Int. J. Cancer: **104,** 579–586 (2003)

Publication of the International Union Against Cancer

SUSCEPTIBILITY OF MULTIDRUG RESISTANCE TUMOR CELLS TO APOPTOSIS INDUCTION BY HISTONE DEACETYLASE INHIBITORS

Maria D. CASTRO-GALACHE[1], Jose A. FERRAGUT[1], Victor M. BARBERA[2], Elena MARTÍN-OROZCO[1], Jose M. GONZALEZ-ROS[1], Pilar GARCIA-MORALES[2] and Miguel SACEDA[2]*

[1]*Centro de Biologia Molecular y Celular, Universidad Miguel Hernandez, Elche (Alicante), Spain*
[2]*Hospital Universitario Elche, Elche (Alicante), Spain*

The main goal of our study has been to analyze the efficiency of new anticancer drugs, specifically histone deacetylase inhibitors, in tumor cells bearing a multidrug resistance phenotype. We report that the histone deacetylase inhibitors, Trichostatin A and Suberoylanilide Hydroxamic Acid (SAHA), dramatically reduce cell viability and promote apoptosis in different drug-resistant cells, affecting in a much lesser extent to their parental drug-sensitive counterparts. The differential effects induced by Trichostatin A and SAHA between drug-sensitive and drug-resistant cells are reflected on the main characteristics of the resistant phenotype. Thus, reverse transcription-PCR and Western immunoblots confirm that both histone deacetylase inhibitors promote endogenous down-regulation of P-glycoprotein, which is overexpressed in the drug-resistant cells. Transfection of drug-sensitive cells with the P-glycoprotein cDNA ruled out the *a priori* possible association between apoptosis and down-regulation of P-glycoprotein induced by the histone deacetylase inhibitors. The results suggest a therapeutic potential of histone deacetylase inhibitors in the treatment of cancers with acquired resistance.

Key words: *P-glycoprotein; apoptosis; histone-deacetylase inhibitors*

The acquisition of the multidrug resistant (MDR)1 phenotype by tumor cells represents an undesirable process of clinical relevance that is associated with poor prognosis and a major impediment for the success of cancer chemotherapy.[1] MDR cells are characterized by cross reactivity against different families of antineoplastics including anthracyclines, *Vinca* alkaloids and epipodophilotoxins,[2] which accumulate intracellularly to a much lesser extent in MDR cells than in drug-sensitive tumor cells.[1,3,4] The most consistent molecular alteration associated to MDR is the overexpression of the transmembrane protein Pgp,[3,5] a finding that has been extended to other proteins such as the multidrug resistant associated protein (MRP), the lung resistance protein (LRP) and the breast cancer resistance protein (BCRP).[1] The genes encoding for Pgp (the mdr gen family) are represented by 2 closely related genes, *MDR1* and *MDR2* (or *MDR3*) in humans and *mdr1a*, *mdr1b* and *mdr2* in mice. Out of these genes, only the human *MDR1* gene and the mouse *mdr1a* and *mdr1b* genes have been linked to the multidrug-resistance phenomenon.[4] Gros *et al*. have demonstrated that transfection of drug-sensitive cells with the cDNA clone coding for Pgp is sufficient to confer the MDR phenotype.[6]

Pgp is a large (170 kDa) molecule that belongs to the ATP binding casette (ABC) transporter superfamily and that promotes export of toxic xenobiotics or prevents their absorption in normal tissues.[3] The possible physiological roles for Pgp have been recently summarized.[7] Overexpression of Pgp in human tumor cells seems to be related with prior chemotherapy,[8] while in cancer cell cultures, the protein overexpresses upon growing the cells in the continuous presence of sublethal concentrations of selected antitumor drugs. Cells grown in culture display patterns of resistance similar to those seen *in vivo* and when they acquire the drug-resistant phenotype, Pgp accounts for the active drug efflux of several chemically unrelated antitumor drugs, contributing thus to efficiently lowering the intracellular accumulation of such drugs.[3,9]

Several lines of evidence suggest a major transcriptional control of Pgp. Thus, differentiating agents,[10] UV radiation[11] or the pro-apoptotic tumor supressor gene *p53*[4,12,13] have been shown to regulate the *MDR1* expression in different cell lines. More recently, it has been demonstrated that regulation of the chromatin structure through modifications of histones by acetylation/deacetylation represents a very important control of gene transcription.[14–16] In fact, histone hyperacetylation by specific iHDACs may critically affect processes such as apoptosis,[17,18] cell proliferation[19] and differentiation.[20] Particular molecular targets affected by acetylation/deacetylation include the tumor supressor gene *p53*, which activity regulating cell growth and apoptosis seems to be dependent on deacetylation[21] and the *MDR1* gene, which expression appears to be modulated by acetylase and deacetylase activities in a human colon carcinoma cell line.[22]

In the present study, we have analyzed the alterations inflicted by 2 histone deacetylase inhibitors, TSA and SAHA, in different drug-resistant tumor cell lines. The study is aimed to gain new insights on cellular events (histone acetylation/deacetylation), which in affecting MDR, constitute the basis for identification of anticancer drugs able to circumvent the undesirable multidrug resistant phenotype.

MATERIAL AND METHODS

Cell lines and cultures

DNM-sensitive murine leukemia L1210 and P388 cells as well as stable resistant L1210R and P388R sublines (~160-fold and ~100-fold, respectively, resistant to DNM determined by *in vitro* proliferation assays) were obtained by stepwise selection with increasing DNM concentrations and maintained in culture as previously described.[23] Breast carcinoma MCF-7 cells and a adriamycin-resistant MCF-7/Adr cell subline were obtained from the Lombardi Cancer Center (Georgetown University, Washington). RWP1 is a human pancreas carcinoma cell line that expresses Pgp. HL-60 and HL-60/R correspond to wild-type and DNM-resistant, respectively, human promyeolocytic leukemia cell lines. HT29 and HT29/M6 correspond to parental and methotrexate-resistant human colon carcinoma cells, respectively. HT29 cells do not overexpress Pgp.[24]

Abbreviations: CMV, cytomegalovirus; CHX, cycloheximide; DNM, daunomycin; iHDACs, histone deacetylase inhibitors; MDR, multidrug resistance; Pgp, P-glycoprotein; SAHA, Suberoylanilide Hydroxamic Acid; TSA, Trichostatin A; VRP, verapamil.

Grant sponsor: CICYT-FEDER; Grant number: 1FD97-0291-C02-02; Grant sponsor: FISS; Grant numbers: 01/0038-01, 01/0168.

*Correspondence to: Centro de Biologia Molecular y Celular, Ed. Torregaitan, Universidad Miguel Hernandez, 03202 Elche (Alicante), Spain. Fax: +34-96-6658758. E-mail: msaceda@umh.es.

Received 30 July 2002; Revised 29 October 2002; Accepted 24 November 2002

DOI 10.1002/ijc.10998

Measurements of cell growth and viability

L1210 and L1210R cells were plated at 3×10^5 cells/ml, treated in triplicates at specified doses of TSA or SAHA and grown at 37°C in a CO_2 atmosphere. Cells were harvested and counted to determine the cell growth after 24 hr of incubation in the absence (control) or in the presence of TSA or SAHA. Cell viability was determined by the Trypan-blue method.

