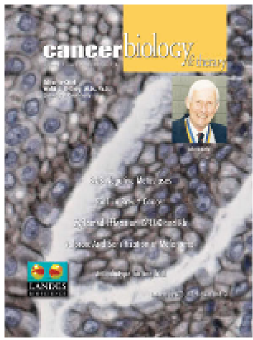

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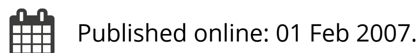
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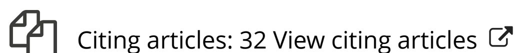
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Research Paper

Valproic Acid Induces Apoptosis, $p^{16INK4A}$ Upregulation and Sensitization to Chemotherapy in Human Melanoma Cells

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KEY WORDS

melanoma, p16[INK4A], valproic acid, M14, apoptosis

ABBREVIATIONS

VPA	valproic acid
HDAC	histone deacetylase
HDACis	histone deacetylase inhibitors
QRT-PCR	quantitative reverse transcription real-time polymerase chain reaction

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ABSTRACT

It is known that melanoma develops as a consequence of multifactorial alterations. To date several studies indicate the effective implication of *p16* as a tumor suppressor gene with a major role in either the development or progression of human melanoma. Deregulation of melanoma cell growth has been widely associated with mutations in the p16-cyclin D/cdk4-pRb pathway. Recently anticancer therapies are focused on restoration of p16 CDK inhibitory function and other proteins unregulated in melanoma cell cycle pathway (e.g., c-myc, p27). A combined strategy for restoration of normal homeostasis in the melanoma skin with targeted delivery of apoptosis-inducing agents does not seems to be far obtained. New class of antitumoral agents are emerging: histone deacetylase (HDAC) inhibitors have attracted much interest because of their ability to arrest cell growth, induce cell differentiation, and in some cases, induce apoptosis of cancer cells. Recently, attention has been focused on the ability of HDAC inhibitors to induce perturbation in cell cycle regulatory protein (e.g., $p21^{CIP1}$) and down-regulation of survival signalling pathway. In the present study, we have examined the effect of valproic acid (VPA) on M14 human melanoma cell line. Here we observed that VPA induces cell cycle arrest and apoptosis sensitising melanoma cells to cis-platin and etoposide treatment. IC_{50} dose (2.99 mM) of VPA was able to induce G_1 arrest (up to 75%) in association with upregulation of p16, p21 and cyclin-D1 related to Rb ipo-phosphorilation. In addition VPA activated apoptosis (50%) in M14 cells, when given alone or in combination with antitumoral agents. The ability of valproic acid to reestablished the G_1 pathway in melanoma cells suggests a potential application of VPA in melanoma therapeutic protocols.

INTRODUCTION

Cutaneous melanoma is a highly aggressive and potentially fatal malignancy of the skin, which is often resistant to currently available therapy and generally has poor prognosis. The transformation of melanocytes to melanoma cells is characterised by abnormal proliferation resulting from alterations in cell cycle regulatory mechanisms. This occurs through alterations in the two major cell cycle regulatory pathways, the retinoblastoma (Rb) and p53 tumour suppressor pathways.

To date several studies indicate the strong implication of *p16* as a tumor suppressor gene with a major role in either the development or progression of a broad range of human cancer, including cutaneous melanoma.[1] The normal levels of *p16* expression is low in tissues and primary cells and are up-regulated with in vitro senescence, ageing, and illegitimate oncogene activation. Hence, the p16 protein operates as a negative regulator in the G_1-S-phase checkpoint playing a role as tumour suppressor protein. Loss of p16[INK4A] has been found in the late stages of melanoma progression.[2,3] Approximately 75% of melanoma cell lines contained either homozygous *p16* deletions or *p16* mutations.[1] Extensive biochemical data are currently available on the effects of mutations in *p16*, especially those mutations that are found in human cancers. To date, three mechanisms that lead to tumor-associated inactivation of *p16* have been characterized. These include point mutation, homozygous deletion and hypermethylation of the CpG island spanning the promoter region of the *p16* gene. Broadly applicable anticancer therapies might be based on restoration of p16 CDK inhibitory function[3] and other proteins unregulated in melanoma cell cycle pathway (c-myc, p27, etc.).[4,5]

