The pan-histone deacetylase inhibitor LBH589 (panobinostat) alters the invasive breast cancer cell phenotype

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Abstract. Triple-negative breast cancer (TNBC) is a very aggressive type of tumour and its aggressiveness is linked to E-cadherin downregulation. In estrogen-sensitive breast cancer, high levels of E-cadherin fit with high levels of ERα and MTA3 (a component of the transcription Mi-2/NuRD complex with intrinsic DAC activity). In TNBC the E-cadherin downregulation could be due to epigenetic silencing of the *CDH1* gene as well as to the lack of a fully functioning ERα-activated pathway. We report that the pan-histone deacetylase inhibitor LBH589, a potent anti-proliferative agent, induced E-cadherin expression on cell membranes of MDA-MB-231 cells (TNBC), determining a reduction of cell invasion and migration. Even though E-cadherin expression in breast cancer is also regulated by estradiol and the ERα/MTA3/Snail/Slug pathway, LBH589 is able to increase E-cadherin without affecting the estrogen pathway. In fact, no expression of ERα, PR and FoxA1 was observed in MDA-MB-231 cells before and after LBH589 treatment; furthermore, the drug caused an increase in Snail and Slug expression with a concomitant reduction of MTA3 levels. Taking into consideration its anti-proliferative and anti-invasive properties, we suggest the use of LBH589 in aggressive breast cancer refractory to hormonal therapy.

Introduction

Breast cancer, the most common cancer in women all over the world (1), is generally managed with success thank to available treatments (2). Regrettably, resistance to conventional therapy and increasing in metastatic and aggressive disease are emerging problems (3,4). A particular aggressive subtype of breast cancer is the triple-negative breast cancer (TNBC) that is defined by the absence of ER, PR and Her2/neu (5,6). TNBC generally occurs more frequently in younger women, frequently presents early metastatic spread and has poor overall prognosis (7,8).

In breast cancer, as well as in other tumour types, epigenetic modifications are considered a crucial mechanism involved in cancer growth, dedifferentiation and aggressiveness; indeed, epigenetic deregulation can alter a number of molecular pathways involved in the control of cell function. Epigenetic drugs, able to restore normal epigenome in cancer cells, are under extensive pharmacological research (9). In breast cancer, histone acetylation state is considered of great importance. Histone acetylation and deacetylation, controlled by the enzymes histone acetyltransferases (HATs) and histone deacetylases (HDACs), affect chromatin conformation and, therefore, gene transcription, DNA repair and replication, and cell cycle checkpoints (10). Altered expression of HDACs has been reported in breast cancer by several authors (11,12). Therefore, HDAC inhibitors (DCI) are considered valuable therapeutic tools and have been under extensive evaluation. DCI vorinostat (13), for example, in association with paclitaxel and bevacizumab induced a partial or complete response in >50% of patients with metastatic breast cancer. The anti-proliferative and re-differentiating effect of several DCI was reported *in vitro* by a number of laboratories (14-17). In recent years, the pan-deacetylase inhibitor panobinostat (LBH589) has been taken into consideration. Our laboratory demonstrated for the first time that LBH589 in nanomolar concentration is a potent antiproliferative agent in ER-positive and -negative breast cancer cells, and that its anticancer activity is sustained by H4 histone acetylation (18).

It is so well known that ERα is the hallmark of breast cancer estrogen sensitivity and good response to tamoxifen therapy (19) and that it can be lost during disease progression, giving rise to hormone-independent and aggressive phenotype (20). Epigenetic deregulation has also been considered one of the possible causes of ERα loss in breast cancer (e.g., histone tail deacetylation and methylation or methylation of the CpG islands in the ER promoter) (21). Hence, DCI were exhaustively studied also for their ability to restore ERα and its pathway in ER-negative breast cancer cells. VPA, for

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Table I. Primers for real-time PCR.

CDH1	Sense:	5'-TTGAAAGAGAAACAGGATGGCTG-3'
	Antisense:	5'-TCATTCTGATCGGTTACCGTGAT-3'
ERα	Sense:	5'-TGT GTC CAG CCA CCA ACC AG-3'
	Antisense:	5'-TTC AAC ATT CTC CCT CCT CTT CGG-3'
PR	Sense:	5'-GCA TCA GGC TGT CAT TAT GGT GTC-3'
	Antisense:	5'-CAT AAG TAG TTG TGC TGC CCT TCC-3'
FoxA1	Sense:	5'-GGG TGG CTCCAG GAT GTT AGG-3'
	Antisense:	5'-GGG TCA TGT TGC CGC TCG TAG-3'
MTA3	Sense:	5'-GTCGGAGATTATGTCTACTTTGAG-3'
	Antisense:	5'-CAGTCTTGTTGAGTTCTTCTATCC-3'
SNAIL	Sense:	5'-CCTGCGTCTGCGGAACCTG-3'
	Antisense:	5'-GGAGCGGTCAGCGAAGGC-3'
SLUG	Sense:	5'-AAACTACAGCGAACTGGACACAC-3'
	Antisense:	5'-GTGGTATGACAGGCATGGAGTAAC-3'
β-ACT	Sense:	5'-GCG AGA AGA TGA CCC AGA TC- 3'
	Antisense:	5'-GGA TAG CAC AGC CTG GAT AG-3'
β2-microglobulin	Sense:	5'-AGA TGA GTA TGC CTG CCG TGT G-3'
	Antisense:	5'-TCA ACC CTC CAT GAT GCT GCT TAC-3'
L13A	Sense:	5'-GCA AGC GGA TGA ACA CCA ACC-3'
	Antisense:	5'-TTG AGG GCA GCA GGA ACC AC-3'

