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Histone Deacetylase Inhibitors Enhance Retinoid Response in Human Breast Cancer Cell Lines

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Abstract. *Solid tumors develop resistance to retinoids during carcinogenesis. One of the strategies to overcome this resistance may include the combination of these molecules with other differentiating, cytotoxic or chromatin-remodelling agents. We analysed the anti-proliferative activity of two histone-deacetylase inhibitors (HDACIs), Trichostatin A (TSA) and sodium phenylbutyrate (PB), alone or combined with retinoids, all-trans retinoic acid (ATRA) and Ro 41-5253, on two human breast cancer cell lines: the hormone-dependent MCF-7 and the hormone-independent MDA-MB-231. These lines responded differently to retinoids: MCF-7 were sensitive, whilst MDA-MB-231 were rather resistant. When the retinoids were combined with HDACIs, these molecules potentiated the retinoid activity on growth inhibition, especially for the association Ro 41-5253 and TSA. By FACS analysis, we observed that the anti-proliferative effects were only partially due to pro-apopotic mechanisms, suggesting a cell-cycle block. The efficacy of the retinoids/HDACIs combinations could represent a new strategy in breast cancer chemotherapy, allowing inhibition of both ER+ and ER- cell populations.*

Retinoids exert profound effects on many biological processes (*i.e.* vision, reproduction and development) and possess anti-proliferative, differentiative and immunomodulatory properties (1,2). The biological activities of retinoids are mediated by two classes of nuclear receptors: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), each of which is encoded by three distinct genes (α, β and γ) and includes different isotypes, depending on different promoters and alternative splicing (3-5). RARs and RXRs modulate the transcription of important genes through their binding to specific DNA sequences, the "retinoic acid response elements" (RAREs) and "retinoic X response elements" (RXREs), which are located in the promoters of responsive genes (6). In therapy, the presence of altered retinoid receptors can be the primary cause of a reduced retinoid response, with the consequent accumulation of retinoid-resistant cells (7,8). Many studies have suggested that this resistance could be correlated with abnormal histone-acetylation and DNA-hypermethylation of the RAR promoters, because of the well-known effects of these epigenetic modifications (9,10). Recent reports indicate that the gene silencing by methylation passes through changes of chromatin structure associated with histone deacetylation. In fact, DNA methylation and histone deacetylation are repression mechanisms which are strictly connected (10-12). For this reason histone deacetylase inhibitors (HDACIs) and demethylating agents were tested *in vitro* on tumoral cells in order to re-express silenced genes and revert the malignant phenotype (10,13-14). Trichostatin A (TSA) and sodium phenylbutyrate (PB) have been proven to promote cytostasis and differentiation in a wide variety of tumors and PB, in particular, has been used in clinical trials as a monotherapy or combined with other differentiating agents (15-18). Moreover, it is already reported that the combinations of HDACIs with retinoids are efficacious in patients with highly resistant acute promyelocytic leukemia (19). In our experiments, we observed the poor anti-proliferative efficacy of retinoids on ER- cells, in comparison with the high efficacy on ER+ cells. However, the heterogeneity is typical of breast tumors, in which hormone-dependent and hormone-independent cells coexist and

Abbreviations: ATRA, all-*trans* retinoic acid; Ro, Ro 41-5253; HDACs, histone deacetylases; HDACIs, histone deacetylase inhibitors; TSA, trichostatin A; PB, sodium phenylbutyrate; ER+, estrogen receptor-positive; ER-, estrogen receptor-negative; RAR, retinoic acid receptor; RARE, retinoic acid response element.

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respond differently to retinoids and chemotherapeutic agents in general. We tried to reproduce this heterogeneity *in vitro*, by working in parallel on the ER+ MCF-7 and the ER- MDA MB 231 cell lines. Amongst the retinoids previously analysed, we selected the natural panagonist ATRA and the synthetic RAR-alpha selective antagonist Ro 41-5253, which in previous studies was demonstrated to be the most efficacious of the synthetic retinoids (20). Their efficacy was tested both as single agents and in combination with two well known HDACIs, TSA and PB.