Measurement of cell cycle arrest and apoptosis

L1210 and L1210R cells incubated with TSA or SAHA at selected times were centrifuged at 500*g* for 8 min, the supernatant was discarded and fresh medium without those inhibitors was added to the pellet of cells. Then, cells were cultured again for an additional time up to 24 hr. After harvesting the cells, 1×10^6 cells were centrifuged as above and washed with cold 10 mM phosphate buffer, pH 7.4, supplemented with 2.7 mM KCl and 137 mM NaCl (PBS) and centrifuged again. The pelleted cells were resuspended in 75% cold ethanol, fixed for 1 hr at −20°C, centrifuged and resuspended in 0.5 ml of PBS supplemented with 0.5% Triton X-100 and 0.05 % RNase A. Then, cells were incubated for 30 min at room temperature, stained with propidium iodide and the distribution of cellular DNA content was analyzed by flow cytometry in an Epics XL instrument (Beckman Coulter Co. Miami, Florida). Non-viable cells were excluded from the analysis on the basis of their abnormal size. TSA-induced apoptosis was also determined by using a caspase-3 colorimetric protease assay kit (MBL Co., Ltd., Japan) following the manufacturer′s instructions. Finally, fluorescent apoptotic cells were observed by staining with an Annexin V-FITC commercial kit (Calbiochem, San Jose, CA) under a fluorescent light microscope. CD95 expression was determined by flow cytometry using specific monoclonal antibodies according to the manufacturer′s instructions (Calbiochem). Caspase-8 activity was measured by using a colorimetric assay and a cell permeable caspase-8 inhibitor was used as control (caspase 8 inhibitor II from Calbiochem).

Drug accumulation studies

Steady-state intracellular accumulation assays of the fluorescent DNM in the absence or in the presence of VRP was determined as previously described.[25]

Pgp determination

Pgp expression by Western immunoblots was carried out using the monoclonal antibody (MAb) C-219 (Centocor, Inc.) as described previously[23,26] followed by enhanced chemiluminiscence (ECL) (Amersham International, Buckinghamshire, UK) to develop protein bands. Protein determinations were carried out by the Bradford method (Bio-Rad, Richmond, CA).

To determine the levels of Pgp mRNA, total RNA from non-treated or TSA-treated L1210 and L1210R cells was isolated by using the TRI reagent (Sigma-Aldrich, Co., St. Louis, MO). To eliminate potential DNA contamination, total RNA was treated with RQ1 DNase (Promega Corp., Madison, WI) for 30 min at 37°C, followed by 2 min at 94°C. The samples were then analyzed for Pgp and β;-actin mRNAs using the one-step 7 RT-PCR Kit (Promega Corp.). Then, 1–2 μg of total RNA were analyzed according to the manufacturer′s instructions. Briefly, 45 min at 48°C for reverse transcription, a 2 min cycle at 94°C followed by 35 cycles of 40 sec at 94°C, 40 sec at 59°C, 1 min at 68°C and a final 7 min cycle at 68°C. PCR products were resolved by electrophoresis on 2% (w/v) agarose gels. Bands were visualized with ethidium bromide and then quantified by optical densitometry. The primers used for Pgp RT-PCR were 5′-CTCATTGTATTGGGCGG-3′ and 5′-CAAAGACAACAGCAGAAAATAC-3′, which yield a product of 353 bp. The primers used for β-actin RT-PCR were obtained from Promega Corp.

Transfection experiments

A solution containing 1 μg of DNA dissolved in TE, pH 7.4, was diluted with cell growth medium devoided of serum, protein and antibiotics up to 60 μl. Four microliters of superfect reagent (Qiagen GmbH, Germany) were added to the DNA solution and the mixture was incubated for 10 min at room temperature to allow complex (DNA/Superfect) formation. Then, 100 μl of cell growth medium (containing serum and antibiotics) were added. The total 164 μl of the mixture was added to each of the wells containing cells that had been plated at 5×10^5 cells/well. Cells were incubated for 2 hr at 37°C, washed with PBS and incubated again with

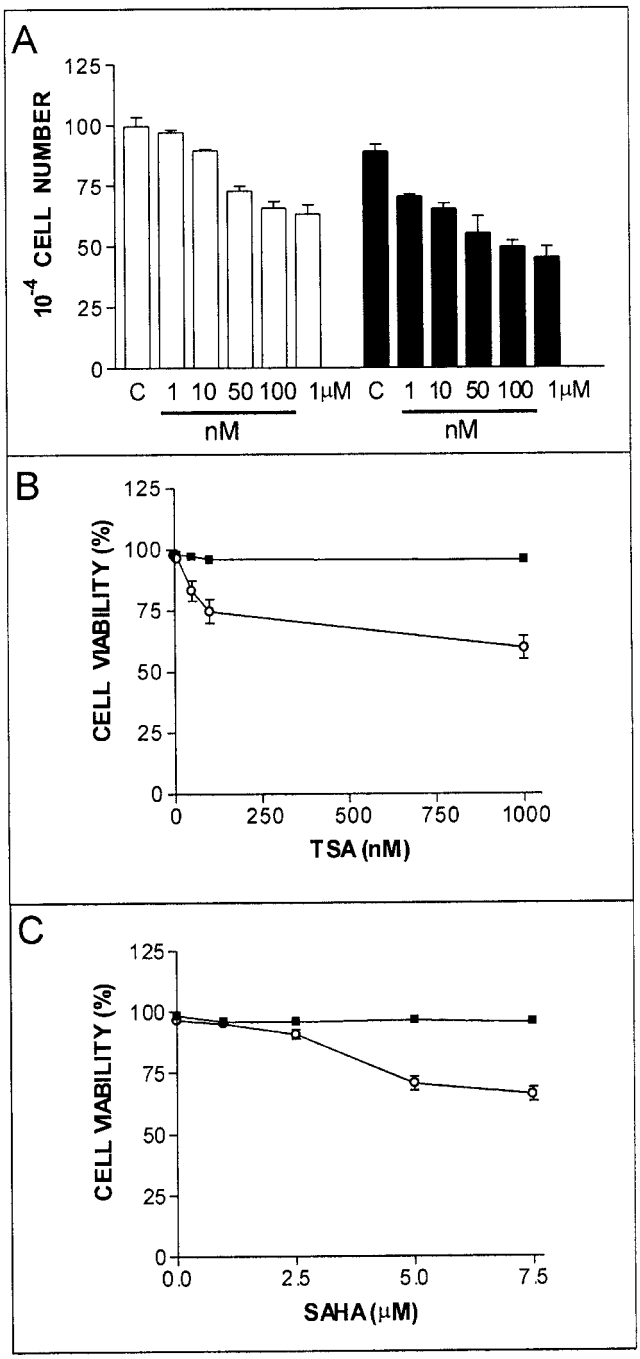

FIGURE 1 – TSA-effect on the growth of L1210 (white bars) and L1210R cells (black bars) (*a*) The cells were incubated for 24 hr with TSA at the different concentrations indicated. Cell viability of L1210 (closed square) and L1210R cells (open circle) after 24 hr of incubation with TSA (*b*) or SAHA (*c*) at indicated concentrations. Data plotted mean ± SEM ($n \geq 5$).