example, was demonstrated by us (16) and by other authors (14) to restore estrogen and, thus, antiestrogen sensitivity in MDA-MB-231 cells considered a good model of TNBC; LBH589 was shown either to induce ERα in MDA-MB-231 cells (22), or not to have any detectable effect (23,24).

Estradiol sensitivity is also strictly linked to aggressiveness of breast cancer cells. Aggressive breast tumours are characterized by E-cadherin downregulation that switches on the phenomenon called epithelial-to-mesenchimal transition (EMT), the corner stone of tumour spreading and metastasis (25). E-cadherin downregulation is mainly due to epigenetic alterations of *CDH1* gene promoter (26,27) or by epigenetic controlled overexpression of several E-cadherin transcriptional repressors, such as Snail/SNAI1, Slug/SNAI2, SIP1/ZEB2 or Twist (28-32).

In breast cancer, estradiol (E_2)/ERα and MTA3/Snail/E-cadherin signalling pathways are intimately linked (33). Estradiol-activated ERα induces MTA3, a member of the histone deacetylase Mi-2/NuRD macro-complex, that downregulates Snail, upregulating E-cadherin (34). The absence of estrogen receptor or MTA3 leads to aberrant expression of the transcriptional repressor Snail and consequent inhibition of E-cadherin (30).

E-cadherin and its regulators are considered attractive therapeutic targets, in order to inactivate cell invasion and metastasis.

The aim of the present study was to evaluate the effect of the pan-deacetylase inhibitor panobinostat (LBH589) on expression and function of ERα and its cognate proteins PR (progesterone receptor) and FoxA1 (forkhead box A1), and of E-cadherin and its repressors Snail and Slug, in TNBC MDA-MB-231 cells.

Materials and methods

Cell lines and reagents. Triple-negative breast cancer cell line MDA-MB-231 and estrogen receptor-positive MCF-7 cells were purchased from ECACC (Salisbury, UK), which certifies the origin and identity of the cells. Moreover, none of the cell lines are included in the database of cross-contaminated or misidentified cell lines (http://www.hpacultures.org.uk/services/celllineidentityverification/misidentifiedcelllines.jsp). Cells were routinely maintained at 37˚C, in 5% CO_2 and 95% humidity, in RPMI-1640 (Sigma, St. Louis, MO, USA) with 100 IU/ml penicillin and 100 μg/ml streptomycin added, supplemented with 10% heat-inactivated FCS (Euroclone, Wetherby, UK). LBH589 was provided by Novartis Pharma AG (Basel, Switzerland), prepared as a 5 mM stock solution in DMSO and stored at -20˚C.

Gene expression: evaluation with real-time PCR. Cells ($1x10^6$) were seeded in 75-cm^2 flasks and treated with LBH589 (5-50 nM). Total RNA was extracted using TRIzol reagent (Invitrogen Ltd., Paisley, UK), as previously described. DNase I was added to remove remaining genomic DNA. Total RNA (1 μg) was reverse-transcribed with iScript cDNA Synthesis

kit (Bio-Rad Laboratories, Inc.), following the manufacturer's protocol.

Primers (Table I) were designed using Beacon Designer 5.0 software according to parameters outlined in the Bio-Rad iCycler manual. Specificity of primers was confirmed by BLAST analysis. Real-time PCR was performed using a Bio-Rad iQ iCycler Detection system (Bio-Rad Laboratories, Inc.) with SYBR green fluorophore. Reactions were performed in a total volume of 25 μl, including 12.5 μl IQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.), 1 μl of each primer at 10 μM concentration, and 5 μl of the previously reverse-transcribed cDNA template. Protocol for primer set was optimized using seven serial 5X dilutions of template cDNA obtained from cells in basal conditions.

The protocol used is as follows: denaturation (95˚C for 5 min), amplification repeated 40 times (95˚C for 15 sec, 60˚C for 1 min). A melting curve analysis was performed following every run to ensure a single amplified product for every reaction. All reactions were carried out at least three times for each sample. Every gene expression level was normalized on the expression level of three house-keeping genes (β-actin, L13A, β-2-microglobulin).

