Epigenetic Modifications with DZNep, NaBu and SAHA in Luminal and Mesenchymal-like Breast Cancer Subtype Cells

ASLIHAN DAGDEMIR[1,2], GAËLLE JUDES[1,2], ANDRÉ LEBERT[3], MAUREEN ECHEGUT[1,2], SEHER KARSLI-CEPPIOGLU[1,2,4], KHALDOUN RIFAÏ[1,2], MARINE DAURES[1,2], MARJOLAINE NGOLLO[1,2], LUCAS DUBOIS[1,2], FRÉDÉRIQUE PENAULT-LLORCA[2,5], YVES-JEAN BIGNON[1,2] and DOMINIQUE BERNARD-GALLON[1,2]

[1]*Department of Oncogenetics, Centre Jean Perrin, CBRV, Clermont-Ferrand, France;*
[2]*EA 4677 "ERTICA", University of Auvergne, Clermont-Ferrand, France;*
[3]*University Blaise Pascal, Institute Pascal UMR 6602 CNRS/UBP, Aubière, France;*
[4]*Department of Toxicology, Faculty of Pharmacy, Marmara University, Istanbul, Turkey;*
[5]*Department of Biopathology, Centre Jean Perrin, Clermont-Ferrand, France*

Abstract. *Background/Aim: Numerous studies have shown that breast cancer and epigenetic mechanisms have a very powerful interactive relation. The MCF7 cell line, representative of luminal subtype and the MDA-MB 231 cell line representative of mesenchymal-like subtype were treated respectively with a Histone Methyl Transferase Inhibitors (HMTi), 3-Deazaneplanocin hydrochloride (DZNep), two histone deacetylase inhibitors (HDACi), sodium butyrate (NaBu), and suberoylanilide hydroxamic acid (SAHA) for 48 h. Materials and Methods: Chromatin immunoprecipitation (ChIP) was used to observe HDACis (SAHA and NaBu) and HMTi (DZNep) impact on histones and more specifically on H3K27me3, H3K9ac and H3K4ac marks with Q-PCR analysis of BRCA1, SRC3 and P300 genes. Furthermore, the HDACi and HMTi effects on mRNA and protein expression of BRCA1, SRC3 and P300 genes were checked. In addition, statistical analyses were used. Results: In the MCF7 luminal subtype with positive ER, H3k4ac was significantly increased on BRCA1 with SAHA. On the contrary, in the MDA-MB 231 breast cancer cell line, representative of mesenchymal-like subtype with negative estrogen receptor, HDACis had no effect. Also, DZNEP decreased significantly H3K27me3 on BRCA1 in MDA-MB 231. Besides, on SRC3, a significant increase for H3K4ac was obtained in MCF7 treated with SAHA. And DZNEP had no effect in MCF7. Also, in MDA-MB 231 treated with DZNEP, H3K27me3 significantly decreased on SRC3 while H3K4ac was significantly increased in MDA-MB-231 treated with SAHA or NaBu for P300. Conclusion: Luminal and mesenchymal-like breast cancer subtype cell lines seemed to act differently to HDACis (SAHA and NaBu) or HMTi (DZNEP) treatments.*

Correspondence to: Dominique Bernard-Gallon, Department of Oncogenetics - Centre Jean Perrin, EA 4677, CBRV, 28 Place Henri Dunant, 63001 Clermont-Ferrand, France. Tel: +33 0473178358. dominique.gallon-bernard@clermont.unicancer.fr

Key Words: Breast cancer cell lines, histone methyl transferase inhibitor, histone deacetylase inhibitor.

Breast cancer is the most frequently diagnosed malignant neoplasia and a leading cause of cancer-related death in females worldwide (1). Breast cancer is a heterogeneous disease, also known as a hormone-dependent cancer. Subsequently, it has become clear that epigenetic modifications play a key role in breast cancer development. Abnormal methylations have been described in breast cancer oncosuppressors (2, 3), while lately post-translational histone modifications are being investigated in cancer (4), including breast cancer (5-7).

Another study acknowledged that BRCA1 also directly regulated the *p300* gene and quantitatively influences P300 expression (8). Also, SRC3 is expressed in the ER-negative cell line, MDA-MB 231, and has been recognized to modulate invasiveness (9).

Further studies on epigenetic mechanisms have always been needed in breast cancer research to improve therapy. Knowing that HDAC inhibitors (HDACis) cause transcriptional up-regulation of genes (10), the anticancer therapeutic action of suberoylanilide hydroxamic acid (SAHA) (11) and sodium butyrate (NaBu) (12) were studied in breast cancer cell lines (13).

Moreover, another drug on HAT mechanism (HMTi) was tested for its therapeutic action, 3-deazaneplanocin A (DZNep) that was known to inhibit the associated histone H3 lysine 27 trimethylation (14, 15).

Althogether, in this study we investigated histone deacetylase inhibitors (HDACis) and a histone methyl

Table I. *Primer and probe sequences use in the study.*

Genes	Forward primers	Reverse primers	Taqman Probes
P300	CGATGGCACAGGTTAGTTTCG	GCGCACCGAGTAGAAAAGATTAA	6FAM-CAGCCCCGGCCTTCCACGTT-TAMRA
SRC3	AAAATTAAGGGCAGGGCTAGGA	GTGCGGCCGCTTTCG	6FAM-TCCGGATCCCGAGGGAGCTCC-TAMRA
BRCA1	CCCCGTCCAGGAAGTCTCA	GCGCGGGAATTACAGATAAATT	6FAM-CGAGCTCACGCCGCGCAG-TAMRA

transferase inhibitor (HMTi) as potential anticancer therapeutics on luminal and mesenchymal-like breast cancers.

Materials and Methods

Breast cancer cell lines and treatments. MCF7 and MDA-MB 231 breast cancer cell lines were used in the study. MCF-7 is an estrogen receptor (ER)-positive breast cancer cell line (16), representative of luminal subtype and MDA-MB-231 is an ER-negative breast cancer cell line (17, 18), representative of mesenchymal-like subtype. All cell lines were purchased from the ATCC (American Type Culture Collection, Manassas, VA, USA). MCF7 were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), gentamycin (20 μg/ml; Panpharma, Luitré, France), 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and insulin (0.04 U/ml; Novo Nordisk, Bagsværd, Denmark) in a humidified atmosphere at 37˚C containing 5% CO_2. MDA-MB-231 cells were cultured in Leibovitz's L-15 medium with 10% fetal bovine serum (Invitrogen), gentamycin (20 μg/ml; Panpharma) and 2 mM L-glutamine in a 37˚C humidified atmosphere without CO_2. Cells (1×10^6 per T75 flask) were treated during 48 h with 5 μM DZNep, 2 mM NaBu or 1 μM SAHA provided by Sigma-Aldrich (Saint-Louis, MI, USA) and dissolved respectively in water, ethanol and dimethyl sulfoxide. For controls, each cell line was conditioned in the medium without any treatment.

ChIP assays. Cells at 80% confluence were trypsinized and counted by Millipore Scepter 2.0 Cell (Fisher Scientific, 67403 Illkirch-Graffenstaden, France). For each treatment and cell controls, the DNA-protein crosslinking was realized with formaldehyde (36.5%; Sigma-Aldrich) diluted to 1% in the culture medium on 1×10^6 cells and 15 min-incubation was performed at room temperature. The Cross-linking was stopped by adding 0.125 M glycin during 5 min. Then, cells were washed with PBS-protease inhibitor and cell membranes were lysed by HighCell ChIP Kit (Diagenode) before chromatin shearing. The chromatin was prepared in TPX tubes (Diagenode) with shearing buffer (S1) and 1× volume protease inhibitor (Diagenode, Liège, Belgium). The samples were sonicated with Bioruptor® UCD-300 (Diagenode) and cooled to 4˚C with a Bioruptor® Water cooler (Diagenode) during sonication. Five runs of 5 cycles were performed with each cycle containing 30 sec "ON" and 30 sec "OFF" at 200 Watt. Between each run, samples were vortexed after a short spin. The sheared chromatin was frozen at –80˚C for later use. The sonication allowed performing chromatin fragments. The appropriate size was around 100 to 200 bp that was checked by migration on 1.5% agarose gel. The ChIP reactions were carried-out on SX-8G IP-Star® Compact Automated System (Diagenode) and realized with 2 μg of Antibodies (Abs), respectively, anti-H3K27me3 (pAb-069-050, Diagenode), anti-H3K9ac (pAb-103-050, Diagenode), anti-H3K4ac (pAb-165-050, Diagenode)] and non-immune rabbit IgG (Kch-504-250, Diagenode) (*e.g.* negative control). They were finalized in 200 μl volume using Auto Histone ChIP-seq kit reagents and incubated for 2 h with correspondent Abs coated on protein A-magnetic beads, and 10 h for IP reactions for 8 strips, at 4˚C. At the end, the input was prepared with 2 μl chromatin in 100 μl of DIB-buffer (19).