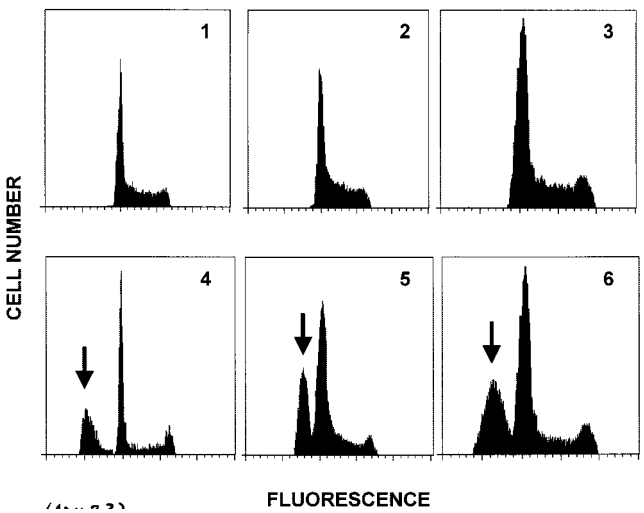

FIGURE 2 – Distribution of cellular DNA content obtained by flow cytometry in different drug-resistant cell lines. (1), (2) and (3) correspond to non-treated (controls) L1210R, P388R and HL-60R cells; (4), (5) Iand (6) correspond to L1210R, P388R and HL-60R cells, respectively, treated with 1 μM TSA for 24 hr. Apoptotic cells corresponding to the sub-G_1 peak are indicated by an arrow.

fresh medium with G-418 (range: 0.4–4 mg/ml) to initiate stable selection of gene expressing cells.

Plasmids

CBMC-6 (stands for Centro de Biologia Molecular y Celular) cells were obtained by transfecting L1210 cells with the plasmid pcDNA 3-mpgp that contains the mouse *mdr1a* Pgp cDNA under the control of the CMV promoter.

RESULTS

The effects of 2 iHDACs, TSA (ranging from 1 nM to 1μM) and SAHA (1–7.5 μM) on different parental and multidrug resistant cell lines were assessed in terms of cell growth and viability. To simplify, we describe the results obtained in the murine leukemia L1210 cells though most of them have also been probed, as specified, in other cell systems. Figure 1*a* shows that TSA treatment inhibits cell growth in a dose-dependent manner in both DNM-sensitive L1210 and DNM-resistant L1210R cells. In addition, increasing concentrations of TSA or SAHA progressively decrease the viability of the L1210R cells with no effect on the L1210 cells (Fig. 1*b* and 1*c*, respectively). This effect was also time-dependent, since at the maximum concentration of TSA assayed (1 μM), cell viability was 60–70% after 24 hr of treatment and decreases up to 5–10% after 48 hr in the L1210R cells but remained practically unaffected (viability ≥ 90%) in the L1210 cells. As shown in Figure 2, the decrease in the viability of the L1210R cells upon TSA treatment for 24 hr seemingly parallels the amount of apoptotic cells (30–40%) induced by TSA (hipohaploid, sub-G_1 peak). Apoptosis in the L1210R cells is confirmed by an approximately 10-fold increase in caspase-3 activity (7.64 ±1,64 and 12.82 ±0.94 after 3 hr and 24 of incubation with TSA, respectively). Figure 2 also shows the TSA-induced apoptosis in other drug-resistant cell lines when compared to nontreated controls. Figure 3 illustrates the apoptosis inflicted by TSA and SAHA in different drug-resistant cell lines including the MCF-7/Adr, RWP1 and L1210R cells detected by staining with Annexin V-FITC. In the case of MCF-7 cells, the chemoresistant MCF-7/Adr cells exhibited TSA-induced apoptosis at very low dosis (10 nM) and displayed massive apoptosis at 100 nM in less than 24 hr of treatment. Meanwhile, the parental MCF-7 cells needed considerable higher concentrations (100nM–1 μM) and longer periods of

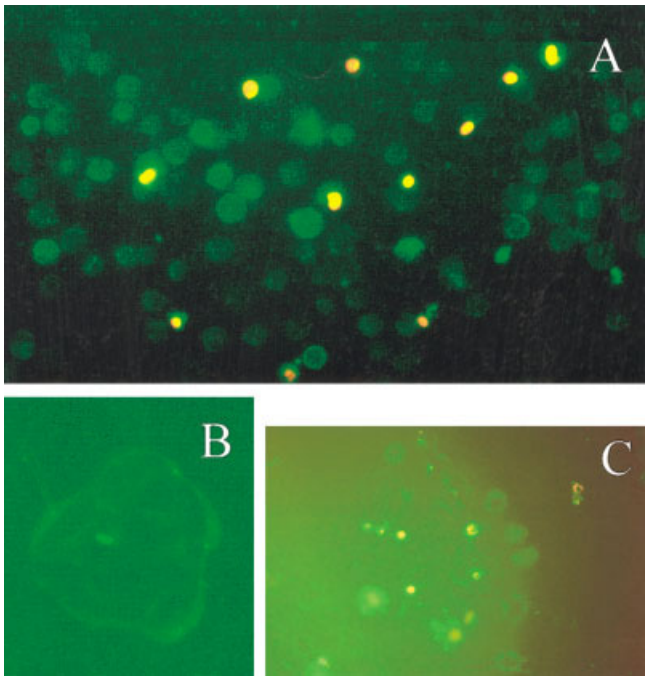

FIGURE 3 – Apoptotic cells stained with Annexin V-FITC after treatment with 1 μM TSA for 24 hr. L1210R cells (*a*). RWP-1 cells (*b*). MCF-7/Adr cells (*c*). Note some non-viable cells stained (yellow) with propidium iodide.

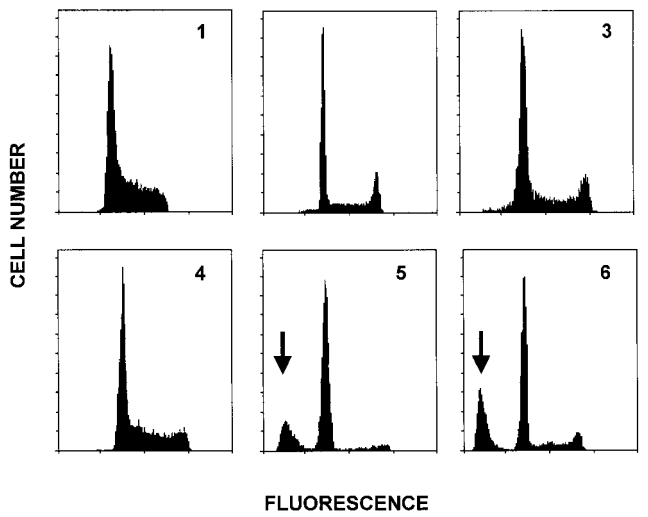

FIGURE 4 – Distribution of cellular DNA in the L1210 (1, 2 and 3) and L1210R cells (4, 5 and 6) induced by treatment for 3 hr (2 and 5) or 24 hr (3 and 6) with 1 μM TSA. Control of nontreated L1210 (1) and L1210R (4) cells, respectively.

incubation (1–3 days) with TSA to achieve measurable apoptosis. TSA induced also apoptosis in the methotrexate-resistant human colon carcinoma HT29/M6 cell subline. In contrast to the observation in the L1210 or P388 cells, the parental HT29 cells were similarly affected by incubation for 24 hr with 1 μM TSA than HT29/M6 (20 ± 6.5% and 18.2 ± 4.7% of apoptotic cells, respectively).