Immunoblotting. Sub-confluent breast cancer cells were treated with LBH589 (25 nM) for 24 h and then they were harvested and lysed in the presence of lysis buffer (0.5% Triton X-100, 2.5 mM EGTA, 5 mM $MgCl_2$, 50 mM NaH_2PO_4) for 2 min on ice. Soluble cytosolic fraction was recovered and stored at -80˚C. The insoluble membrane fraction was dissolved in SDS sample buffer (TrizmaBase 0.2 M, glycerol 50%, SDS 10%), recovered and stored at -80˚C.

SDS-PAGE was performed on gels, loading 30 μg protein/well. Separated proteins were electro-transferred onto PVDF membrane and probed with anti-E-cadherin antibody (1:1,000 dilution, Sigma). PVDF membrane was then stripped and re-probed with an anti-α-tubulin antibody (clone 6-11B-1, 1:2,000 dilution, Sigma) to check protein loading. Proteins were detected with Pierce Super Signal chemiluminescent substrate following the manufacturer's instructions. Bands were photographed and analyzed using Kodak 1D Image analysis software.

Immunofluorescence microscopy. Cells ($4x10^3$) were seeded in 96-well plates and treated with 25 nM LBH589 for 24 h. After treatment, cells were fixed in PFA 1% and incubated with polyclonal anti-E-cadherin antibody (1:100 dilution, Sigma) at 4˚C overnight. Then cells were washed with PBS containing 0.5% Triton and 0.05% NaN_3 followed by detection with anti-rabbit Cy3-conjugated secondary antibody (1:1,000 dilution, GE Healthcare Europe, GmbH, Milan, Italy) in PBS plus 0.5% Triton and 0.05% NaN_3 for 2 h. Nuclear staining was obtained by treating cells with Hoechst 33258 (500 ng/ml in DMSO) in PBS. Cells were washed twice with distilled water and mounted with 50% glycerol-PBS media.

Scratch wound assay. Cells ($2x10^5$) were seeded in 6-well plates. Cells were treated with 25 nM LBH589 for 24 h and afterwards cell monolayer was gently wounded by scratching. The cells were washed twice with cooled PBS and incubated for further 24 h either in the absence or presence of 25 nM LBH589. For each wound, pictures were taken in the same field and the distance between the wound edges was analyzed using the ImageJ 1.42 software. For each condition, the percentage of the wound recovery in respect to the wound area at 0 h, was calculated.

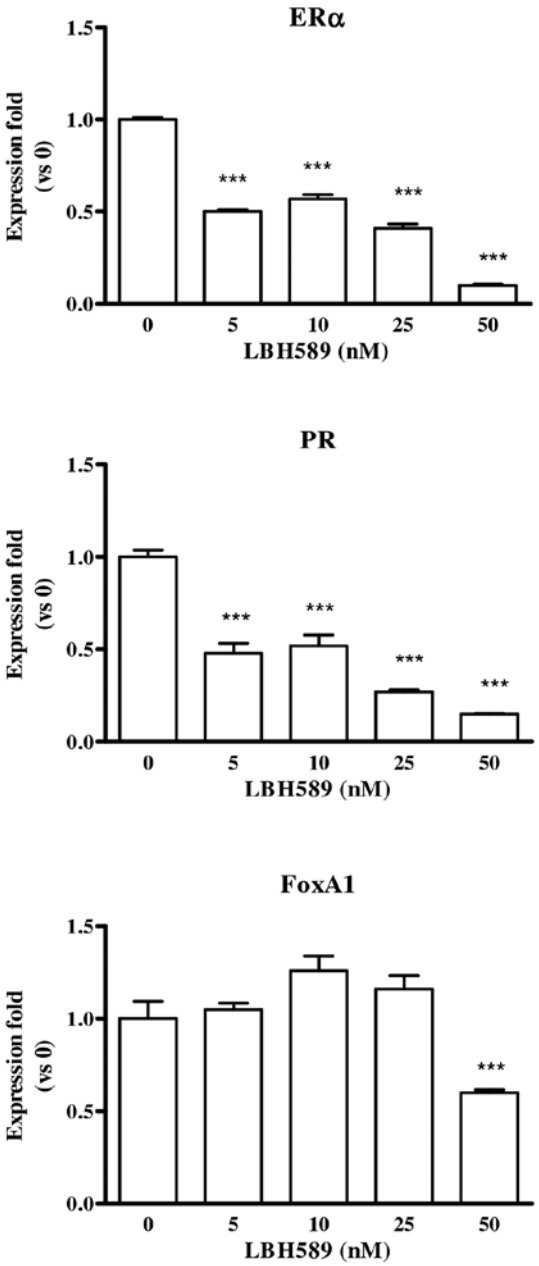

Figure 1. Effect of LBH589 on ERα, PR and FoxA1 gene expression in MCF-7 cells. ERα, PR and FoxA1 mRNA was evaluated in MCF-7 cells treated with increasing doses of LBH589 with real-time PCR. Results were normalized for three different housekeeping genes (β-actin, β2-microglobulin and L13A) and expressed as relative expression fold vs untreated controls (0). Results are expressed as the means ± SD; n=3; ***P<0.001. The evaluation was performed also in MDA-MB-231 cells; no expression of ERα, PR and FoxA1 mRNA was detectable either in basal condition or after LBH589 treatment.