ChIP-qPCR. After the ChIP, real-time PCR was performed in triplicate using a ninety-six-well optical tray with optical adhesive film at a final reaction volume of 25 μl containing DNA IP (*e.g.* immunoprecipited DNA) or DNA input (*e.g.* total DNA) (5 μl), 1X SYBR Green Supermix (Applied Biosystem, Foster City, California, USA) and 200 nM each of *C-FOS* (pp-1004-500, Diagenode) (positive control for acetylation) or *TSH2B* (pp-1041-500, Diagenode) (positive control for methylation) promoters. For other genes, Taqman Universal PCR Master Mix was used at 1×, 400 nM each of forward and reverse primers and 250 nM of probe. Initial denaturation at 95˚C for 10 min to activate DNA polymerase was followed by fifty cycles of denaturation at 95˚C for 15 sec and annealing and extension at 60˚C for 1 min (7900HT, Real-Time PCR System; Applied Biosystems). Primer and probe sequences were selected with the help of Primer Express software (ABI) and expressed in Table I. The amount of 6-FAM fluorescence released during the PCR was measured by the real-time PCR system and was directly proportional to the amount of the PCR generated product. The cycle number at which the fluorescence signal crosses a detection threshold is referred to as Ct. The level of methylation or acetylation was disclosed by the rate of IP relative to Input. The efficiency of chromatin immunoprecipitation of particular genomic locus can be calculated from qPCR data and reported as a percentage of starting material: %(ChIP/Total Input)= 2^[(Ct(x%input) – log(x%)/log2) – Ct(ChIP)] ×100% (19, 20). Before any ChIP-QPCR analysis, we had to check the presence of activator and repressive marks for MCF7 and MDA-MB 231 cells. Hence, for acetylation modification, the Fold-enrichment of *C-FOS* (positive control) over *TSH2B* (negative control) demonstrated that H3K4ac and H3K9ac marks were increased in MCF7 and MDA-MB 231 cell lines treated with anti-HDAC, HMTi or even without any treatment (Figure 1A). Likewise, for methylation marks like H3K27me3, the Fold-enrichment of *TSH2B* (positive control) over *C-FOS* (negative control) was increased in both cell lines (Figure 1B).

Statistical analysis. All statistical analyses were performed using the R 3.0.1 software (21) and the statistical packages agricolae (22), HH (23) and multcomp (24). All gathered data were statistically analyzed by three-way ANOVA to test the level of statistical significance of cell lines, treatments and H3 histone marks and their interactions on the three studied genes. Post-hoc procedures were used when the F-test was significant ($p<0.05$). Multiple comparisons among means were examined by a Tukey's test for cell lines and treatments. The level of statistical significance was set at $p<0.05$.

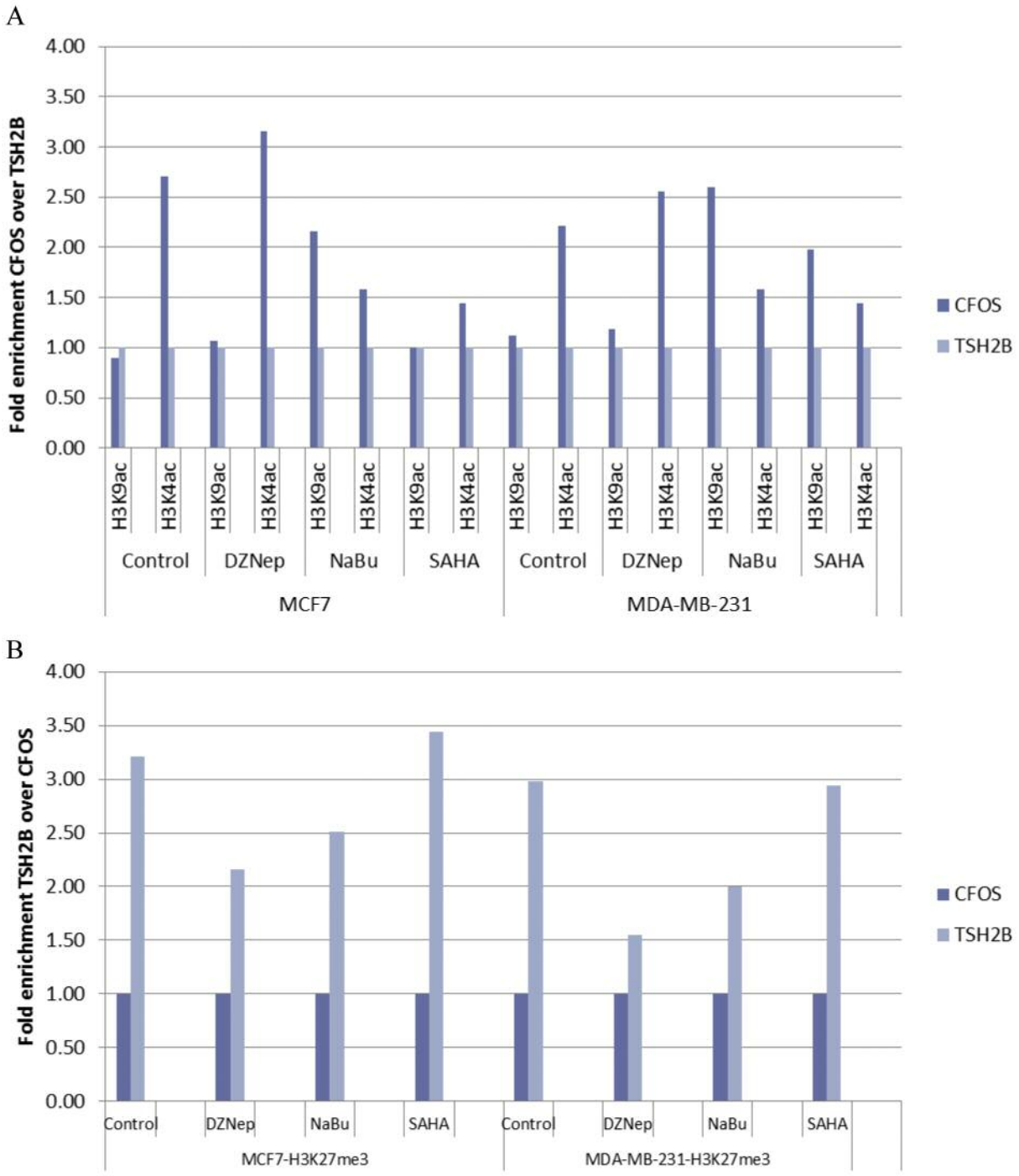

Figure 1. *(A) Fold-enrichment of C-FOS (positive control) over TSH2B (negative control) demonstrated that H3K4ac and H3K9ac marks were increased with C-FOS. (B) Fold-enrichment of TSH2B (positive control) over C-FOS (negative control) for methylation mark of H3K27me3 was increased with TSH2B.*

Reverse transcription and q-PCR. For each cell line, the RNA extraction was performed in cells, firstly washed in flask, three times with PBS. Afterwards, cells were lysed with 5 ml of RNA B™ according to manufacturer's protocol (BioProbe). RNA samples were verified using a NanoDrop ND-8000 Spectrophotometer. Five micrograms of total RNA were reverse transcribed in a 15 μl total volume using the First-Strand DNA Synthesis Kit according to the manufacturer's protocol (GE Healthcare Life Science, Piscataway, NJ,