Modulation in the expression of cancer-related genes silenced by changes in DNA methylation and histone deacetylase inhibitors has been shown to exert antitumor effects

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in vitro and in vivo models.[6] Hence it has been proposed that histone acetylation plays an important role in transcriptional regulation. Acetylation loosens histone-DNA bonds and reduces the interaction of histones with ATP-dependent chromatin-remodelling complexes, which facilitates the binding of transcription factors.[7] The enzyme histone deacetylase (HDAC) catalyses the removal of acetyl groups on the amino-terminal lysine residues of core nucleosomal histones. Various natural and synthetic compounds that inhibit HDAC activity have been identified to date. HDAC inhibitors (HDACis) from diverse origins arrest cell growth and induce differentiation in various in vitro and in vivo models including acute promyelocytic leukaemia and in cell lines derived from colon, lung and prostate carcinomas.[8,9]

HDAC inhibitors have emerged as a potentially promising new class of anticancer drugs based on their ability to activate a variety of genes implicated in the regulation of cell survival, proliferation, differentiation and apoptosis.[9] These drugs have both cytostatic and cytotoxic activities, depending on the dose of drug used. At low doses, these drugs have cytostatic activity characterised by a G_1 phase cell cycle arrest that is associated with the increased expression of the cyclin-dependent kinase (cdk) inhibitor p21.[10] Specifically, HDACis can cooperate with DNA methylation inhibitors to reactivate a silenced, aberrantly methylated *p16 INK4A* promoter.[11] In a different context, HDACis can up-regulate p16 INK4A in rat synovial fibroblasts from arthritic joints, but not from normal ones[12] and in mice induced *p16* expression through acetylation of its promoter region.[13]

Valproic acid (VPA) is a short-chain branched fatty acid commonly used as antiepileptic agent, recently VPA has been shown to inhibit histone deacetylase at therapeutic concentrations.[14,15] Studies in the last 2 years have corroborated that the short chain fatty acid VPA and VPA-analogs potently modulate the biology of diverse tumour cell types by inducing differentiation, inhibiting proliferation, increasing apoptosis, and immunogenicity and by decreasing metastatic and angiogenetic potency. The first evidence that VPA downregulates HDAC-activity in teratocarcinoma and neuroblastoma cells was provided by Phiel et al., Cinatl et al., and Göttlicher et al.[16] Novel data provide evidence that prostate cancer may particularly benefit from VPA[17] and regarding melanoma, valproic acid can be effective in inducing cell death modulating in particular surviving expression molecules.[18] Therefore it has been demonstrate that leukaemia cell line engineered to inducible express cdk inhibitor p16 is refractory to the effects of HDACis treatment when arrested in G_1 phase by p16 induction.[19]

In this study, we investigate the effect of VPA in inducing melanoma cell growth arrest and apoptosis. Here we observed that in melanoma cells, VPA induces cell cycle arrest through upregulation of p16 cell cycle inhibitor and apoptosis sensitising to cis-platin and etoposide treatment.

MATERIALS AND METHODS

HDAC assay. HDAC activity was measured by using HDAC Activity Assay Kit (Upstate, colorimetric detection). Experimental procedures were designed and performed according to the protocol provided within the kit. In brief, cell lysates (prepared according to the same procedure described in Western blot analysis section) from untreated or treated cells (48 h of exposure to different doses of VPA) were used as sources for HDAC activity. The absorbance (405 nm) was measured using Sirio-S (SEAC, Radim Group). HDAC assay buffer plus 4 μM Trichostatin A (TSA) has been used as control.