The inhibition of cell growth and the different cell viability promoted by TSA or SAHA between drug-sensitive cells and their drug-resistant counterparts prompted us to analyze the cell cycle distribution after exposure of the cells to the iHDACs. Thus, the

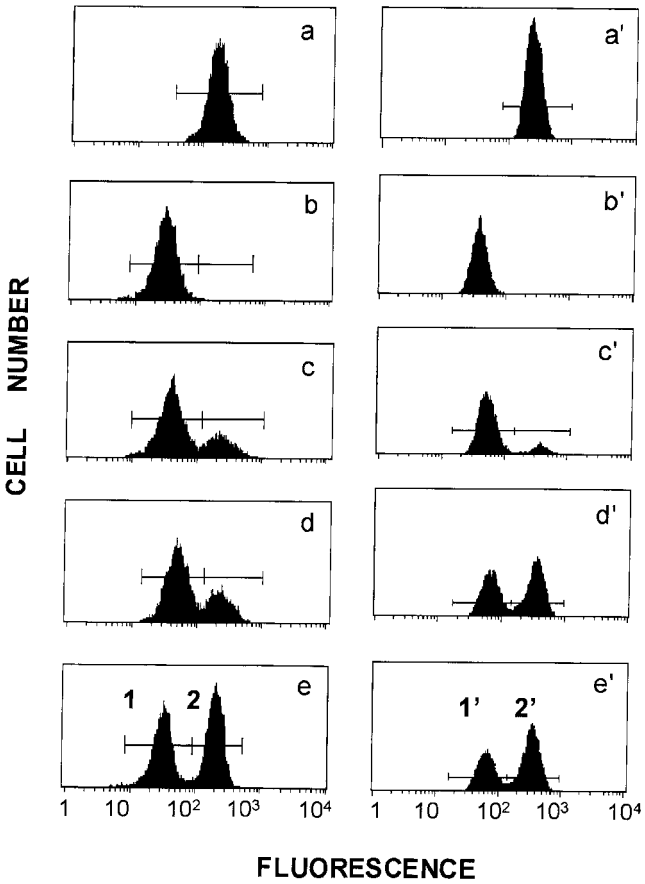

FIGURE 5 – Steady-state intracellular accumulation of DNM in L1210 and L1210R cells incubated with TSA or SAHA for 24 hr analyzed by flow cytometry. Untreated L1210 and L1210R cells (*a*,*b*); L1210R cells treated with 10 nM, 100 nM or 1 μM TSA (*c*,*d* or *e*), respectively. Untreated L1210 and L1210R cells (a′ and b′); L1210R cells treated with 2, 5 or 7.5 μM SAHA (c′, d′ or e′), respectively.

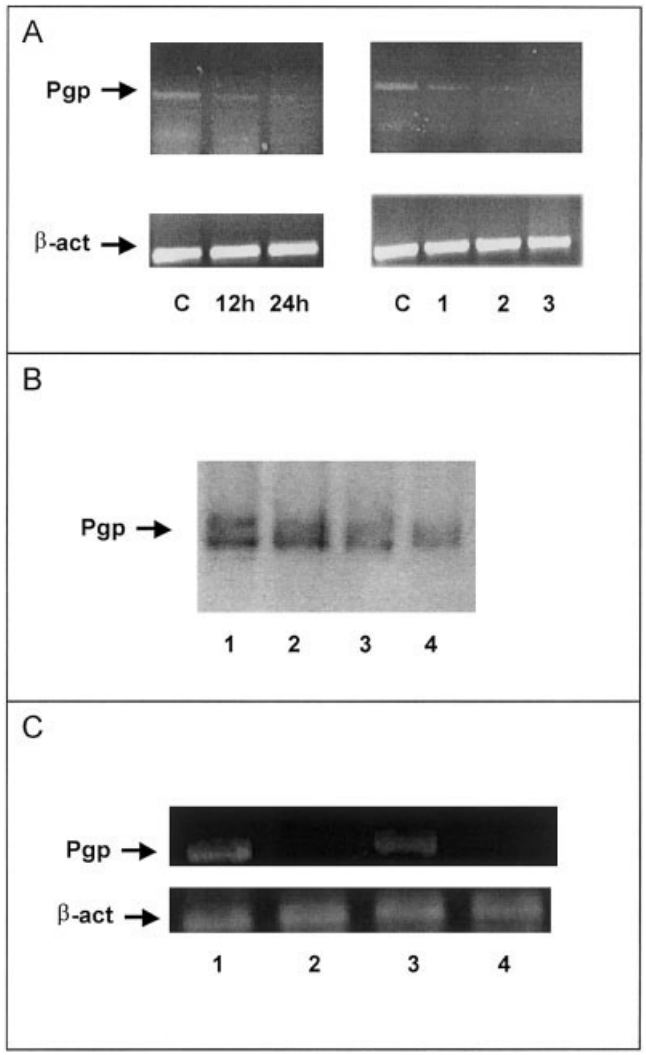

FIGURE 6 – Time-dependent (left) and concentration-dependent (right) reduction of Pgp mRNA levels in L1210R cells treated with TSA analyzed by RT-PCR (*a*). mRNA bands were resolved on 2 % agarose gel and visualized by ethidium bromide. Left panel: untreated cells (control) and 1 μM TSA-treated cells for 12 or 24 hr, respectively. Right panel: untreated cells (control); lanes 1, 2 and 3 correspond to L1210R cells incubated with 10 nM, 100 nM and 1 μM TSA, respectively. As control for loading and RNA quality, parallel RNA samples were analyzed with β-actin primers. Detection of Pgp by Western blot (*b*) of untreated L1210R cells (lane 1) or cells treated with 100 nM (lane 2), 500 nM (lane 3) or 1 μM (lane 4) TSA for 24 hr using the anti-Pgp MAb C219 in crude membrane extracts containing 30 μg of protein. Pgp mRNA levels (*c*) corresponding to peaks 1, 2, 1′ and 2′ (Fig. 5) isolated by cell sorting flow cytometry after treatment of L1210R cells with 1 μM TSA or 7,5 μM SAHA for 24 hr.

L1210R cells treated with TSA or SAHA undergo an initial arrest in the G_1 phase (panel 5, Fig. 4), which population decreased concomitant to the raise in apoptosis after 24 hr of incubation (panel 6, Fig. 4). On the other hand, the L1210 cells do not undergo apoptosis and tend to accumulate in the G_2-M phase (panels 2 and 3, Fig. 4). These observations indicate that apoptosis in the drug-resistant cells occurs at the G_1-S phase transition.

Since drug-resistant cells appear to be more susceptible than drug-sensitive cells to iHDACs-induced apoptosis, we decided to study if other characteristics of the MDR phenotype had also been affected. It is well established that MDR cells display decreased intracellular accumulation of anticancer drugs with respect to their drug-sensitive counterparts.[3] Because Pgp is overexpressed in the L1210R cells[23] and represents a very efficient mechanism responsible for lowering chemotherapeutic drug in the MDR cells, we analyzed the effect of TSA and SAHA on the intracellular accumulation of the fluorescent anthracycline DNM along with its reversion by VRP, as a functional assay for Pgp activity.[3] Flow cytometry experiments demonstrated that treatment with both iHDACs did not affect the accumulation of DNM in the drug-sensitive cells (the histograms of DNM accumulation in these cells after incubation with the inhibitors overlap with those corresponding to untreated L1210 cells (Fig. 5*a* or 5*a*′), but originated the division of the drug-resistant cells into 2 subpopulations distinguished by differential accumulation of DNM. The new second subpopulation, gradually appears in a dose- and time-dependent manner as the TSA or SAHA concentration increases (peak 2 or 2′, respectively, in Fig. 5) and represents more than 50% of the cells at the maximum concentration used. This cell subpopulation is characterized by a level of DNM accumulation indistinguishable from that of parental L1210 cells (Fig. 5*a* or 5*a*′) and remains unaffected after preincubation with VRP. The previous results suggest that the new emerging cell subpopulation has undergone either loss of Pgp expression or inhibition of its drug efflux pump activity. The following experiments evidentiated that TSA promoted down-regulation of Pgp expression. Thus, Figure 6*a* shows that TSA considerably reduced the levels of Pgp mRNA in the L1210R cells as determined by RT-PCR. Comparative analysis of Western blotting between untreated and TSA-treated L1210R cells