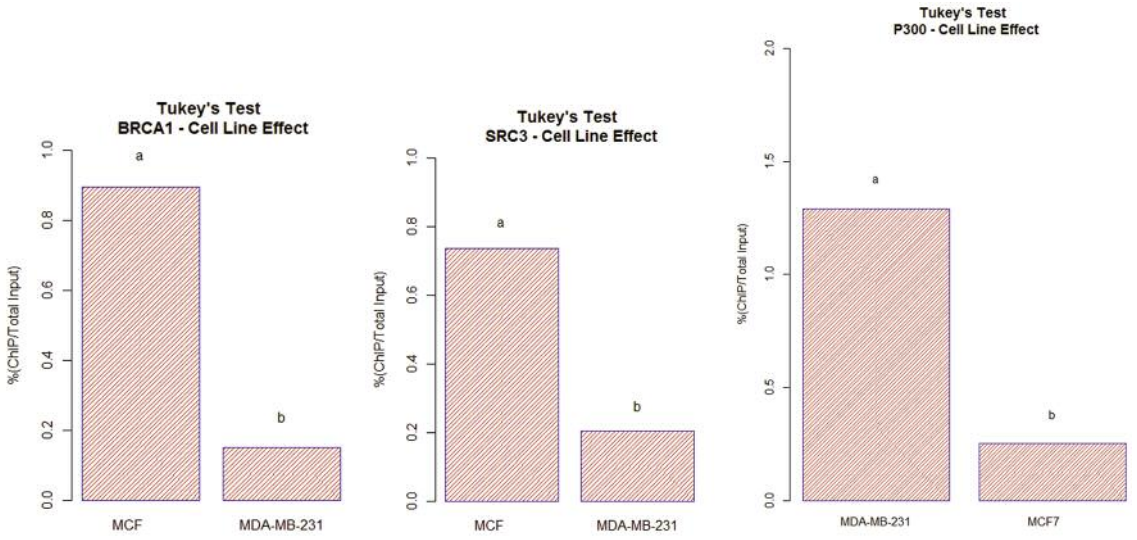

Figure 2. *Summary of the status of studied genes in breast cancer cell lines (MCF7 and MDA-MB 231) after HMTi (DZNep) and HDACi (NaBu, SAHA) treatments and ChIP with Abs against the H3K9ac, H3K4ac and H3K27me3 marks. The efficiency of ChIP was calculated by qPCR for BRCA1, P300 and SRC3 genes and reported as a percentage of starting material %(ChIP/Total Input) on the Y-axis. Anova analysis demonstrated the significant difference between the two cell lines with Tukey's test ($p<0.05$).*

USA). Reverse transcriptase was thermally inactivated (95˚C, 10 min). Then, the cDNA was quantified with PCR, carried-out in 96-well plates using 15 ng of cDNA in a reaction mix of 25 μl containing 12.5 μl TaqMan® gene expression Master Mix 1X (Applied Biosystems), 1.25 μl of each 200 nM assay on demand corresponding respectively to the studied genes, and 0.125 μl of 18S rRNA primers (10 μM) and 0.25 μl of *18S* TaqMan probe (5 μM). For studied genes, assay-on-demand, primers and Taqman® probes were purchased from Applied Biosystems as follows: *BRCA1*:Hs01556193_m1, *P300*:Hs00914223_m1 and *SRC3*: Hs01105251_m1 and for 18S as followed, forward: 5'-CGG CTA CCA CAT CCA AGG AA-3', reverse: 5'-GCT GGA ATT ACC GCG GCT-3', probe: 5'-TGC TGG CAC CAG ACT TGC CCT C-3' (VIC). Data were collected using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems) for 50 cycles (95˚C for 15 s, 60˚C for 1 min) after an initial step (50˚C for 2 min, 95˚C for 10 min). The fold change in expression of each gene was calculated using the $\Delta\Delta C_T$ method, with the ribosomal *18S* rRNA as an internal control. All data were generated in triplicate and expressed as mean±SD.

Western blotting. MCF-7 and MDA-MB-231 cells treated with DZNep, NaBu and SAHA, after 48 h they were washed three times with PBS and lysis buffer containing 20 mM Tris (pH 8), 50 mM EDTA, 0.8% NaCl, and 0.1% Triton X-100. Protease Inhibitor and Phosphatase Inhibitor Cocktails (Sigma) were added. Proteins were quantified using the Bradford Method. Twenty five mg proteins were loaded onto 10% gels for SDS-PAGE (except for BRCA1 and P300 proteins we used 4% gels) and electrophoresed. Then, separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane during 1hour. After the membranes were incubated overnight at 4˚C with Saline Tween 0.1% (TBST) containing 5% dry milk, washed 3 times with TBST, incubated 2 h with respectively monoclonal anti-BRCA1 Abs (1:50 [Ab-1], (Calbiochem); polyclonal anti-P300 Abs (1:100,[N-15], (Santa Cruz Biotechnology); polyclonal anti-SRC-3 Abs (1:500, NCOA3 [N1N2], (GeneTex) or monoclonal anti-actin Abs (1:120,000, Calbiochem). Membranes were washed three times with TBST and incubated for 2 h with alkaline phosphatase (AP) -conjugated secondary antibody (1:2,000 anti-mouse IgG - AP conjugate or 1:2,000 anti-rabbit IgG-AP conjugate, Promega). Detection was performed with Western Blue® Stabilized Substrate for AP (Promega).

Results

H3 histone modifications on BRCA1, SRC3 and P300 genes after HMTi (DZNep) and HDACi (NaBu, SAHA) treatments in MCF7 and MDA-MB-231 breast cancer cell lines. In Figure 2, statistical analysis by three-way ANOVA exhibited a significant difference between the two breast cancer cell lines (MCF7 and MDA-MB 231) with the combined effects of the HMTi (DZNep) and HDACi (NaBu, SAHA) treatments on the 3 histone marks (H3K9ac, H3K4ac and H3K27me3) for *BRCA1*, *SRC3* and *p300* genes. Mean comparisons of %(ChIP/Total Input) for the 3 different genes were examined by a Tukey's test between the two breast cancer cell lines.

For the *BRCA1* gene, the interactions between marks and treatments were less important in MDA-MB 231 breast cancer cell line than for the MCF7 breast cancer cell line. For *SRC3* gene, we also found a significant difference between MCF7 and MDA-MB 231 breast cancer cell lines. Percentage (ChIP/Total Input) for *SRC3* gene was higher in

MCF7 compared to MDA-MB 231. At the opposite, for the *p300* gene, the difference was found higher in MDA-MB 231 breast cancer cell line than in MCF7 breast cancer cell line.

H3 histone modifications (H3K4ac, H3K9ac and H3K27me3) for BRCA1 gene in breast cancer cell lines (MCF7 and MDA-MB 231) after HMTi (DZNep) and HDACi (NaBu, SAHA) treatments.

Mark effects: Firstly, with Tukey's test, in Figure 3, means for the different effects of marks (H3K4ac, H3K9ac and H3K27me3) were examined in two breast cancer cell lines (MCF7 and MDA-MB 231). Columns with different letters exhibited a significant difference ($p<0.05$). In MCF7 cells, for *BRCA1* gene, H3K4ac mark effect (a) was more important by comparison to H3K9ac (b) and H3K27me3 (b) marks for which their effects can be differentiated. For MDA-MB 231 cell line, for *BRCA1* gene, we demonstrated three different effects with the 3 marks, H3K9ac (a), H3K27me3 (b) and H3K4ac (c), by Tukey's test that exhibited different letters.

Treatment effects: As presented in Figure 3, in MCF7 breast cancer cell line for *BRCA1* gene, with Tukey's test, the SAHA (HDACi) treatment (a) was found more important and distinct from the control (b), the DZNEP (HMTi) (b) and the NaBu (HDACi) (b) seemed to have the same effects. In MDA-MB 231 cell line, for *BRCA1* gene, NaBu (HDACi) (a) and SAHA (HDACi) (a) were not different from the control (a). On the opposite, the DZNEP (HMTi) (b) exhibited a significant decrease by comparison with the control (a).

Mark and treatment effects: When we combined mark and treatment effects for the *BRCA1* gene with Tukey's test, in breast cancer cell lines, the examination of the means showed for MCF7 cells, an increase in H3K4ac with SAHA treatment (a) by comparison to the control (b). For *BRCA1*, there was no difference for H3K4ac and DZNEP treatment (b), H3K4ac and NaBu treatment (b) and H3K4ac with control treatment (b). On the other hand, there were no effects for *BRCA1* gene with Tukey's test regarding H3K27me3 with control treatment (c) or treated with HMTi (c) or HDACi (c) and H3K9ac with control treatment (c) or treated with HMTi (c) or HDACi (c).

In the MDA-MB 231 cell line, for *BRCA1*, the examination of means showed a significant difference with H3K27me3 with DZNEP treatment (c) compared to the correspondent H3K27me3 with any treatment or control (ab). With SAHA-H3K27me3 (a) and NaBu-H3K27me3 (a), Tukey's test revealed just an increased trend, but insignificant when compared to the control - H3K27me3 (ab).

In addition we did not find any significant effects with the acetylated marks and treatments or control for *BRCA1* gene. With H3K9ac-NaBu (a) and also H3K9ac-SAHA, a tendency was exhibited but not a significant difference with H3K9ac-control (ab). And the DZNEP (ab) has no effect on the H3K9ac mark by comparison with H3K9ac-control (ab). For *BRCA1* gene and H3K4ac, Tukey's test demonstrated a trend for H3K4ac-NABu (c), H3K4ac-SAHA (c), H3K4ac-DZNEP (c) by comparison with H3K4ac-control (bc).