Materials and Methods

Reagents. ATRA, TSA (Sigma-Aldrich,Italy) and Ro 41-5253 (F. Hoffmann-La Roche Ltd., Switzerland) were dissolved in absolute ethanol at a concentration of 10^{-2} M and stored in the dark at –20˚C. PB (kindly supplied by Fyrklövern Scandinavia AB, Sweden) was dissolved in PBS 1X at a concentration of 1M and stored at +4˚C. Stock solutions were then diluted to the desired concentrations in fresh medium just prior to use. Media, antibiotics and amino acids were supplied by Sigma-Aldrich.

Cell culture. MCF-7 and MDA MB 231 lines were supplied by the American Type Culture Collection and grown in monolayer culture in DMEM, supplemented with 10% FBS, 1% penicillin-streptomycin and 1% non-essential amino acids, in a 5% CO_2 humidified atmosphere at 37˚C. The cell medium was changed every 2-3 days.

Proliferation analysis. The cells were seeded in duplicate on 6-well tissue culture plates at a density of 1 x 10^4 cells/well. After two days of culture, the medium was added with HDACIs used as single agents (PB 1, 2.5 and 5mM or TSA 10^{-8}, 10^{-7} and 10^{-6} M) or alternatively with the selected combination of HDACIs (TSA 10^{-8} M and PB 1 mM) and retinoids (10^{-8} and 10^{-5} M). The untreated controls received an equivalent volume of the solvent. The cells were harvested and counted at the established time periods and cell viability was determined by trypan blue dye exclusion. The percentage of viable cells was calculated in comparison to untreated cells. The experiments were repeated three times in duplicate. Results are expressed as mean ± SD.

Flow cytometry analysis. This assay was performed as previously described (20).

Histone acetylation. The average acetylation of nuclear chromatin was evaluated by immunofluorescence labelling of the acetylated H4, using anti-H4ac IgG as primary antibody (Upstate Biotechnology, UK) and IgG-FITC conjugated as the secondary one (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were washed with PBS 1X and fixed for 45 minutes in 2% glutaraldehyde, 0.75% formaldehyde, 2 mM $MgCl_2$ and 2 mM EGTA in PBS 1X, pH 7.35. After a brief permeabilisation with 1% Triton, the cells were washed and incubated overnight with the primary antibody. The secondary antibody was added for one hour in the dark the day after and the fluorescence was measured by a FACScan flow cytometer (Becton Dickinson, Milan, Italy) and analysed using Cell Quest Software (Becton Dickinson).

Results

Effects of HDACIs and retinoids on cell proliferation inhibition. In the initial phase of our work, we recorded the growth curves for the selected cell lines, as a function of the treatment with the two retinoids at low (10^{-8} M) and high (10^{-5} M) concentrations. The treatment with the natural retinoid ATRA inhibited MCF-7 proliferation in a dose- and time-dependent manner. Also the synthetic retinoid, Ro 41-5253, reduced the MCF-7 growth, but only the highest dose gave an inhibition comparable to ATRA. Under the same conditions, neither retinoids showed significant anti-proliferative effects on MDA MB 231 (data not shown). After treatment with the two HDACIs as single agents, MCF-7 showed an early inhibition even at the lowest dose: on the 4th day, TSA 10^{-8} M and PB 1 mM produced 41% and 39% of growth suppression, respectively. The highest concentration (TSA 10^{-6} M and PB 5 mM) produced a stronger efficacy, with TSA showing effects earlier than PB (Figure 1A). Similar effects were observed for MDA MB 231 cells too. At the highest dose, TSA induced an 89% inhibition as early as the 2nd day, while PB gave 62% inhibition only after 3 days of treatment (Figure 1B). We can, therefore, say that, when retinoids were tested as single agents, ATRA showed a better anti-proliferative efficacy in respect to Ro 41-5253 on the ER+ MCF-7 cells, while both retinoids were almost ineffective on the ER- MDA MB 231. When HDACIs were tested as single agents, TSA appeared more efficacious than PB on both cell lines. Next, we verified the possibility of reaching an appreciable efficacy by combining ATRA with the lowest dose of TSA and/or PB. At the same time, we evaluated the possibility that the combinations could have earlier effects with respect to the same drugs administered as single agents. Similar tests were performed using Ro 41-5253 in association with the two HDACIs. In fact, this synthetic retinoid has been proven to have a lower toxicity with respect to the natural molecule, thus being an interesting candidate for clinical application (20,21). When MCF-7 were treated with the various combinations of retinoids and HDACIs at the lowest concentration, we observed an improvement of the efficacy in respect to the drugs used alone. In fact, all the co-treatments provoked a marked growth reduction at a very early stage: as early as the 4th day, the cell growth was reduced by 54% (for ATRA + HDACIs) and 60% (for Ro 41-5253 + HDACIs) (Figures 1C, 1E). When the same associations were tested on MDA MB 231, they appeared able to overcome the retinoid-resistance. Within five days, Ro 41-5253 10^{-5} M + TSA 10^{-8} M showed a growth inhibition of 47% and ATRA 10^{-5} M + PB 1mM gave an inhibition of 42%. Neither of the combinations reached 50% growth inhibition, but they were more efficacious than the drugs administered as single agents. Moreover, they