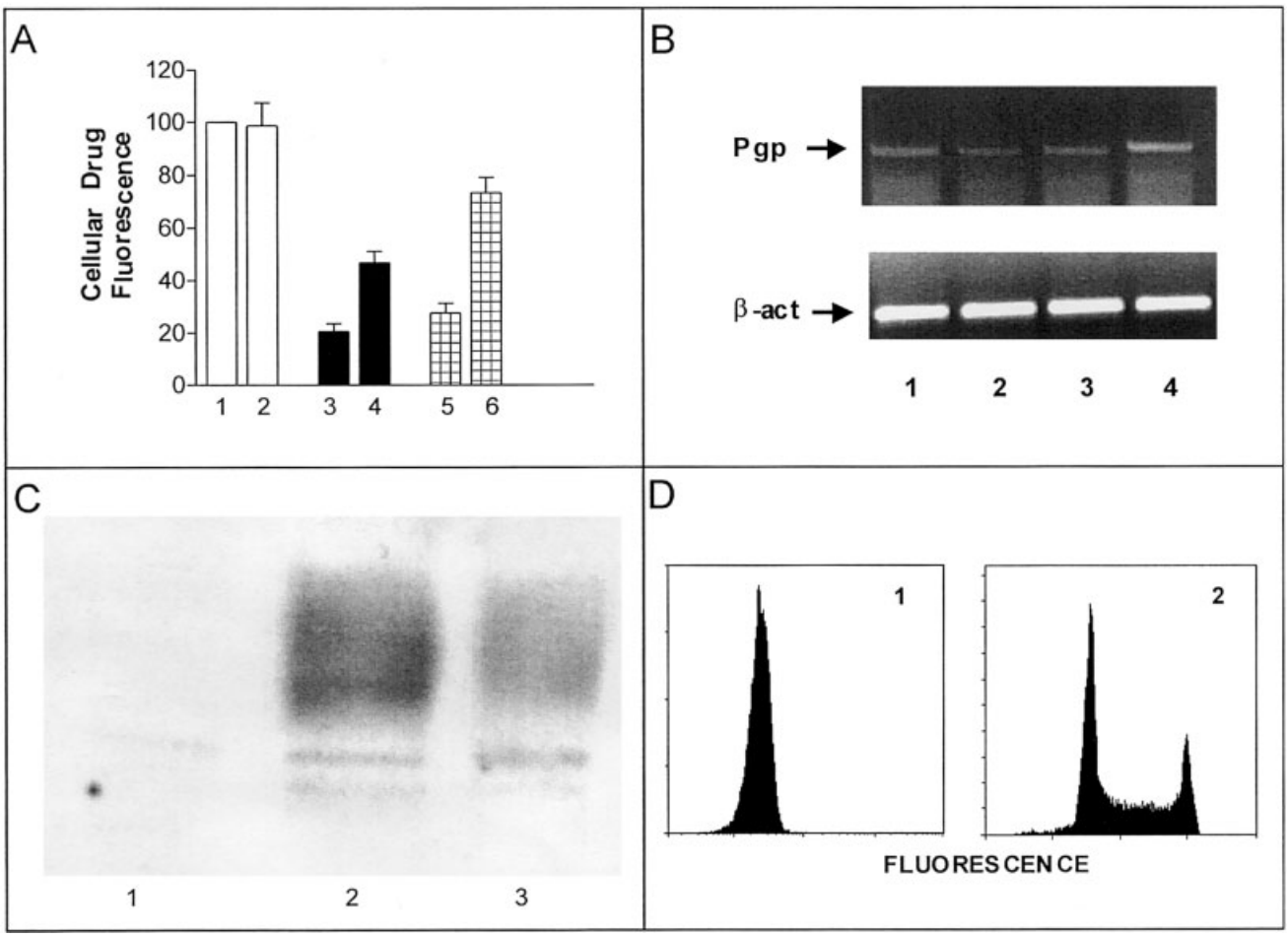

FIGURE 7 – Characterization of L1210 cells transfected with mouse Pgp cDNA (CBMC-6 cells). Relative accumulation of DNM (*a*) estimated by flow cytometry in L1210 (1), L1210R (3) and CBMC-6 (5) cells and its reversion by 5 μM VRP in L1210 (2), L1210R (4) and CBMC-6 (6) cells. Data plotted mean ± SEM (n = 7). RT-PCR analysis of Pgp mRNA (*b*) in the CBMC-6 cells (lane 1). Lanes 2, 3 and 4 correspond to CBMC cells treated with 5 μg/ml CHX for 24 hr, 1 μM TSA for 24 hr and CHX plus TSA, respectively. Detection of Pgp by Western blot (*c*) in the CBMC-6 cells (lane 3). Lanes 1 and 2 correspond to L1210 and L1210R, respectively. DNM accumulation in the CBMC-6 cells (*d*) treated with 1 μM TSA (1). Distribution of cellular DNA content after treatment of CBMC-6 cells with 1 μM TSA for 24 hr (2). Note the absence of apoptotic cells in comparison with panel 4 in Figure 2 or panel 6 in Figure 4.

also revealed a progressive dose-dependent reduction of Pgp expression. As expected, individual RT-PCR analysis of the 2 cell populations (Fig. 5) isolated by cell sorting flow cytometry demonstrated that the loss of Pgp expression was mostly due to the complete absence of the Pgp mRNA in the second cell subpopulation (Fig. 6*c*), characterized by high accumulation of DNM (peaks 2 or 2′, Fig. 5).

In order to further study the specific implication of Pgp on the different effects induced by the iHDACs between drug-sensitive and drug-resistant cells, we stably transfected wild-type L1210 with mouse *mdr1a* Pgp cDNA, originating the CBMC-6 cell subline. The newly generated CBMC-6 cells exhibit a similar pattern of DNM accumulation than the L1210R cells (Fig. 7). This observation indicates functional expression of Pgp in the CBMC-6 cells which was further confirmed by i) the increase of DNM accumulation in the presence of VRP (Fig. 7*a*) and ii) Pgp mRNA levels determined by RT-PCR (Fig. 7*b*) and Western blot (Fig. 7*c*). However, CBMC-6 and L1210R cells behave completely different when treated with TSA since in the former, a) DNM accumulation was not increased, *i.e.*, the appearance of a second cell subpopulation with elevated drug accumulation was not observed (panel 1, Fig. 7*d*), b) apoptosis was not induced (panel 2, Fig. 7*d*) and c) Pgp mRNA levels were not reduced (Fig. 7*b*). The fact that Pgp mRNA levels remain unaffected in the CBMC-6 cells but decrease in the L1210R cells suggests a transcriptional regulation of the endogenous Pgp promoter by TSA that is not operating in the CBMC-6 cells because in these cells, the Pgp mRNA is under control of a heterologous promoter. On the other hand, since the CBMC-6 cells do not enter in apoptosis upon treatment with TSA or SAHA, it seems clear that the apoptosis mediated by these agents is independent on Pgp activity and therefore the sole functional expression of Pgp results insufficiently to explain the differences exerted by TSA or SAHA between drug-resistant and drug-sensitive cells.