H3 histone modifications (H3K4ac, H3K9ac and H3K27me3) for SRC3 gene in breast cancer cell lines (MCF7 and MDA-MB 231) after HMTi (DZNep) and HDACi (NaBu, SAHA) treatments.

Mark effects: In the MCF7 breast cancer cell line for *SRC3* gene, Tukey's test demonstrated by mean comparison a high effect for H3K4ac mark (a) and no difference between H3K9ac (b) and H3K27me3 (b) (Figure 4). In MDA-MB 231 cell line, for *SRC3* gene, we found 3 different effects with the 3 marks, H3K9ac (a), H3K27me3 (b) and H3K4ac mark (c). And these effects were more important with H3K9ac and H3K27me3 than H3K4ac.

Treatment effects: Mean comparison with Tukey's test in MCF7 breast cancer cell line for *SRC3* gene, identified a more important effect of SAHA (HDACi) (a) compared to the control (b). NaBu (HDACi) (ab) showed just an increased trend compared to the control (b). DZNEP (HMTi) (b) was found without any effect by comparison with the control (b) (Figure 4).

NaBu (HDACi) (a) and SAHA (HDACi) (a) were without any effect by comparison with the control (a) by Tukey's test in MDA-MB 231 cell line, for *SRC3* gene. Except, DZNEP (HMTi) (b) presented a difference with the control (a) (Figure 4).

Mark and treatment effects: For the *SRC3* gene in MCF7 breast cancer cell line, Tukey's test exhibited %(ChIP/Total Input) mean increased significantly for H3K4ac-SAHA (a) compared to H3K4ac-control (b) (Figure 4). Besides, H3K4ac and NaBu treatment (ab) presented a trend to an increase by comparison with H3K4ac-control (b) even though this difference was not significant. In addition, H3K4ac-DZNEP (b) was without any effect compared to H3K4ac-control (b). Moreover H3K27me3 and H3K9ac marks combined with all treatments were found with no effect when compared to the correspondent control.

In MDA-MB 231 cell line, for *SRC3* gene, H3K9ac-NaBu (a) and H3K9ac-SAHA (a) were without any effect compared to the mean exhibited by H3K9ac-control (a) (Figure 4). For H3K9ac-DZNEP (b) the mean decreased significantly compared to H3K9ac-control (a). Furthermore, H3K27me3-SAHA (a) and H3K27me3-NaBu (a) showed no modification compared to H3K27me3-control (a). At the opposite, H3K27me3-DZNEP (cd) presented a significant decrease by comparison to H3K27me3-control (a). For the three treatments DZNEP (HMTi) (bc), NaBu (HDACi) (d) and SAHA (HDACi) (d) combined with H3K4ac, the compared means showed no difference with H3K4ac - control (cd).

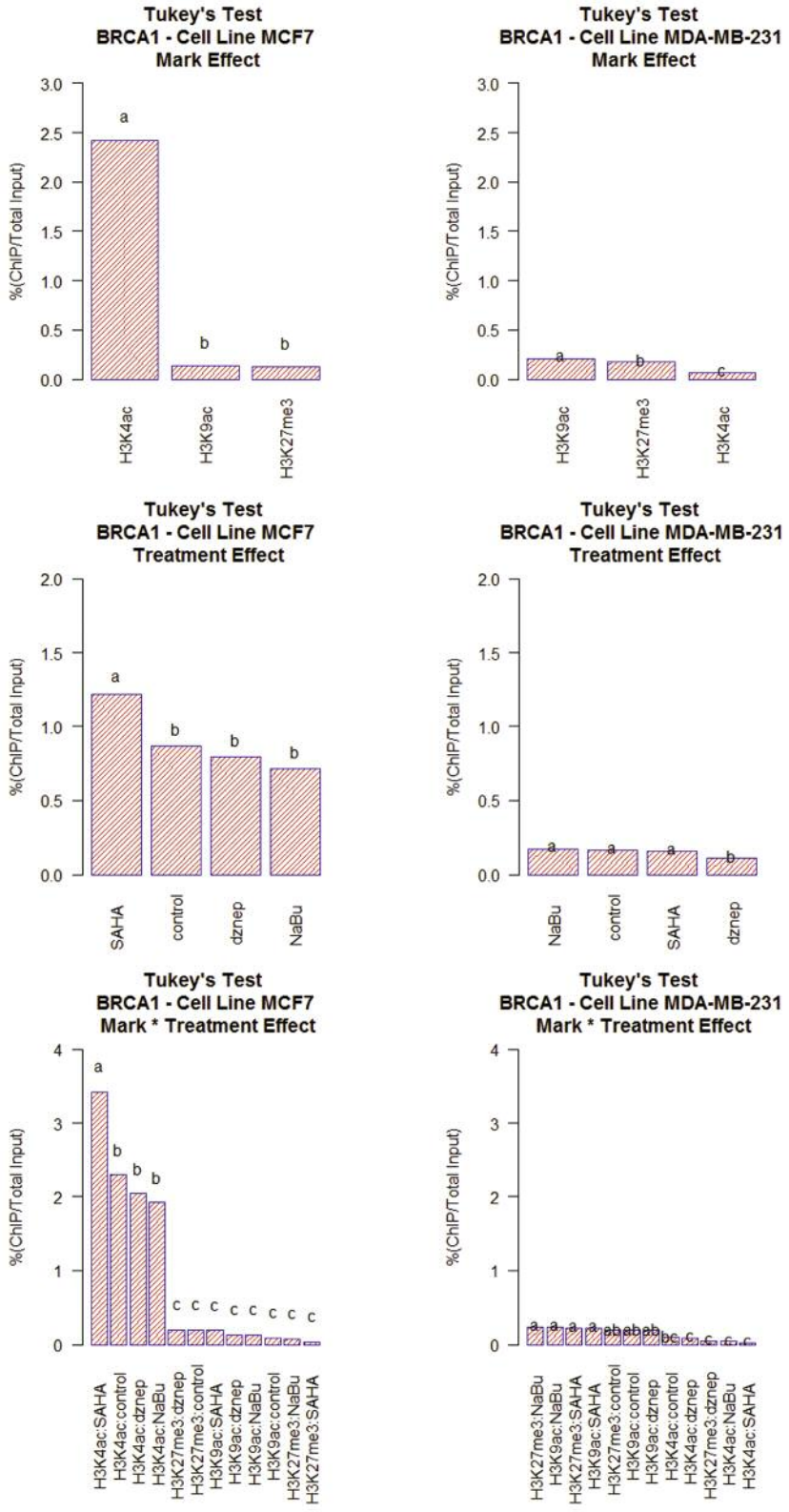

Figure 3. *H3 histone mark modifications (H3K4ac, H3K9ac and H3K27me3) for BRCA1 gene in breast cancer cell lines (MCF7 and MDA-MB 231) after HMTi (DZNep) and HDACi (NaBu, SAHA) treatments. The efficiency of ChIP was calculated by qPCR for each gene and reported as a percentage of starting material %(ChIP/Total Input) on the Y-axis. Columns with different letters exhibited a significant difference ($p<0.05$) with Tukey's test.*