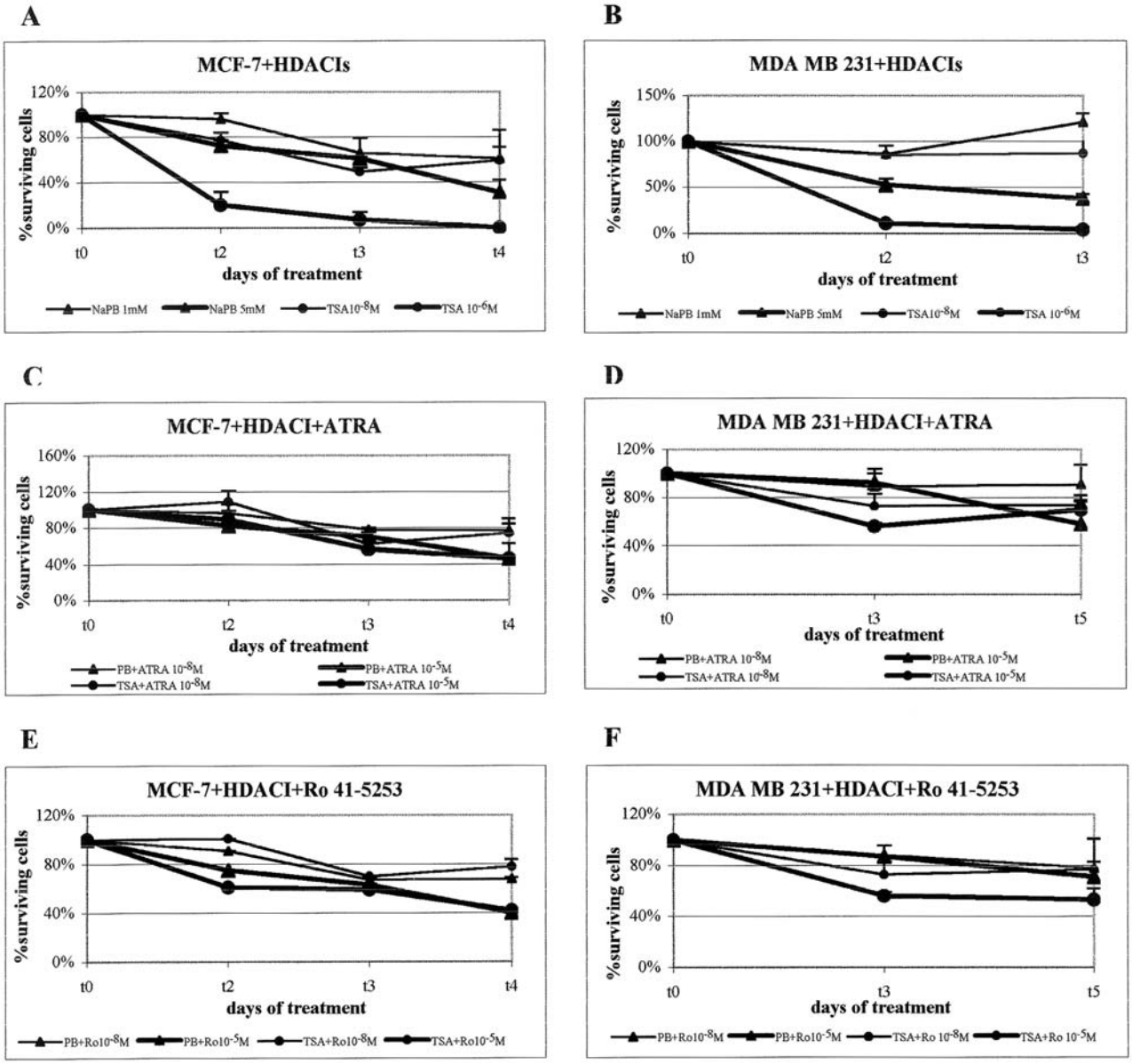

Figure 1. *Antiproliferative effects of PB and TSA, either alone, or in combination with ATRA or Ro 41-5253 on the two breast cancer cell lines. (A, B) Inhibition of MCF-7 (A) and MDA MB 231 (B) cell growth by HDACIs. Cells (10000 cells/well) were treated with 1mM (–▲–) and 5 mM (–▲–) PB and 10^{-8} M (–●–) and 10^{-6} M TSA (–●–). The intermediate dose has not been shown in the figure in order to mantain its clarity. (C-F) Inhibitory effect of the different combinations. MCF-7 (C, E) and MDA MB 231 (D, F) were treated with PB 1mM + ATRA/ Ro 41-5253 10^{-8} M (–▲–); PB 1 mM + ATRA/ Ro 41-5253 10^{-5} M (–▲–); TSA 10^{-8} M + ATRA/ Ro 41-5253 10^{-8} M (–●–); TSA 10^{-8} M +ATRA/ Ro 41-5253 10^{-5} M (–●–). The percentage of viable cells was calculated in comparison to untreated cells. The experiments were repeated three times each consisting of duplicates. Results are expressed as mean ± SD.*

acted earlier than the single treatments, allowing a reduction in the toxic effects of the prolonged treatments (Figures 1D, 1F).

Cytofluorimetric analysis. In order to better clarify the mechanisms through which the retinoids exerted their cytotoxicity, we quantified, for each treatment, the fraction of sub-G0/G1 cells as an indirect estimation of the apoptotic index (22). The exposure of MCF-7 cells to TSA 10^{-8} M for 3 days produced 9% of sub-G0/G1, which increased to 24% when it was combined with ATRA 10^{-5}M. Normalising the 24% of sub-G0/G1 to the 44% total growth inhibition recorded by the growth curves, we deduced that half of the inhibition (54%), induced by the combination TSA 10^{-8} M + ATRA 10^{-5} M, is probably due to an apoptotic mechanism. When ATRA 10^{-5} M was combined with PB 1mM, an