Chemical, cell culture and cytotoxicicity assays. M14 human melanoma cell lines were grown in RPMI-1640 (Gibco) supplemented with 10% of fetal bovine serum (GIBCO), 5% of L-Glutamine (GIBCO) and antibiotics, under standard conditions (37°C temperature, 5% CO_2 in a humidified atmosphere). For valproic acid cytotoxicity assays, cells were seeded in 96-well plates (Falcon, CA) in 100 μl of culture medium. Valproic Acid (Sigma, St.Louis, MO, USA) was dissolved in sterile water. For each experiment drugs were serially diluted in cell culture medium to the desired concentrations and an equal volume of the diluted solution (100 μl/well) was added to the cells. Each treatment was performed in triplicate in three independent experiments. Cells were continuously exposed to valproic acid for 72 h. To determine the cytotoxic effect at the end of drug incubation, MTS solution (Promega, 20 μl/well) was added to the cells. The plates were incubated 2 h at 37°C and then the absorbance at 490 nm was measured using Sirio-S (SEAC, Radim Group). IC_{50} dose was calculated by using GraFit32 program.

Cytoxicity of chemotherapy plus valproic acid: cells were seeded into 96-well plates in 100 μl of culture medium. The next day, cells were treated for 72 h in complete medium with *cis*-platin 2.5-5-10 μM, or etoposide 0.5-1-5 μM and valproic acid at 1 mM. Combination index was evaluated as described in the following equation:

$$CI = \frac{C_{A,I}}{IC_{I,A}} + \frac{C_{B,I}}{IC_{I,B}}$$

which provides qualitative information on the nature of drug interaction (CI of less than, equal to, and more than 1 indicates synergy, additivity, and antagonism, respectively).

Results are expressed as mean ± standard deviation (SD) for n = *3* independent experiments.

Cell growth, cell cycle and TUNEL assay. Cell growth inhibition and viability of melanoma cells were evaluated following the growth curves by trypan blue exclusion test. For cell cycle analysis, cells were incubated to indicated dose of VPA and after 24 h, 48 h and 72 h cells were fixed in ice-cold 75% ethanol and stored at least 1 h at -20°C. After rinsing, cells were resuspended in PBS containing 50 Units/ml RNAse and 50 μg/ml propidium iodide and incubated 1 h in the dark at room temperature. Analysis was performed on a FACScan flow cytometer (Becton Dickinson, CA) using Cellquest and ModFit software.

For TUNEL assay (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling), cells were harvested and fixed in 4% paraformaldeyde for 45 min at room temperature, after exposure to IC50 concentrations of VPA for 72 h. After rinsing with PBS, the cells were permeabilised in a solution of 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. Samples were washed with PBS and incubated in the TUNEL reaction mixture (Boehringer Mannheim-Roche) for 1 h at 37°C in the dark. After a final wash with PBS samples were analysed using a FACScan flow cytometer (Becton Dickinson, USA). The percentage of FITC-positive in the overall cell population was determined using the Cell Quest software package (Becton Dickinson).

Western blotting. Total cell lysates were obtained resuspending the cells in buffer containing 1% Triton, 0.1% SDS, 2 mM $CaCl_2$, 100 μg/ml phenylmethyl sulfonyl fluoride. Protein content was determined using the Protein Assay Kit 2 (Bio-Rad Laboratory, Hercules, CA, USA). 30 μg of proteins were electrophoresed in 10% SDS-polyacrylamide gel and then electro-transferred to nitrocel-