Invasion assay. Cells ($5x10^5$) were seeded in 75-cm^2 flasks and treated with LBH589 (25 nM). After 24 h, $2x10^5$ cells were seeded in the BD BioCoat™ BD Matrigel™ invasion chamber (BD Biosciences Discovery Labware, Two Oak Park, Bedford,

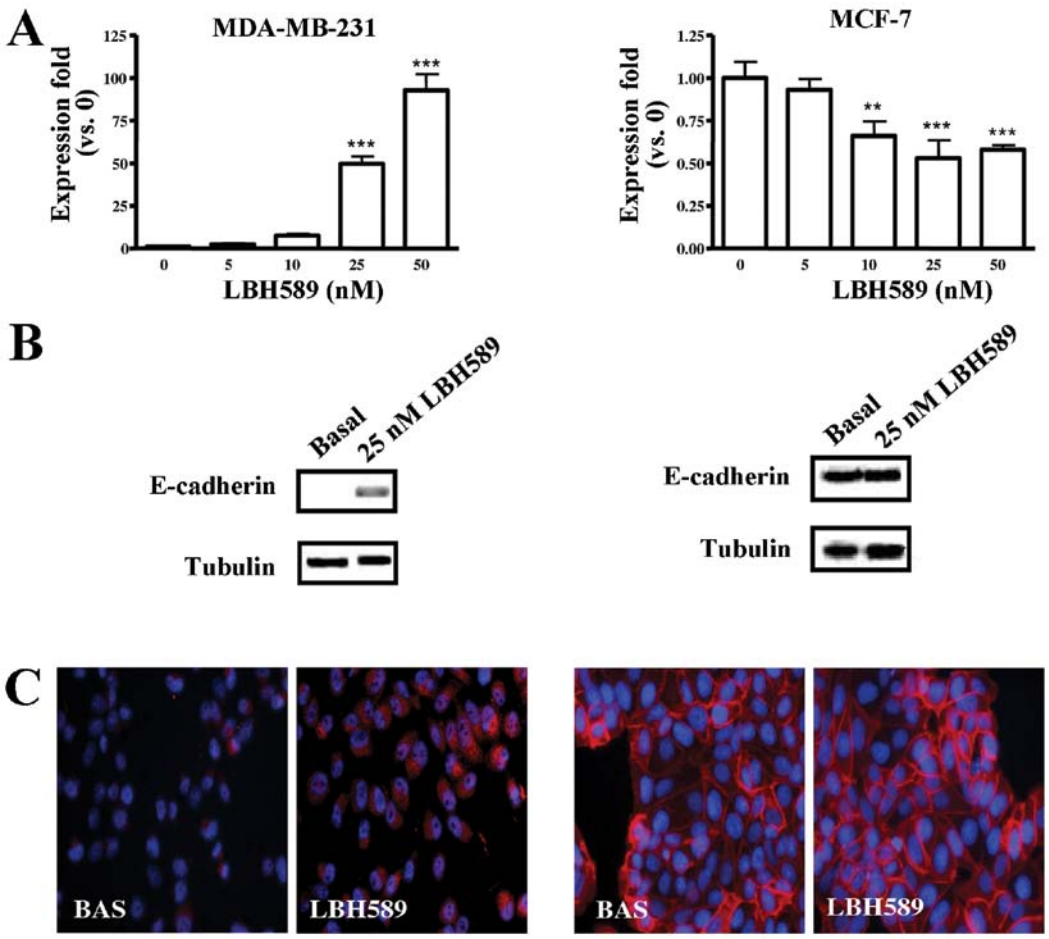

Figure 2. Effect of LBH589 on E-cadherin expression and localization. (A) E-cadherin mRNA was evaluated in MDA-MB-231 and MCF-7 cells with real-time PCR. Results were normalized for three different housekeeping genes (β-actin, β2-microglobulin and L13A) and expressed as relative expression fold vs untreated controls (0 nM LBH589). Results are expressed as the means ± SD; n=3; **P<0.01; ***P<0.001. (B) Western blotting for E-cadherin was performed on membranes obtained from MDA-MB-231 and MCF-7 cells before and after treatment with 25 nM LBH589. Equal loading and transfer were verified by re-probing the blot with anti-tubulin antibodies. The image shows a typical experiment. (C) E-cadherin protein localization in MDA-MB-231 and MCF-7 cells. Immunofluorescence for E-cadherin was performed on cells before and after 25 nM LBH589 treatment. Magnification, x400.

MA, USA) and stimulated with 25 nM LBH589 for 24 h. At completion, the lower surfaces of the membrane were fixed with methanol and stained with crystal violet solution, to point out cells that migrated across the Matrigel and invaded the inferior face of the membranes. The number of cells that had migrated to the basal side of the membrane was quantified by counting 12 independent fields under the microscope.