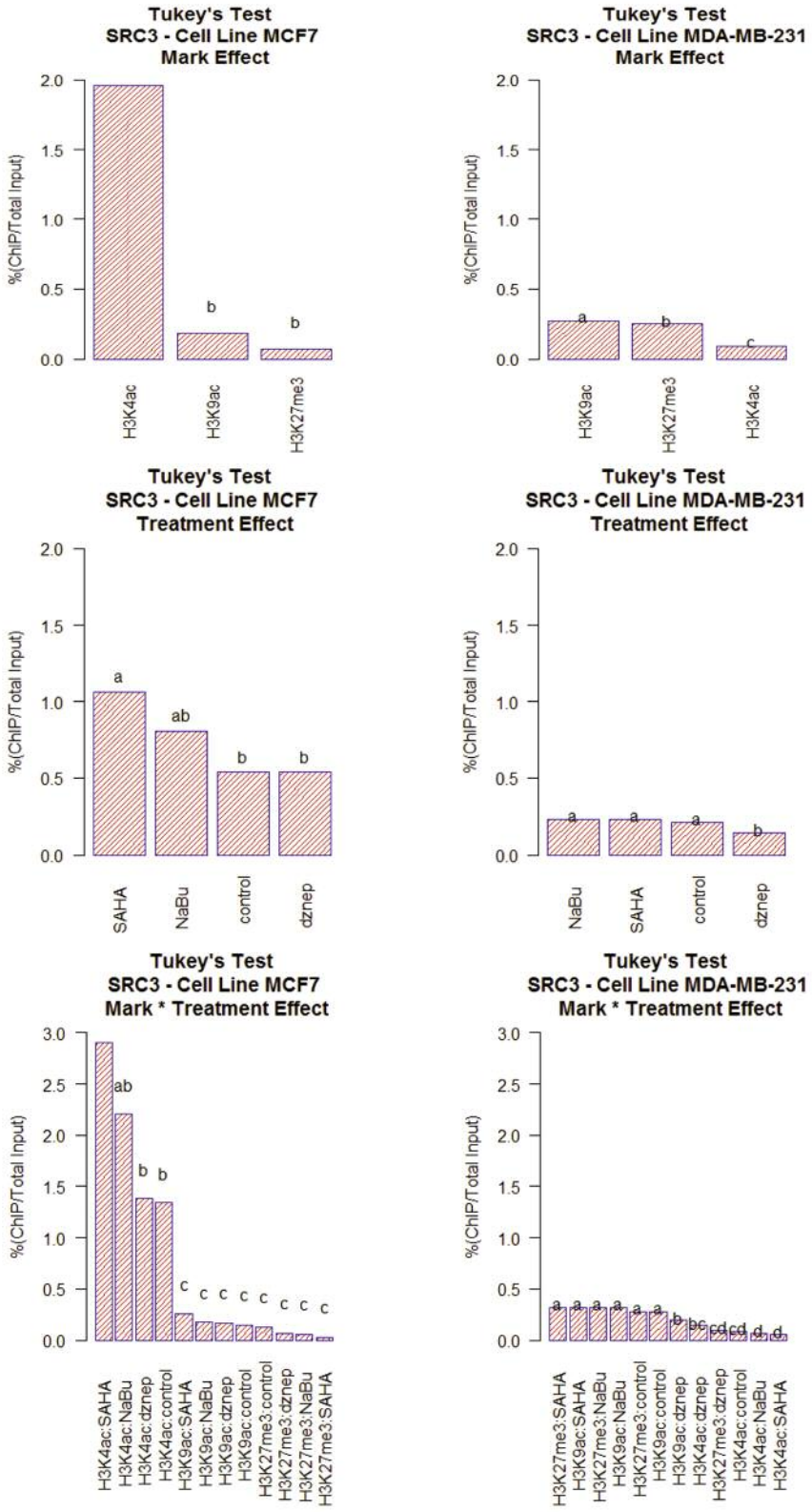

Figure 4. *H3 histone modifications (H3K4ac, H3K9ac and H3K27me3) for SRC3 gene in breast cancer cell lines (MCF7 and MDA-MB 231) after HMTi (DZNep) or HDACi (NaBu, SAHA) treatments. The efficiency of ChIP was calculated by qPCR for each gene and reported as a percentage of starting material %(ChIP/Total Input) on the Y-axis. Columns with different letters exhibited a significant difference ($p<0.05$) with Tukey's test.*

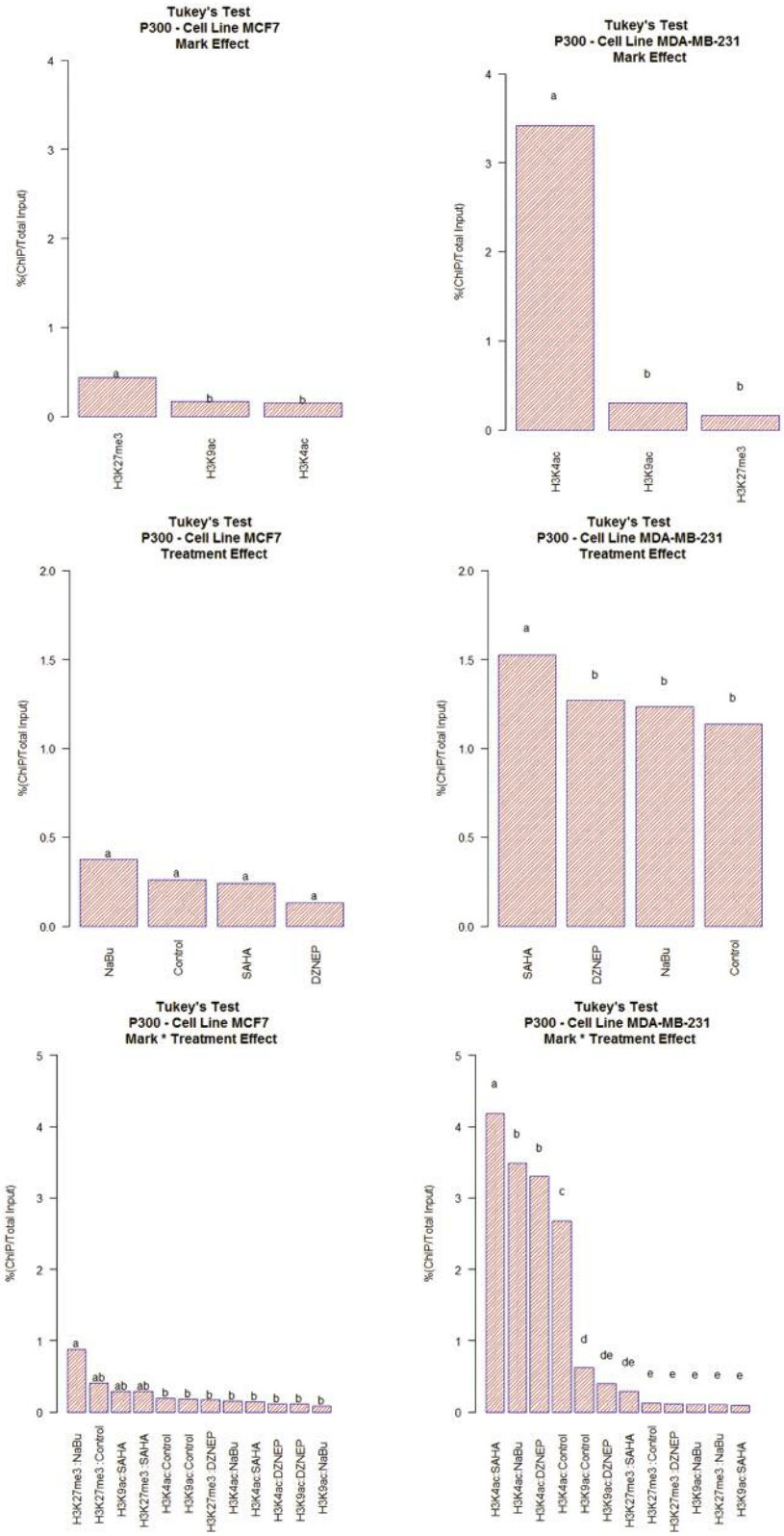

Figure 5. *H3 histone modifications (H3K4ac, H3K9ac and H3K27me3) for p300 gene in breast cancer cell lines (MCF7 and MDA-MB 231) after HMTi (DZNep) or HDACi (NaBu, SAHA) treatments. The efficiency of ChIP was calculated by qPCR for each gene and reported as a percentage of starting material %(ChIP/Total Input) on the Y-axis. Columns with different letters exhibited a significant difference ($p<0.05$) with Tukey's test.*