As noted above, TSA and SAHA were able to induce apoptosis and also to reduce Pgp expression in the drug-resistant cells. In order to explore whether these effects were mediated by some protein component(s), L1210R cells were incubated with the protein synthesis inhibitor CHX. Because of a 24 hr incubation with CHX induced apoptosis in L1210 and L1210R cells, we performed time-course experiments aimed to determine the minimal time needed for TSA to reproduce its effects. Treatment of L1210R cells with 1 μM TSA for 1 hr, followed by 23 hr of incubation with fresh medium without TSA, was sufficient to induce the expected effects (Fig. 8). Control experiments under these conditions revealed that CHX (5 μg/ml) induced low levels of apoptosis *per se* (Fig. 8*a*). Incubation of L1210R cells in the presence of both TSA

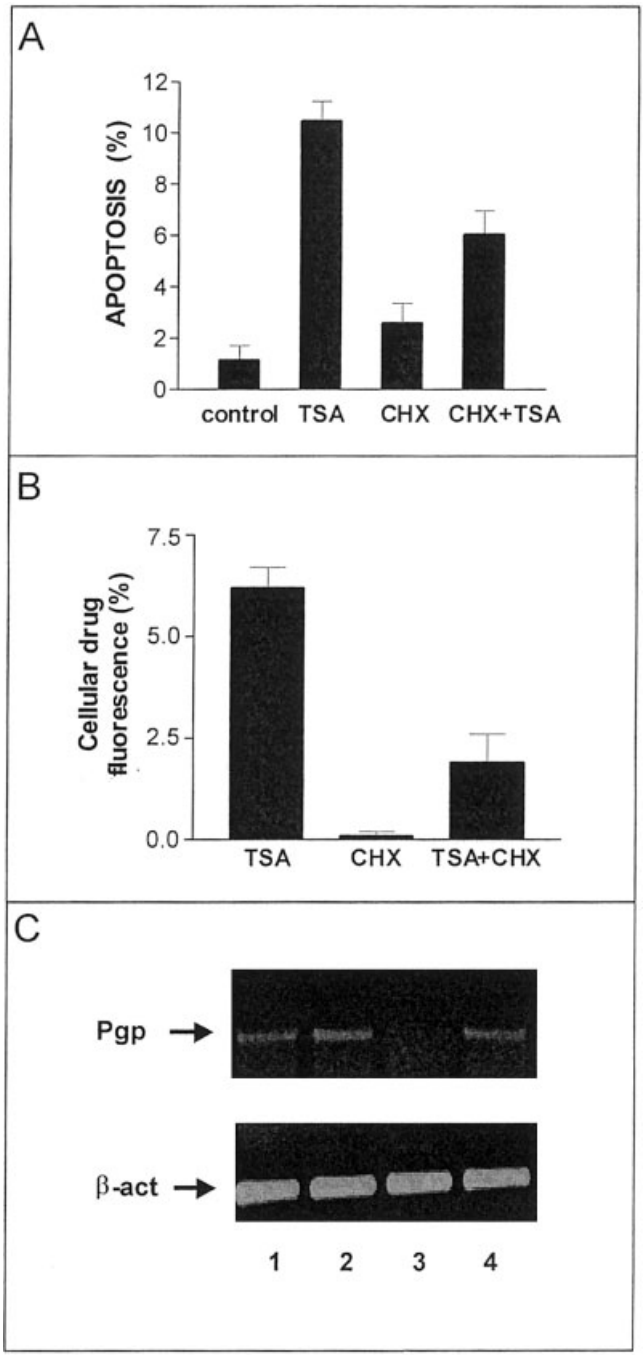

FIGURE 8 – Preventing action of CHX on the effects induced by treatment with TSA in the L1210R cells. Experiments were performed incubating the cells with TSA for 1 hr followed by incubation in fresh medium up to 24 hr. Apoptosis of cells (*a*) treated with 1 μM TSA, 5 μg/ml of CHX or with (TSA + CHX). Control: untreated cells. Apoptosis is determined by the number of cells within the sub-G_1 peak (in the cell cycle analysis by flow cytometry). DNM accumulation (*b*) corresponding to peak 2 in Figure 5*e* upon treatment of L1210R cells with TSA, CHX or both, under the conditions described in (*a*). Pgp mRNA levels (*c*) determined by RT-PCR in untreated cells (1) and in cells treated for 24 hr with CHX (2), TSA (3) and CHX plus TSA (4) as above. As loading and RNA quality control, parallel RNA samples were analyzed with β-actin primers.

and CHX significantly abolished the TSA-induced apoptosis (Fig. 8*a*), the emergence of a new 12-cell subpopulation with high DNM accumulation (Fig. 8*b*) and the decrease in Pgp mRNA levels (Fig. 8*c*). Clearly, these data indicate that TSA induces the expression of a protein effector(s), which is (are) mediating apoptosis and down-regulation of Pgp in drug resistant cells.

DISCUSSION

In the past few years, it has been suggested that histone acetylation is involved in transcriptional regulation.[27,28] The pleiotropic nature of the MDR phenomenon comprises alteration of multiple genetic events,[29,30] thus providing an excellent cellular situation to study activation or repression of endogenous genes, whose transcription involves acetylation/deacetylation of histones. In this regard, a question arises as to what extent the main characteristics of the drug-resistant tumor cells are affected by drugs that, altering the acetylated/deacetylated status of the cells, could efficiently overcome the MDR phenotype.

In the present study, we report that the histone deacetylase inhibitors TSA and SAHA are promoting 2 major effects in the drug-resistant cells: down-regulation of Pgp and induction of apoptosis. With respect to the former, we observed that TSA and SAHA significantly increased the accumulation of DNM in the L1210R cells with no effect on the drug-sensitive counterparts. This finding raised the following question: is it the decrease of Pgp activity or is it the loss of Pgp expression responsible for the increase of DNM accumulation observed in the drug-resistant cells? The former possibility does not seem plausible since Pgp activity was unaffected by TSA in the Pgp-transfected CBMC-6 cells. On the other hand, RT-PCR and Western blot analysis consistently demonstrated that TSA markedly reduced Pgp expression in the L1210R drug-resistant cell lines. In contrast, some studies report no changes in the expression of Pgp in human CEM cells by SAHA[31] or an increase in the steady-state level of *MDR1* mRNA in the human colon carcinoma SW620 cells upon treatment with TSA.[22] Although the basis for this discrepancy is not known, possible explanations are a) the DNA sequences of mouse and human MDR1 promoters are different[3] and therefore they are most likely subjected to differential regulation and b) the regulation of Pgp expression is tissue-specific[32] since epithelial cells such as colon cells constitutively express high levels of Pgp as compared to hematopoietic cells (with the exception of CD34-positive bone marrow stem cells[9] that, as in the case of the L1210R cells, have been selected under pressure to overexpress Pgp (see Experimental Procedures). In this context, while differentiating agents increased the expression of Pgp in colon carcinoma SW620 cells,[22] the opposite effect was found in human pluripotent stem cells.[33]

It is noteworthy that iHDACs did not affect Pgp expression in the CBMC-6 cells, probably because the expression of Pgp in these cells is under the control of the heterologous CMV promoter. This, in turn, suggests that regulation of Pgp expression by these inhibitors in the L1210R cells is due to a transcriptional control of the endogenous Pgp promoter.