Luciferase assay. Cells ($2.5x10^5$) were seeded in 6-well plates. After 24 h, cells were transfected with 1 μg of pGL2Basic-EcadK1 plasmid/well using Lipofectin reagent (Invitrogen Ltd.). The plasmid (Promega Italia, Milan, Italy) contains the human *CDH1* promoter sequences from -108 to +125 linked to the luciferase gene as reporter (17). After transient transfection, cells were treated with 25 nM LBH589 for 24 h. Luciferase activity was assayed with the Luciferase assay system (Promega Corp., Madison, WI, USA).

Statistical analysis. Data are expressed throughout as the means ± SD, calculated from at least three different experiments. Comparison between groups was performed with analysis of variance (one-way ANOVA) and the threshold of significance was calculated with the Bonferroni test. Statistical significance was set at P<0.05.

Results

LBH589 effect on ERα, PR and FoxA1. First of all, the effect of LBH589 on the expression of ERα and cognate PR (progesterone receptor) and FoxA1 (forkhead box A1) was studied. In estrogen-sensitive MCF-7 cells, LBH589 reduced the level of expression of all the three genes (Fig. 1), while MDA-MB-231 cells did not express any of the genes under study, neither in basal condition nor after LBH589 treatment.

LBH589 effect on E-cadherin expression. E-cadherin was not expressed in untreated MDA-MB-231 cells; LBH589 treatment increased E-cadherin gene expression, its effect being evident at doses ≥25 nM; a slight reduction of gene expression in E-cadherin positive MCF-7 cells was also observed (Fig. 2A). Western blotting for E-cadherin, reported in Fig. 2B, confirmed that increased gene expression resulted in the appearance of the protein in MDA-MB-231 cells; on the other hand, the reduction of E-cadherin gene expression