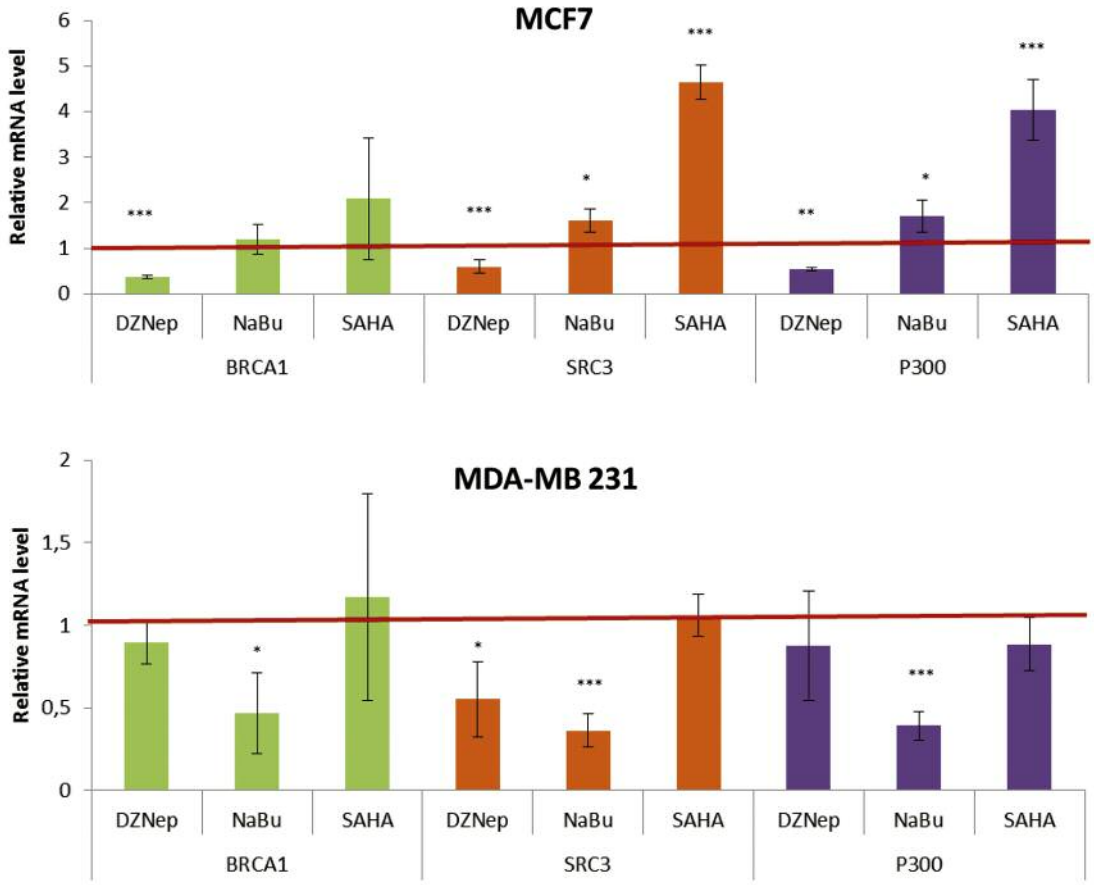

Figure 6. *BRCA1, SRC3 and P300 mRNA relative expression quantified by real time quantitative RT-QPCR in MCF7 and MDA-MB231 breast cancer cell lines after treatment with DZNep, NaBu or SAHA. Target gene expression values were expressed as fold exchanges compared to untreated cells (defined as 1). Data represented the average of the 3 replicates with respective error bars; (*p<0.05; ** p<0.01; *** p<0.001).*

H3 histone modifications (H3K4ac, H3K9ac and H3K27me3) for p300 gene in breast cancer cell lines (MCF7 and MDA-MB 231) after HMTi (DZNep) and HDACi (NaBu, SAHA) treatments.

Mark effects: For the *p300* gene in MCF7 breast cancer cell line, with Tukey's test, %(ChIP/Total Input) mean increased significantly for H3K27me3 (a) compared to the two other acetylated H3K4ac (b) and H3K9ac (b) marks. The two acetylated marks, H3K4ac (b) and H3K9ac (b) were not distinguishable with the different treatments (Figure 5). In MDA-MB 231 cell line, for *p300* gene, Tukey's test demonstrated by mean comparison, an important effect with H3K4ac marks (a). H3K9ac (b) and H3K27me3 (b) showed no modification at all (Figure 5).

Treatment effects: Mean comparison with Tukey's test in MCF7 breast cancer cell line for *p300* gene, demonstrated no effect of SAHA (HDACi) (a), NaBu (HDACi) (a) and DZNEP (HMTi) (a) and by comparison with the control (a) with all the marks. Just a trend was found with NaBu (Figure 5). In MDA-MB 231 cell line, for the *p300* gene, there was a significant increase with SAHA (a) with all the marks, by comparison with any treatment - the control (b). At the opposite, DZNEP (HMTi) (b) and NaBu (HDACi) (b) showed no difference with the control without any treatment (b) (Figure 5).

Mark and treatment effects: For *p300* gene in MCF7 breast cancer cell line, with Tukey's test, the calculated mean corresponding to %(ChIP/Total Input) for H3K9ac-SAHA (ab) showed just a higher trend by comparison to the H3K9ac-control (b). For H3K9ac-NaBu (b) and H3K9ac-DZNEP (b) there was no difference with the H3K9ac control (b). For H3K4ac-SAHA (b), H3K4ac-NaBu (b) and H3K4ac-DZNEP (b), no difference was found by comparison with the H3K4ac-control (b). For H3K27me3-DZNEP (b) just a low trend was found by comparison with the H3K27me3-control (ab). With H3K27me3-NaBu (a) a trend to increase compared to the H3K27me3-control (ab) was exhibited. With H3K27me3-SAHA (ab), no difference was found with the H3K27me3-control (ab) (Figure 5).

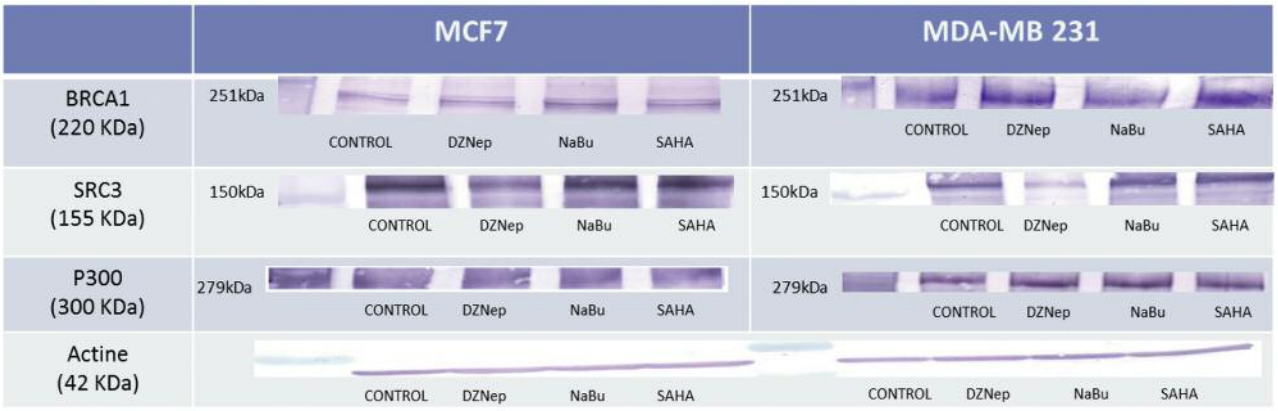

Figure 7. *BRCA1, SRC3 and P300 protein expressions in MCF7 and MDA-MB231 breast cancer cell lines treated with DZNep, NaBu or SAHA. Western blot analyses were performed using anti Anti-BRCA1 (1/50), Anti-P300 (1/100) and Anti-SRC-3 (1/500).*

In Figure 5, for *p300* gene in the MDA-MB 231 breast cancer cell line, Tukey's test demonstrated a significant increase for H3K4ac-SAHA (a), H3K4ac-NaBu (b) and H3K4ac-DZNEP (b) compared to the H3K4ac-control (c). H3K9ac-SAHA (e) showed a significant decrease by comparison to the H3K9ac- control (d). The H3K9ac-DZNEP (de) presented only a trend toward decrease by comparison to the H3K9ac-control (d). On the opposite, H3K9ac-NaBu (e) mean decreased significantly by comparison with the H3K9ac- control (d). For H3K27me3-DZNEP (e) and H3K27me3-NaBu (e), comparison of means exhibited no difference with the H3K27me3-control (e). With H3K27me3-SAHA (de) just a trend of increase was found by comparison to the H3K27me3 control (e).

BRCA1, SRC3 and p300 mRNA expression in MCF7 and MDA-MB231 breast cell lines after treatment with DZNep, NaBu or SAHA. In Figure 6, in MCF7, the DZNep (HMTi) decreased significantly the mRNA expression of *BRCA1* ($p<0.001$), *SRC3* ($p<0.001$) and *p300* ($p<0.01$) compared to the control corresponding to untreated cells and defined as 1. Whereas NaBu (HDACi) increased significantly compared to the control, the expression of *SRC3* ($p<0.05$), and *p300* ($p<0.05$) mRNA and for *BRCA1* just a trend to increase was found. In addition, SAHA (HDACi) increased significantly the mRNA expression for SRC3 ($p<0.001$) and *p300* ($p<0.001$) genes compared to the control. For *BRCA1* mRNA expression, the increase was insignificant. In the MDA-MB 231 cell line, in Figure 6, DZNep (HMTi) decreased significantly the mRNA expression of *SRC3* ($p<0.05$) compared to the control. A trend to decrease was just found for *BRCA1* and *p300* mRNAs. NaBu (HDACi) seemed to reduce significantly the mRNA expression of *BRCA1* ($p<0.05$), *SRC3* ($p<0.001$) and *p300* ($p<0.01$) compared to the control. On the other hand, SAHA (HDACi) was without any effect on the mRNA expression for the 3 genes.