The second highlight of this report is concerned the greater susceptibility to apoptosis induced by TSA and SAHA in several drug-resistant cell lines than in their corresponding drug-sensitive counterparts. This aspect was marked in the Pgp-expressing L1210R, P388R (murine leukemia) and MCF-7/ADR (human breast carcinoma) cell lines and less noticeable in the MRP-expressing HL60/R cell line. In a recent article, Ruefli *et al.*[34] have reported similar sensitivity to SAHA-mediated apoptosis in drug-sensitive and drug-resistant cells, a finding also observed in our HT29 cells. It seems reasonable to assume that tumor cells of diverse origins might have dissimilar responses to certain apoptotic stimuli. In addition to having diverse origin, a particular situation can be attained in tumor cells upon acquiring the complex drug-resistant phenotype. Taken together with the at-the-moment reported susceptibility of tumor cells to iHDACs-mediated apo-

ptosis[34] and the data here observed, it is possible to classify the cells into the 3 following categories: first, cell lines in which drug-resistant cells such as L1210/R and P388/R cells are susceptible to iHDACs-mediated 15 apoptosis, while their parental counterparts are not. In the case of P388 cells, Ueda *et al*.[35] found in mice bearing ascitic tumors of P388 cells treated with FR901228, a potent inhibitor of histone deacetylase, that this agent was more effective against mitomycin C, cyclophosphamide, vincristine and 5-fluorouracil resistant P388 leukemias than against non-resistant P388 cells.

A second category can be represented by the human breast carcinoma MCF/7 cell line characterized by a dose-dependent sensitivity to the iHDACs-mediated apoptosis. In these cells, the chemoresistant MCF-7/Adr cells were considerable more susceptible to TSA-induced apoptosis than the parental MCF-7 counterparts.

Finally and in close agreement with the report by Ruefli *et al*.,[34] TSA induced apoptosis in a similar extent in both drug-sensitive and drug-resistant human colon carcinoma HT29 cells. In this scenario, it seems plausible that along with the acquisition and development of drug resistance, different experimental conditions (drug selection, viral transfections, type of drug(s) for selection, degree of resistance, etc.) could decide if the resulting drug-resistant cells retain the original susceptibility of the parental, drug-sensitive cells to apoptotic stimuli. In any case, it must be pointed out that with the reported exception of K562 cells,[34] drug-resistant cells are susceptible to iHDAC-mediated apoptosis and that the situation can be also extended to other drug-resistant non-mdr cells, since methotrexate-resistant HT29 cells, which do not overexpress Pgp,[24] undergo apoptosis when treated with TSA.

In agreement with the reported role for iHDACs as apoptosis inductors in other cell systems,[17,18] we observe that inhibition of cell viability in the drug-resistant cells after incubation with TSA or SAHA was related to apoptosis in these cells. As stated in Figure 4, distribution of cellular DNA content upon treatment with TSA was different in drug-sensitive and drug-resistant L1210 cells and it has been recently reported that the final destination of the cells, *i.e*, apoptosis or cell survival, could be dependent on the phase of the cell cycle to which cells tend to accumulate.[36]

Pgp represents the most consistent molecular alteration of the MDR phenotype.[3,5] Previous studies have reported delay or protection to different apoptotic stimuli by Pgp[37,38] and therefore it was pertinent to evaluate the possible contribution of Pgp to the apoptosis induced by TSA or SAHA in our drug-resistant cell lines. Transfection of the drug-sensitive L1210 cells with the Pgp cDNA rendered the CBMC-6 cells with functional expression of Pgp. However, the CBMC-6 cells did not undergo apoptosis upon treatment with TSA or SAHA, indicating that the apoptosis induced by these agents in the drug-resistant cells was irrelevant to Pgp overexpression. Also, we have observed that low concentrations of TSA or SAHA do not induce apoptosis (Fig. 1*b*) but do decrease Pgp mRNA levels (Fig. 6*a*) in the L1210R cells, supporting separation between both processes.

The 2 major effects inflicted by TSA or SAHA, apoptosis and reduction of Pgp expression were abolished in the presence of the protein synthesis inhibitor cycloheximide, indicating the requirement of protein effectors for both processes to occur. Identification of specific molecules responsible for mediating the effects promoted by both DHACs inhibitors is under way in our laboratory. However, it seems reasonable to speculate that, apart from Pgp-expression, the pleiotropic nature of MDR could originate a different balance of acetylase/deacetylase activities between drug-sensitive and drug-resistant cells. This in turn, could determine the response of the cells to changes in the acetylation/deacetylation of histones and other cellular targets.[39] In such a case, the hyperacetylation induced by iHDACs, such as TSA or SAHA, could result in a different activation or derepression of specific cell death genes[36] as reported here in drug-resistant cells, increasing their susceptibility to apoptotic stimuli. A putative candidate to mediate TSA-induced apoptosis in the L1210R cells is CD95, which expression increases by treatment with histone deacetylase inhibitors.[36] However, we ruled out this possibility in our cells because, first, TSA induced CD95 expression in both L1210 and L1210R cells the concentration of TSA needed for CD95 induction (5 nM) was considerably smaller than that required for apoptosis induction (50 nM) and third, a cell permeable inhibitor of caspase 8 was able to block TSA-induced caspase 8 activity but failed to prevent TSA-induced apoptosis in the L1210R cells (data no shown).

Finally, TSA reduced Pgp expression and "sensitized" drug-resistant L1210 cells to DNM, suggesting that in addition to its proposed antitumor action in the treatment of carcinomas,[40,41] iHDACs might have a therapeutic potential against tumors with acquired resistance phenotype.

ACKNOWLEDGEMENTS

We are grateful to J. Bauza (M.D, Centro de Transfusiones, GV) for his help with the flow cytometry experiments and to Dr. A.V. Ferrer-Montiel for critically reading the article.