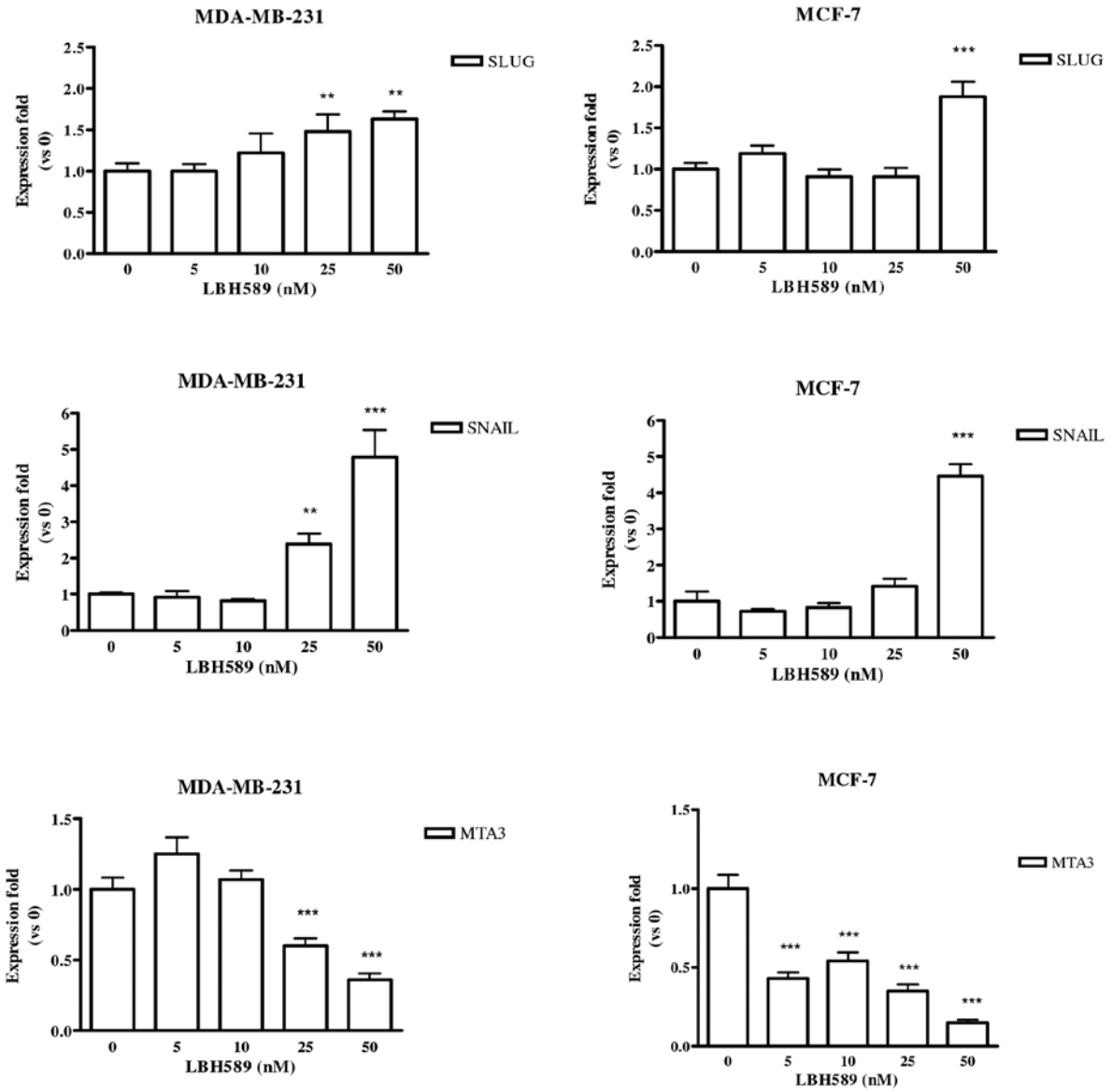

Figure 3. Effect of LBH589 on Snail, Slug and MTA3 gene expression. Snail, Slug and MTA3 mRNA was evaluated in MDA-MB-231 and MCF-7 cells treated with increasing doses of LBH589 (5-50 nM) with real-time PCR. Results were normalized for three different housekeeping genes (β-actin, β2-microglobulin and L13A) and expressed as relative expression fold vs untreated controls (0 nM LBH589). Results are expressed as the means ± SD; n=3; **P<0.01; ***P<0.001.

observed in MCF-7 cells was not followed by a significant reduction of the protein level. LBH589 treatment determined also a correct expression of E-cadherin on the MDA-MB-231 surface (Fig. 2C).

LBH589 effect on Slug, Snail and MTA3. Both E-cadherin repressors, Slug and Snail, were evaluated. As reported in Fig. 3, the expression of Slug and Snail was significantly enhanced by LBH589 treatment in both cell lines. Lastly, the Mi-2/NuRD macro-complex member MTA3 was studied. We observed that LBH589 significantly reduced the level of expression of MTA3 in both cell lines (Fig. 3, last row).

LBH589 effect on cell migration and invasion. To determine whether LBH589 had a role in MDA-MB-231 cell migration, a scratch wound assay was carried out. As shown in Fig. 4, the migration of treated cells was significantly reduced with respect to untreated controls. After 24 h, cells treated with 25 nM LBH589 occupied only 15% of the wound compared to 33% occupation of untreated cells (P<0.001). Moreover, the role of LBH589 on the invasive potential of MDA-MB-231 cells was assayed through the use of a transwell invasion chamber (Fig. 5). We observed that invasion of treated cells was markedly reduced compared with untreated cells after 24 h (P<0.001). As reported also in Figs. 4 and 5, migration and invasion of MCF-7 cells were not affected by LBH589 treatment.

LBH589 effect on CDH1 promoter. To evaluate whether LBH589 directly acts on *CDH1* promoter inducing transcription, a luciferase assay using a construct containing *CDH1* promoter was performed. We observed a significant increase in CDH1 promoter transcription level homogeneous with the increase of E-cadherin we reported above in MDA-MB-231