BRCA1, SRC3 and p300 protein expressions in MCF7 and MDA-MB231 breast cancer cell lines treated with DZNep, NaBu or SAHA. To investigate BRCA1, SRC3 and p300 protein expression, the effects of HMTi (DZNep) and HDACi (NaBu and SAHA) in MCF7 and MDA-MB231 breast cancer cell lines were performed by western blotting using anti-BRCA1, anti-P300 and anti-SRC-3 antibodies, respectively. Figure 7 demonstrated that DZNep (HMTi) scarcely decreased the expression of the studied proteins, and NaBu (HDACi) or SAHA (HDACi) showed a trend to increase BRCA1, SRC3 and p300 protein expression in MCF7 and MDA-MB231 breast cancer cell lines.

Discussion

The profile of *BRCA1* gene in the breast cancer cell lines (MCF7 and MDA-MB 231) was examined by the three-way ANOVA analysis with the combined effects of the HMTi (DZNep) and HDACi (NaBu, SAHA) treatments on the 3 histone marks (H3K9ac, H3K4ac and H3K27me3). For the *BRCA1* gene, the interaction marks and treatments displayed a significant difference and the effects were less important in MDA-MB 231 breast cancer cell line than in MCF7 breast cancer cell line. This can be explained by the fact that these 2 breast cancer cell lines had a *BRCA1* allelic loss and were wild-type for *BRCA1* mutation status, but the identified sequence variants were different and the *BRCA1* promoter region might be un-methylated in the breast cancer cell lines (25). Moreover, Rice and Futscher, demonstrated in MCF7 that the functional 5' regulatory region of *BRCA1* CpC islands was not methylated like normal cells like HMEC

normal breast cell line. They also demonstrated that HMEC and MCF7 breast cell lines were acetylated at histones H3 and H4. These data seemed to indicate that active transcription of *BRCA1* coincided with a non-methylated and histone acetylated promoter (26). This can explain the results of SAHA treatment on H3K4ac that was higher in MCF7 breast cancer cell lines by comparison to MDA-MB 231. In MCF7, the amount of % (ChIP/Total Input) for H3K4ac-control is important and after treatment with SAHA (HDACi) an increase of the % of H3K4ac-SAHA was induced.

At the opposite, for the *p300* gene, that is a member of the mammalian histone acetyl transferase (HAT) family, with the three-ways ANOVA analysis, the significant difference was found higher in MDA-MB 231 breast cancer cell line by comparison with MCF7 breast cancer cell line. Yang *et al*., 2013 reported that *p300* was expressed in MDA-MB 231 and seemed to play a critical role in driving its invasive growth (27).

However, in MDA-MB 231 breast cancer cell line, Tukey's test demonstrated a significant increase in H3K4ac marks on *p300* gene after treatment with SAHA and NaBu. Besides, the expression of *p300* was found increased at the mRNA and protein levels. These results go along with other results reported by Ogryzko *et al*., they demonstrated that acetylation levels by p300 were very close to those of hyperacetylated histones prepared from HeLa nuclei treated with NaBu (HDACi) (28). In the same way, Davie *et al*. also reported that inhibition of HDAC activity with NaBu allowed the HAT activity of p300 to increase the histone acetylation levels at the promoter and nearby regions (29).

Therefore, SAHA and NaBu seemed to have a critical impact in the mesenchymal-like breast cancer due to the overexpression of *p300*. These observations suggested that *p300* might be a potential therapeutic target for treating cancer. A new anticancer agent that targets *p300* called LOO2 notably suppressed histone H3 (H3ac) and H4 (H4ac) acetylation in cancer cell lines, including MDA-MB 231 (27). In addition, Fermento *et al*., also demonstrated that inhibitors of p300 acetyltransferase activity are potent anticancer agents and that p300 inhibition is an effective strategy for treating triple-negative and mesenchymal-like breast cancers (30). As a matter of fact, SRC3 expression was found to correlate significantly to other co-factors like p300/CBP (30). The up-regulation of SRC3 and p300/CBP in normal and malignant tissue was consistent with mRNA level findings (31). The observed correlation suggests a combined function as well as a functional link for these proteins (9, 32).

For *SRC3* gene, the combined effects were found significantly higher in MCF7 compared to MDA-MB 231, with the three-way ANOVA analysis. MCF-7 is an ER-positive breast cancer cell line and MDA-MB-231 is an ER-negative breast cancer cell line. SRC3 is known as an ER-coregulator and is amplified in breast cancer (33, 34). Moreover, high SRC3 expression was associated with poor DFS in patients with ER-positive tumors treated with adjuvant tamoxifen (35). SRC3 is expressed in the ER-negative breast cancer cell line, MDA-MB-231 and has been shown to modulate the invasiveness of this cell line (9).

Additionally, with DZNEP, H3K27me3 showed a significant decrease by comparison with H3K27me3-control. The DZNEP was known to inhibit histone methylation by down-regulation of PRC2 complex. Other studies demonstrated that DZNEP treatment regulated gene expressions that were transcriptionally repressive in breast cancer, by inversion of PRC2 and histone methylation-mediated gene silencing (5, 36).

However, in MCF-7 breast cancer cell line, Tukey's test demonstrated a significant increase for all the acetylated marks on *SRC3* gene after HDACi treatment with SAHA or NaBu. We also demonstrated an increase in *SRC3* mRNA and protein expressions. Nevertheless, further studies had already demonstrated that an overexpression of *SRC3* gene was associated with a poor prognosis in breast cancer (37). As noted earlier, *SRC3* is a key co-regulator of Erα activity and has been shown to play a role in breast cancer. Further investigations were required to define the potential diagnosis and prognosis role of *SRC3* and its possible value as a therapeutic target.

Concerning, HDAC inhibitors (HDACis) that have potential as anticancer therapeutics in various tumors. HDACis induce transcriptional up-regulation of genes by interfering with the catalytic domain of HDACs to block substrate recognition of these enzymes (38). So far, two HDACi vorinostat (suberoylanilide hydroxamic acid-SAHA, Zolinza) and depsipeptide (romidepsin, Istodax) have been recognized for cutaneous T-cell lymphoma and peripheral T-cell lymphoma treatment (39).

HDACis are more effective on breast tumor cells that are altered with vorinostat treatment in comparison with normal cells (40). However, they have the potential to modulate ER expression and provides novel opportunity to reverse the resistance to hormone therapy in breast cancer.

HDACi sodium butyrate is active on inhibition of cell growth and apoptosis. It was shown that sodium butyrate induced the activity of caspase-3,-8,-10 in a time- and dose-dependent manner in human breast cancer cell line MRK-nu-1. Moreover, sodium butyrate treatment induced DNA fragmentation in a dose-dependent manner (41).

Epigenetic drugs, which are effective on HAT mechanism, were also tested for their therapeutic potential. 3-Deazaneplanocin A (DZNep) is an inhibitor of S-adenosylhomocysteine (AdoHcy) hydrolase and inhibits histone methylation by downregulation of PRC2 complex (42). Tan *et al*. demonstrated that DZNep regulates gene expression in breast cancer, by inversion of PRC2 and histone methylation-mediated gene silencing (36). They clarified that DZNep induces apoptosis of PRC2 target genes (5).

Conclusion

Luminal and mesenchymal-like breast cancer subtype cell lines exhibited two different responses to HDACi treatments (SAHA and NaBu). In luminal subtype, MCF-7 (ER-positive cell line), an increase of BRCA1 was found with HDACis. On the contrary, in MDA-MB-231, representative of mesenchymal-like subtype and ER-negative breast cancer cell line, the HDACis stayed without any effect, perhaps due to the breast cancer cell line resistant status.

Acknowledgements

Aslihan Dagdemir was supported by Protema Saglik Hizm A.S (Istanbul, Turkey). Seher Karsli-Ceppioglu was supported by the Scientific and Technology Research Council of Turkey (TUBITAK-2219) project grants. Khaldoun RIFAÏ was supported by Hariri Foundation (Beyrouth, Liban).

This work was supported by La Ligue Contre Le Cancer (Comité du Puy de Dôme).

References

1 Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin *61*: 69-90, 2011.

2 Bosviel R, Michard E, Lavediaux G, Kwiatkowski F, Bignon YJ and Bernard-Gallon DJ: Peripheral blood DNA methylation detected in the BRCA1 or BRCA2 promoter for sporadic ovarian cancer patients and controls. Clin Chim Acta *412*: 1472-1475, 2011.