REFERENCES

1. Tan B, Piwnica-Worms D, Ratner L. Multidrug resistance transporters and modulation. Curr Opin Oncol 2000;12:450–8.
2. Ford JM, Hait WN. Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol Rev 1990;42:155–99.
3. Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 1993;62:385–427.
4. Bellamy WT. P-Glycoproteins and multidrug resistance. Annu Rev Pharmacol Toxicol 1996;36:161–83.
5. Nielsen D, Skovsgaard T. P-glycoprotein as multidrug transporter: a critical review of current multidrug resistance cell lines. Biochim Biophys Acta 1992;1139:169–83.
6. Gros B, Ben Neriah Y, Croop J, Housman DE. Isolation and expression of a complementary DNA that confers multidrug resistance. Nature 1986;323:728–31.
7. Johnstone RW, Ruefli AA, Smyth MJ. Multiple physiological functions for multidrug transporter P-glycoprotein? TIBS 2000;25:1–6.
8. Grogan TM, Spier CM, Salmon SE, Matzner M, Ribski J. P-glycoprotein expression in human plasma cell myeloma: correlation with prior chemotherapy. Blood 1993;81:490–5.
9. Ambudkar SW, Dey S, Hrycyna CA, Ramachandran M, Pastan I, Gottesman MM. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol 1999;39:361–98.
10. Mickley LA, Bates SE, Richert ND, Currier S, Tanaka S, Foss F, Rosen N, Fojo T. Modulation of the expression of a multidrug resistance gene (*MDR1*/Pglycoprotein) by differentiating agents. J Biol Chem 1989;264:18031–40.
11. Uchiumi T, Khono K, Tanimura H, Matsuo K, Sato S, Uchida Y, Kuwano M. Enhanced expression of the human multidrug resistance 1 gene in response to UV light irradiation. Cell Growth Diff 1993;4:147–57.
12. Thottassery JV, Zambetti GP, Arimori K, Schuetz EG, Schuetz JD. *p53*- dependent regulation of *MDR1* gene expression causes selective resistance to chemotherapeutic agents. Proc Natl Acad Sci USA 1997;94:11037–42.
13. Chin KV, Ueda K, Pastan I, Gottesman MM. Modulation of activity of the promoter of the human MDR1 gene by Ras and *p53*. Science 1992;225:459–62.
14. Grunstein M. Histone acetylation in chromatin structure and transcription. Nature 1997;389:349–52.
15. Brownell JE, Zhou J, Ranalli T, Kobayashi R, Edmondson DG, Roth SY, Allis CD. Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell 1996;84:843–51.
16. Ayer DE. Histone deacetylases: transcriptional repression with SINers and NuRDs. Trends Cell Biol 1999;9:193–8.
17. Kwon SH, Ahn SH, Kim YK, Bae GU, Yoon JW, Hong S, Lee HY, Lee HW, Han JW. Apicidin, a histone deacetylase inhibitor, induces apoptosis and Fas/Fas ligand expression in human acute promyelocytic leukemia cells. J Biol Chem 2002;277:2073–80.
18. Glick RD, Swendeman SL, Coffey D.C, Rifkind RA, Marks PA,

Richon VM, La Quaglia MP. Hybrid polar histone deacetylase inhibitor induces apoptosis 21 and CD95/CD95 ligand expression in human neuroblastoma. Cancer Res 1999;59:4392–4399.
19. Richon VM, Emiliani S, Verdin E, Webb Y, Breslow R, Rifkind RA, Marks PA. A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. Proc Natl Acad Sci USA 1998;95: 3003–7.
20. Minucci S, Horn V, Bhattacharyya N, Russanova V, Ogryzco VV, Gabriele L, Howard BH, Ozato K. A histone deacetylase inhibitor potentiates retinoid receptor action in embryonal carcinoma cells. Proc Natl Acad Sci USA 1997;94:11295–300.
21. Luo J, Su F, Chen D, Shiloh D, Gu W. Deacetylation of *p53* modulates its effects on cell growth and apoptosis. Nature 2000;408:377–81.
22. Jin S, Scotto KW. Transcriptional regulation of the *MDR1* gene by histone acetyltransferase and deacetylase is mediated by NF-Y. Mol. Cell Biol 1998;18:4377–84.
23. Soto F, Planells-Cases R, Canaves JM. Ferrer-Montiel AV, Aleu J, Gamarro F, Castanys S, Gonzalez-Ros JM, Ferragut JA. Possible coexistence of two independent mechanisms contributing to anthracycline resistance in leukaemia P388 cells. Eur J Cancer 1993;29A: 2144–50.
24. Kunzelman K, Slotki IN, Klein P, Koslowsky T, Ausiello DA, Greges R, Cabantchick ZI. Effects of P-glycoprotein expression on Cyclic AMP and volumeactivated ion fluxes and conductances in HT-29 colon adenocarcinoma cells. J Cell Physiol 1994;161:393–406.
25. Soto F, Canaves JM, Gonzalez-Ros JM, Ferragut JA. Rapid kinetics of the interaction between daunomycin and drug-sensitive or drug-resistant P388 leukemia cells. FEBS Lett 1992;301:119–23.
26. Aleu J, Ivorra I, Lejarreta M, Gonzalez-Ros J.M, Morales A, Ferragut JA. Functional incorporation of P-Glycoprotein into *Xenopus* oocyte plasma mebrane fails to elicit a swelling-evoked conductance. Biochem. Biophys Res Commun 1997;237:407–12.
27. Van Lint C, Emiliani S, Ott M, Verdin E. Transcriptional activation and chromatin remodelling of the HIV-1 promoter in response to histone acetylation. EMBO J 1996;15:1112–20.
28. Vettese-Dadey M, Grant PA, Hebbes TR, Robinson CC, Allis CD, Workman JL. Acetylation of histone H4 plays a primary role in enhancing transcription factor binding to nucleosomal DNA *in vitro.* EMBO J 1996;15:2508–18.
29. Riordan JR, Ling V. Genetic and biochemical characterization of multidrug resistance. In: Goldman ID, ed. Membrane transport of antineoplastic agents. Oxford: Pergamon Press, 1986. pp 215–39.
30. Simon SM, Schindler M. Cell biological mechanisms of multidrug resistance in tumors. Proc Natl Acad Sci USA 1994;91:3497–504.
31. Ruefli AA, Ausserlechner MJ, Bernhard D, Sutton VR, Tainton KM, Kofler R, Smyth MJ, Johnstone RW. The histone deacetylase inhibitor and chemotherapeutic agent suberoylanilide hydroxamic acid (SAHA) induces a cell-death pathway characterized by cleavage of Bid and production of reactive oxygen species. Proc Natl Acad Sci USA 2001;98:10833–8.
32. Khono K, Sato S, Uchiumi T, Yakano H, Kato S, Kuwano M. Tissue-specific enhancer of the human multidrug-resistance (*MDR1*) gene. J Biol Chem 1990;265:19690–6.
33. Chaundary PM, Roninson IB. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell 1991;66:85–92.
34. Ruefli AA, Bernhard D, Tainton KM, Kofler R, Smyth MJ, Johnstone RW. Suberoylanilide hydroxamic acid (SAHA) overcomes multidrug resistance and induces cell death in P-glycoprotein-expressing cells. Int J Cancer 2002;99:292–8.
35. Ueda H, Manda T, Matsumoto S, Mukumoto S, Nishigaki F, Kawamura I, Shimomura K. FR901228, a novel antitumor bicyclic depsipeptide produced by chromobaterium violaceum Number 968. III. Antitumor activities on experimental tumors in mice. J Antibiot (Tokyo) 1994;47:315–23.
36. Johnstone RW. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nature Rev 2002;1:287–99.
37. Robinson LJ, Roberts WK, Ling TT, Lamming D, Sternberg SS, Roepe PD. Human MDR1 protein overexpression delays the apoptotic cascade in Chinese hamster ovary fibroblasts. Biochemistry 1997;36: 11169–78.
38. Smyth MJ, Krasovskis E, Sutton VR, Johnstone RW. The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumors cells from multiple forms of caspase-dependent apoptosis. Proc Natl Acad Sci USA 1998;95:7024–29.
39. Dangond F, Gullans SR. Differential expression of human histone deacetylase mRNAs in response to immune cell apoptosis induction by Trichostatin A and butyrate. Biochem Biophys Res Commun 1998;247:833–7.
40. Saunders N, Dicker A, Popa C, Jones S, Dahler A. Histone deacetylase inhibitors as potential anti-skin cancer agents. Cancer Res 1999; 59:399–404.
41. Gray SG, Kytola S, Lui W, Larsson C, Ekstrom TJ. Modulating IGFBP-3 expression by trichostatin A: potential therapeutic role in the treatment of hepatocellular carcinoma. Int J Mol Med 2000;5:33–41.