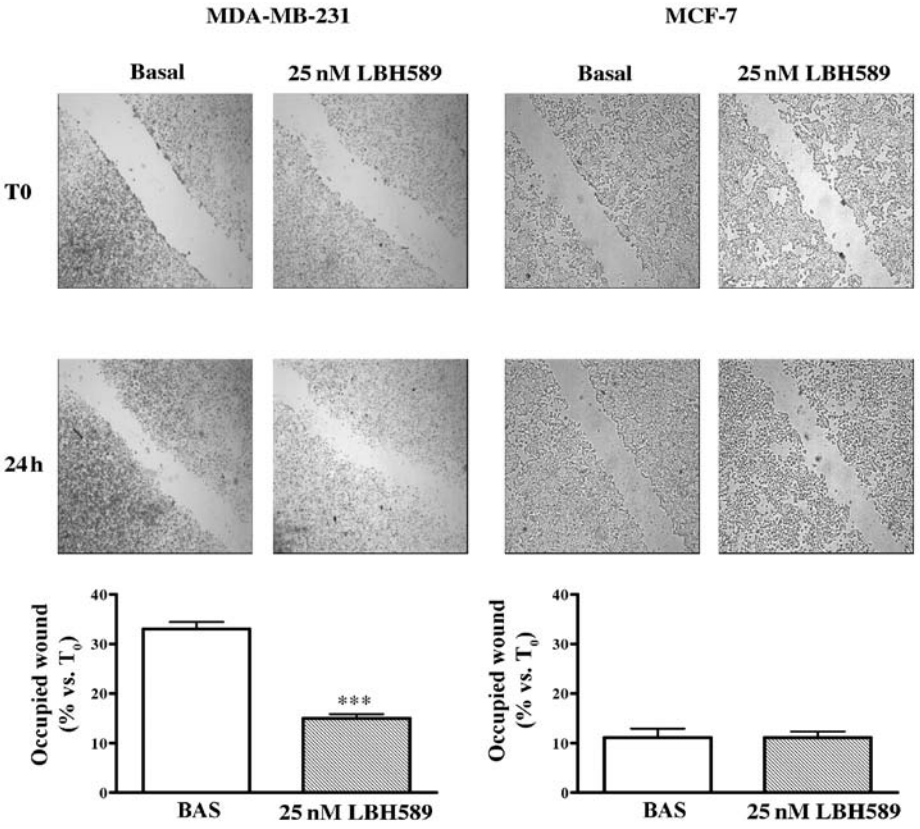

Figure 4. Effect of LBH589 on MDA-MB231 and MCF-7 cell invasion. Scratch wound assay was performed on cells before and after treatment with 25 nM LBH589. A typical experiment is reported in the upper panels. Results, expressed as percent of occupied wound after 24 h with respect to T0, are reported in the lower panels; data are expressed as the mean ± SD; n=3; ***P<0.001.

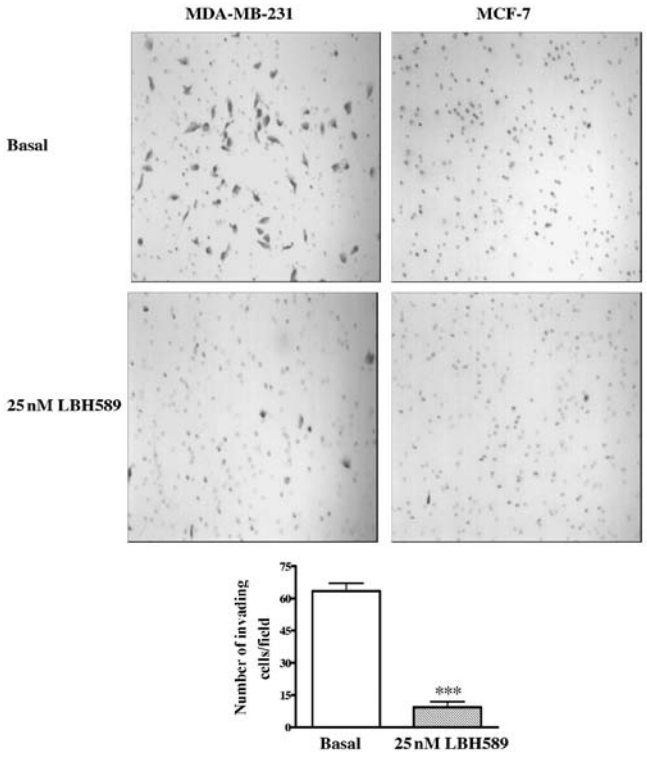

Figure 5. Effect of LBH589 on MDA-MB231 and MCF-7 cell migration. Matrigel invasion assay was performed on cells before and after treatment with 25 nM LBH589. A typical experiment is reported in the upper panels. Results, expressed as number of invading cells/field, are reported in the lower panels; data are expressed as the mean ± SD; n=3; ***P<0.001. No significant migration of MCF-7 cells was observed in either condition.

cells; the direct effect of LBH589 on *CDH1* promoter was observed even in MCF-7 cells (Fig. 6).

Discussion

The pan-deacetylase inhibitor LBH589 is a powerful anti-tumour agent even at low nanomolar concentrations in a number of hematologic and solid tumours. It was, in addition, reported to induce re-expression of previously silenced genes modulating thus the behaviour of cancer cells (35-37).

The present report follows our previous observation in breast cancer cells where we observed a potent cytotoxic activity of LBH589 in both estrogen-sensitive and -insensitive cells (16). Our present data demonstrate that LBH589 induces E-cadherin gene expression in aggressive TNBC cells. E-cadherin gene expression resulted, furthermore, in the correct appearance of the cognate protein on MDA-MB-231 surface and, the re-expression of E-cadherin caused a clear modification of MDA-MB-231 cell behaviour. In fact, after LBH589 treatment, cell migration and invasive potential were greatly reduced.

Our previous observation on the anti-proliferative and pro-apoptotic effects of LBH589 in ER-negative breast cancer cells has been recently confirmed by other authors (38). The same authors reported that the most induced gene by LBH589 treatment in MDA-MB-231 cells is the *CDH1* gene that codes for E-cadherin, in total agreement with our observation on the increase of the *CDH1* gene promoter activity

