection 100) and treated with vehicle alone (control) or with TSA in oestrogen-containing medium. Cell number was quantified as described in Materials and Methods. Results are the means ($\pm$ s.d.) of three quantifications. (A) Proliferation of Ad5- and Ad-hER α -infected MDA-MB-231 cells was analysed between 24 h after infection (day=0) and six days after infection in control conditions or in response to TSA (20 ng/ml). Results are expressed as μ g/plates. (B) The growth inhibitory activity of TSA was analysed at six days in Ad5- and Ad-hER α -infected MDA-MB-231 cells, treated by increasing amounts of TSA (ng/ml). Results are expressed as a percentage of the value obtained in the absence of TSA. * $P < 0.05$, ** $P < 0.001$ compared with values from Ad5-infected control cells.

proliferation blockade in response to HDAC inhibitors has previously been well documented. The use of mouse embryo fibroblasts (MEF) or HCT116 colon cancer cells deleted for the p21^{WAF1/CIP1} gene showed that the growth inhibitory effects of the FR901228 molecule (Sandor *et al.* 2000) or butyrate derivatives (Archer *et al.* 1998) were impaired. When associated with initial high levels, a slight increase in p21^{WAF1/CIP1} gene expression could therefore mediate important modifications of cell proliferation. However, it is clear that the p21^{WAF1/CIP1} gene is not the unique mediator of the antiproliferative properties of HDAC inhibitors, but more likely it is a key protein acting

in combination with other regulators. A differential expression or regulation of these other target genes could explain why, in some cases, p21^{WAF1/CIP1} gene expression is not associated with an increased sensitivity to HDAC inhibitors (Vaziri *et al.* 1998).

With regard to the association between ER α and p21^{WAF1/CIP1} levels, our data are supported by a clinical analysis in breast tumour samples which reported a positive correlation between the expression of these two proteins measured by Western blot (Chen *et al.* 2000). Moreover, the crosstalk between ER α and p21^{WAF1/CIP1} could be even more complex since p21^{WAF1/CIP1} appears to be involved in the activation of the oestrogen-signalling pathway. Transient transfection experiments have shown that the expression of p21^{WAF1/CIP1} amplified transcriptional activation by ER α in a CREB-binding protein-dependent manner (Redeuilh *et al.* 2002).

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 (Saito *et al.* 1999, Saunders *et al.* 1999, Vigushin *et al.* 2001) 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 ER α 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.

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