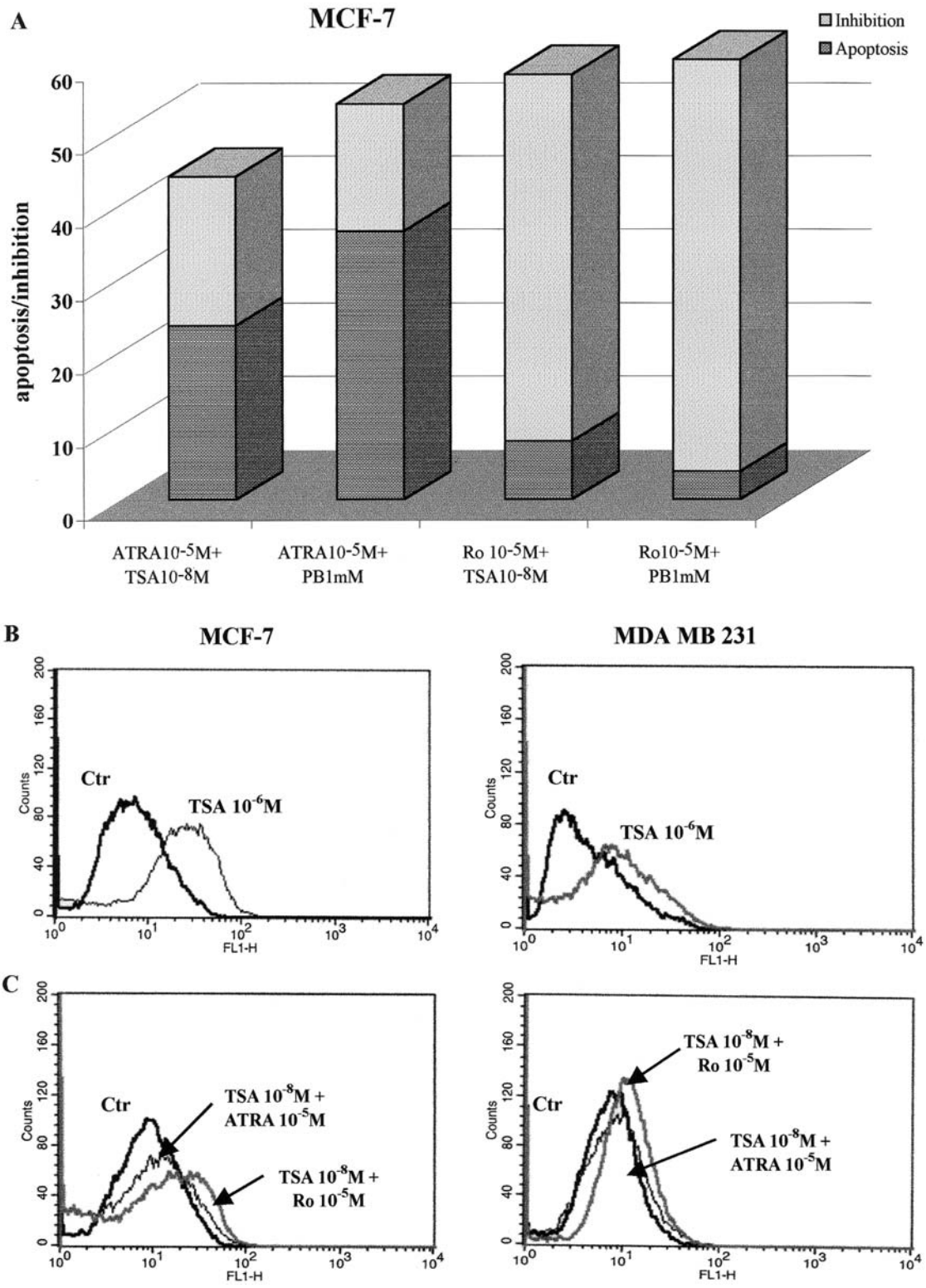

Figure 2. *(A) Histogram representation of the fraction of sub-G0/G1 population as an indirect estimation of apoptosis in the MCF-7 cell line. The percentage of apoptotic cells is expressed with respect to the percentage of growth inhibition. (B, C) Immunofluorescence labelling of the H4 acetylated histone as an evaluation of the average acetylation of nuclear chromatin in MCF-7 and MDA MB 231 cell lines. (B) MCF-7 and MDA MB 231 treated with TSA 10^{-6} M; (C) MCF-7 and MDA MB 231 treated with TSA 10^{-8} M + ATRA 10^{-5} M and TSA 10^{-8} M + Ro 41-5253 10^{-5} M. The X-axis (FL1-H) represents the Histone H4 acetylation levels, and the Y-axis represents the number of events (counts).*

increase of the sub-G0/G1 population (37%) appeared, which corresponded to 68% of the growth inhibition (Figure 2A). Neither the combination Ro 41-5253 10^{-5} M + TSA 10^{-8} M nor Ro 41-5253 10^{-5} M + PB 1 mM showed evidence of a percentage of sub-G0/G1 greater than 15% (Figure 2A). Similar analyses on MDA MB 231 cells did not reveal a relevant apoptotic population, confirming their typical drug resistance. On this cell line, exclusively the exposure to the highest dose of TSA (10^{-6} M) induced a significant proportion of cells in sub-G0/G1 (more than 20%). Neither combinations which showed good efficacy on cell growth (Ro 41-5253 10^{-5} M + TSA 10^{-8} M and ATRA 10^{-5} M + PB 1 mM), produced an appreciable proportion of sub-G0/G1 cells. The highest sub-G0/G1 fraction was registered for the combination ATRA 10^{-5} M + TSA 10^{-8} M: after three days of treatment, the apoptosis was barely 7%, representing only 15% of the growth inhibition (data not shown).