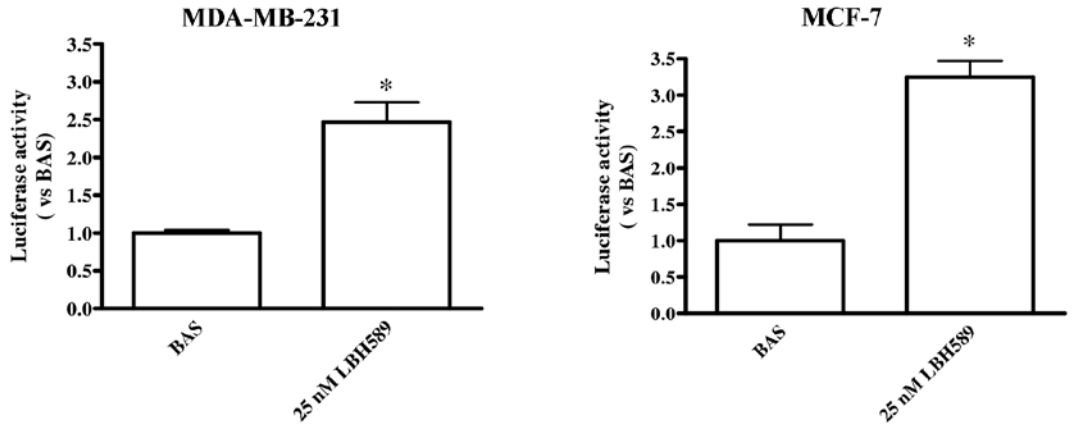

Figure 6. Effect of LBH589 on CDH1 promoter activity. MDA-MB-231 and MCF-7 cells were transiently transfected with a reporter plasmid carrying the luciferase gene under the control of the *CDH1* promoter. The luciferase activity was assayed before and after treatment with 25 nM LBH589. Data are expressed as the mean ± SD; n=3; $^{*}P<0.05$.

we report in the present study and consequent E-cadherin induction. Tate *et al* also noted an increase in the *CDH1* gene expression on the periphery of the primary tumour from MDA-MB-231 xenografts, and hypothesized that the induction of *CDH1* expression by LHB589 at the invasive edge may be indicative of decreased metastatic potential. Actually, our observation *in vitro* of reduced migration and invasion of MDA-MB-231 cells treated with LBH589 substantiates their hypothesis, confirming that E-cadherin induction by LBH589 has a functional meaning and could be exploited for therapeutic application.

It is also interesting to note that in ER-positive MCF-7 cells that express high level of E-cadherin, LBH589 is a clear antiproliferative and proapoptotic agent even at low nanomolar doses (16), but does not modify the E-cadherin expression and cell migration/invasion leaving unaffected the low metastatic potential of these cells.

As reported, in breast cancer MTA3/Snail/E-cadherin and estradiol (E2)/ERα signalling are closely linked (34). The appearance of E-cadherin in MDA-MB-231 cells treated with LBH589 could be due to the reactivation of ERα pathway in TNBC cells since other DCI were reported to act similarly in several studies. For example, DCI in association with demethylating agents like 5-aza-2'-deoxycytidine were shown to reactivate ERα expression in ER-negative breast cancer cells (22,39,40); the DCI valproic acid was demonstrated to enhance the efficacy of the anti-estrogen tamoxifen through increasing ER-mediated transcription (16) and also to induce ERα, PR, pS2 and FoxA1, giving to MDA-MB-231 cells an estrogen-sensitive 'phenotype' and restoring their sensitivity to anti-estrogen therapy (18).

Controversial results on LBH589 effect on ERα expression in MDA-MB-231 cells, either receptor induction (23) or no effect at all (24), have been reported.

The present data confirm that LBH589 does not induce ERα expression and ER-pathway restoration in MDA-MB-231 cells. Moreover, LBH589 reduced MTA3 levels, and significantly increased both Snail and Slug, whose levels are inversely related to E-cadherin expression, either in ER-negative MDA-MB-231 or in ER-positive MCF-7 cells. Furthermore, in MCF-7 cells LBH589 significantly decreased the expression level of ERα, PR and FoxA1, as previously reported (24). The effects of LBH589 on ER pathway are not consistent with those observed on E-cadherin gene and protein expression in both cell lines. It is thus conceivable that LBH589 can control E-cadherin independently of estradiol and this is not affected by the level of expression of Snail, in agreement with recent observations in ovarian carcinoma (41). Snail requires histone deacetylase activity to repress E-cadherin promoter and it has already been demonstrated that treatment with trichostatin A is sufficient to block this effect (42). Our data suggest that LBH589 in MDA-MB-231 breast cancer acts directly on the *CDH1* promoter and does not need to modify E-cadherin transcriptional repressors to induce E-cadherin expression and to modify the cell aggressive attitude. In MCF-7 cells, even though LBH589 smoothens down the ER pathway, reducing MTA3 and increasing Snail and Slug, its direct action on *CDH1* promoter is also present, the final effect on E-cadherin protein expression is irrelevant, so that migration and invasion of MCF-7 cells are not affected.

In conclusion, LBH589 is able to induce E-cadherin in highly aggressive TNBC cells reducing their migration and invasion, by-passing E-cadherin transcriptional repressors such as Snail and Slug and without any detectable effect on ERα expression and pathway. This compound can, therefore, be proposed for treatment of aggressive breast cancer, refractory to hormonal therapy exploiting its antiproliferative and anti-invasive properties. At least eight different trials on advanced breast cancer treatment with LBH589 both in monotherapy and in association with trastuzumab, capecitabine, lapatinib, or paclitaxel, respectively, are now listed on the site www.clinicaltrials.gov. We are looking forward to their conclusions and strongly hope LBH589 will be available soon for advanced breast cancer treatment.

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