3 Bosviel R, Durif J, Guo J, Mebrek M, Kwiatkowski F, Bignon YJ and Bernard-Gallon DJ: BRCA2 promoter hypermethylation in sporadic breast cancer. OMICS *16*: 707-710, 2012.

4 Daures M, Ngollo M, Judes G, Rifai K, Kemeny JL, Penault-Llorca F, Bignon YJ, Guy L and Bernard-Gallon D: The JMJD3 Histone Demethylase and the EZH2 Histone Methyltransferase in Prostate Cancer. OMICS *20*: 123-125, 2016.

5 Karsli-Ceppioglu S, Dagdemir A, Judes G, Ngollo M, Penault-Llorca F, Pajon A, Bignon YJ and Bernard-Gallon D: Epigenetic mechanisms of breast cancer: an update of the current knowledge. Epigenomics *6*: 651-664, 2014.

6 Judes G, Rifai K, Daures M, Dubois L, Bignon YJ, Penault-Llorca F and Bernard-Gallon D: High-throughput << Omics >> technologies: New tools for the study of triple-negative breast cancer. Cancer Lett Mar 7, 2016.

7 Judes G, Rifai K, Ngollo M, Daures M, Bignon YJ, Penault-Llorca F and Bernard-Gallon D: A bivalent role of TIP60 histone acetyl transferase in human cancer. Epigenomics *7*: 1351-1363, 2015.

8 Wang L and Di LJ: BRCA1 and estrogen/estrogen receptor in breast cancer: where they interact? Int J Biol Sci *10*: 566-575, 2014.

9 Gojis O, Rudraraju B, Gudi M, Hogben K, Sousha S, Coombes RC, Cleator S and Palmieri C: The role of SRC-3 in human breast cancer. Nat Rev Clin Oncol *7*: 83-89, 2010.

10 Hsieh CL, Ma HP, Su CM, Chang YJ, Hung WY, Ho YS, Huang WJ and Line RK: Alterations in histone deacetylase 8 lead to cell migration and poor prognosis in breast cancer. Life Sci 2016.

11 Min A, Im SA, Kim DK, Song SH, Kim HJ, Lee KH, Kim TY, Han SW, Oh DY, O'Connor MJ and Bang YJ: Histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA), enhances anti-tumor effects of the poly (ADP-ribose) polymerase (PARP) inhibitor olaparib in triple-negative breast cancer cells. Breast Cancer Res *17*: 33, 2015.

12 Li L, Sun Y, Liu J, Wu X, Chen L, Ma L and Wu P: Histone deacetylase inhibitor sodium butyrate suppresses DNA double strand break repair induced by etoposide more effectively in MCF-7 cells than in HEK293 cells. BMC Biochem *16*: 2, 2015.

13 Wang ZT, Chen ZJ, Jiang GM, Wu YM, Liu T, Yi YM, Zeng J, Du J and Wang HS: Histone deacetylase inhibitors suppress mutant p53 transcription via HDAC8/YY1 signals in triple negative breast cancer cells. Cell Signal *28*: 506-515, 2016.

14 Kurdistani SK: Histone modifications as markers of cancer prognosis: a cellular view. Br J Cancer *97*: 1-5, 2007.

15 Santos-Rosa H and Caldas C: Chromatin modifier enzymes, the histone code and cancer. Eur J Cancer *41*: 2381-2402, 2005.

16 Soule HD, Vazguez J, Long A, Albert S and Brennan M: A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst *51*: 1409-1416, 1973.

17 Cailleau R, Mackay B, Young RK and Reeves WJ, Jr.: Tissue culture studies on pleural effusions from breast carcinoma patients. Cancer Res *34*: 801-809, 1974.

18 Vissac-Sabatier C, Bignon YJ and Bernard-Gallon DJ: Effects of the phytoestrogens genistein and daidzein on BRCA2 tumor suppressor gene expression in breast cell lines. Nutr Cancer *45*: 247-255, 2003.

19 Dagdemir A, Durif J, Ngollo M, Bignon YJ and Bernard-Gallon D: Histone lysine trimethylation or acetylation can be modulated by phytoestrogen, estrogen or anti-HDAC in breast cancer cell lines. Epigenomics *5*: 51-63, 2013.

20 Ngollo M, Lebert A, Dagdemir A, Judes G, Karsli-Ceppioglu S, Daures M, Kemeny JL, Penault-Llorca F, Boiteux JP, Bignon YJ, Guy L and Bernard-Gallon D: The association between Histone 3 Lysine 27 Trimethylation (H3K27me3) and prostate cancer: relationship with clinicopathological parameters. BMC Cancer *14*: 994, 2014.

21 Team RDC: R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria, 2013.

22 Mendiburu F: agricolae: Statistical Procedures for Agricultural Research. package version 114, 2013.

23 Heiberger R: HH:Statistical Analysis and Data Display. R package version 2337, 2013.

24 Torsten H, Bretz F, Westfall P and Heiberger RM: Multcomp: Simultaneous Inference in General Parametric Models. R package version 1218, 2013.

25 Elstrodt F, Hollestelle A, Nagel JH, Gorin M, Wasielewski M, van den Ouweland A, Merajver SD, Ethier SP and Schutte M: BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants. Cancer Res *66*: 41-45, 2006.

26 Rice JC and Futscher BW: Transcriptional repression of BRCA1 by aberrant cytosine methylation, histone hypoacetylation and chromatin condensation of the BRCA1 promoter. Nucleic Acids Res *28*: 3233-3239, 2000.

27 Yang H, Pinello CE, Luo J, Li D, Wang Y, Zhao LY, Jahn SC, Saldanha SA, Chase P, Planck J, Geary KR, Ma H, Law BK, Roush WR, Hodder P and Liao D: Small-molecule inhibitors of acetyltransferase p300 identified by high-throughput screening are potent anticancer agents. Mol Cancer Ther *12*: 610-620, 2013.

28 Ogryzko VV, Schiltz RL, Russanova V, Howard BH and Nakatani Y: The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell *87*: 953-959, 1996.
29 Davie JR: Inhibition of histone deacetylase activity by butyrate. J Nutr *133*: 2485S-2493S, 2003.
30 Fermento ME, Gandini NA, Salomon DG, Ferronato MJ, Vitale CA, Arevalo J, Lopez Romero A, Nunez M, Jung M, Facchinetti MM and Curino AC: Inhibition of p300 suppresses growth of breast cancer. Role of p300 subcellular localization. Exp Mol Pathol *97*: 411-424, 2014.
31 Kurebayashi J, Otsuki T, Kunisue H, Tanaka K, Yamamoto S and Sonoo H: Expression levels of estrogen receptor-alpha, estrogen receptor-beta, coactivators, and corepressors in breast cancer. Clin Cancer Res *6*: 512-518, 2000.
32 Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK and Rosenfeld MG: The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature *387*: 677-684, 1997.
33 Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM and Meltzer PS: AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science *277*: 965-968, 1997.
34 Madak-Erdogan Z, Charn TH, Jiang Y, Liu ET, Katzenellenbogen JA and Katzenellenbogen BS: Integrative genomics of gene and metabolic regulation by estrogen receptors alpha and beta, and their coregulators. Mol Syst Biol *9*: 676, 2013.
35 Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, Fuqua SA, Wong J, Allred DC, Clark GM and Schiff R: Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst *95*: 353-361, 2003.
36 Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, Karuturi RK, Tan PB, Liu ET and Yu Q: Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev *21*: 1050-1063, 2007.
37 York B and O'Malley BW: Steroid receptor coactivator (SRC) family: masters of systems biology. J Biol Chem *285*: 38743-38750, 2010.
38 Ververis K, Hiong A, Karagiannis TC and Licciardi PV: Histone deacetylase inhibitors (HDACIs): multitargeted anticancer agents. Biologics *7*: 47-60, 2013.
39 Campas-Moya C: Romidepsin for the treatment of cutaneous T-cell lymphoma. Drugs Today (Barc) *45*: 787-795, 2009.
40 Huang L and Pardee AB: Suberoylanilide hydroxamic acid as a potential therapeutic agent for human breast cancer treatment. Mol Med *6*: 849-866, 2000.
41 Nohara K, Yokoyama Y and Kano K: The important role of caspase-10 in sodium butyrate-induced apoptosis. Kobe J Med Sci *53*: 265-273, 2007.
42 Chiang PK and Cantoni GL: Perturbation of biochemical transmethylations by 3-deazaadenosine *in vivo*. Biochem Pharmacol *28*: 1897-1902, 1979.

Received January 21, 2016
Revised April 13, 2016
Accepted April 21, 2016