Histone acetylation. When we evaluated the acetylation status of MCF-7 and MDA MB 231 chromatin by immuno-fluorescence labelling, we observed an average chromatin hypoacetylation in the untreated cells. This status changed after the exposure of both cell lines to TSA 10^{-6} M: we observed, in fact, a marked shift of the fluorescence histogram towards higher intensity values (Figure 2B). In Figure 2C can be seen the fluorescence histograms for those combinations of HDACIs and retinoids that supplied a good efficacy on growth inhibition, both on ER+ and ER- cells. The shift of the fluorescence histogram towards higher values demonstrates a change of the acetylation status in nuclear chromatin, occurring as a consequence of these treatments.

Discussion

Several clinical trials have demonstrated the effectiveness of natural retinoids, such as ATRA, in the treatment of acute promyelocitic leukemia, whilst for solid tumors the clinical use of retinoids is controversial. The best results with these molecules were obtained in the treatment of oral pre-malignant lesions and in the chemo-prevention of head and neck cancer (2, 23-26). Since intensive treatment with retinoids is toxic for the host, researchers have been working to identify synthetic analogues of retinoids with good chemo-preventive potential, but with lower toxicity. One of these compounds is the selective RAR-α antagonist, Ro 41-5253, which does not appear to transactivate RA-responsive genes, responsible for the classic pattern of retinoid toxicity seen with other retinoids (27). Another problem encountered in retinoid therapy is that of a prolonged treatment arousing retinoid resistance. Numerous studies have suggested that this resistance could be correlated to epigenetic changes of chromatin, such as acetylation and methylation of control and/or coding regions of the DNA (14). This is the reason why HDACIs are applied as chemotherapeutic agents for several classes of tumors. The hyperacetylation of the histones, in fact, induces a more relaxed chromatin structure, which results in more accessibility to transcription factors, such as the activated retinoid-receptors.

In this study, we tested the possibility of reaching an appreciable efficacy by combining ATRA, or Ro 41-5253, with low concentrations of TSA or PB for short treatments. Our data suggest that retinoids and HDACIs act in synergy on two different breast cancer cell lines with a very short exposure to drugs, and this result may have potential clinical applications for the treatment of breast cancer. When retinoids are administered to MDA MB 231, in combination with the two HDACIs, the retinoid resistance of this ER- cell line is overcome. The efficacy improvement is also evident when the combination is compared with HDACIs as single agents: with respect to TSA 10^{-8} M alone, its association with ATRA or Ro 41-5253 10^{-5} M produces a higher growth inhibition (from 13% to 27%) after five days of treatment. The cytofluorimetric analysis indicates that this growth reduction is only partially due to an apoptotic mechanism. With regards to MCF-7 cells, which are responsive to both retinoids, HDACIs act synergistically with retinoids in repressing cell proliferation. The anti-proliferative effects are already evident after 4 days of exposure to the treatment: 60% inhibition was observed for the combination of Ro 41-5253 10^{-5} M with PB 1 mM, while just 42% was recorded for the retinoid as a single agent. The cytofluorimetric analysis revealed that the growth inhibition induced on MCF-7, by the co-treatments HDACIs plus ATRA, was mostly due to a pro-apoptotic mechanism. For the combinations of Ro 41-5253 plus HDACIs, the observed cytotoxicity could not be explained by cytofluorimetric analysis, since no arrest of the cell cycle was evident. Further studies are required in order to identify the possible mechanisms involved in the proliferation inhibition exerted by these combinations. Since the current research on anti-tumoral therapy was aimed at the entire tumor population, comprising hormone-responsive and hormone-resistant cells, we consider the results of our *in vitro* study to be very promising. In fact, the possibility of blocking the growth of both ER+ and ER- breast cancer cells, using a single specific combination of retinoids plus HDACIs, could represent a marked improvement in breast tumor therapy. Therefore, new clinical protocols including HDACIs and retinoids in breast cancer patients should be justifiable, particularly considering recent clinical study on the use of HDACIs in solid tumors (18).

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