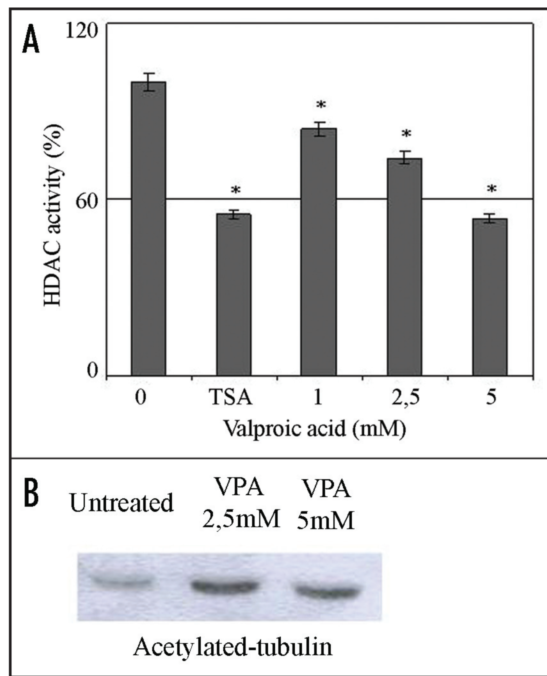

Figure 1. (A) HDAC assay using nuclear extracts from M14 cells treated with VPA in doses of 1 2.5 5 mM. TSA (4 μM) was used as experimental control. All experiments were carried out in triplicate and repeated three times data are represented as the mean ± SD (vertical bar) *p < 0,05. (B) Representative Western blotting illustrating the expression of acetylated tubulin in M14 cells after 48 h of exposure to VPA.

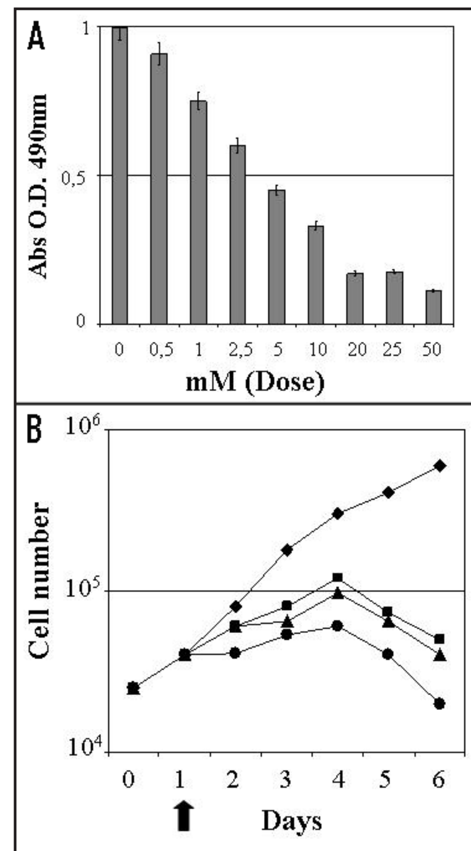

Figure 2. (A) Cytotoxic effect of valproic acid on M14 melanoma cells, evaluated by MTS assay as described in section materials and methods. Data were shown by mean ± SD (vertical bar) of triplicates experiments, *p < 0.05. (B) M14 (1 x 10^4/well in triplicate in 24-well plates) cells were plated and grown under routine growth conditions or after treatment with Valproic acid (♦, untreated cells; ■, 1 mM; ▲, 2.5 mM; ●, 5 mM), black arrow indicates the day of treatment. Cells were counted by trypan blue exclusion method. Data are represented as the mean ± SD (vertical bar) from three independent experiments.

lulose membrane (Amersham biosciences, Piscataway, NJ, USA), which was then blocked overnight with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20 (TBS-T) containing 5% non fat dry milk. The membrane was then incubated with 1 μg/ml of primary antibody in TBS-T [anti acetylated-tubulin (clone 6-11B-1, SIGMA) p16 (clone G175-1239, BD Pharmingen) p21 (clone 2G12, BD Pharmingen) cyclin D1 (Ab-3, Calbiochem San Diego CA, USA) pRb (clone G3-245, BD Pharmingen) cyclin-E (clone HE12, Oncogene res.) c-myc (clone 9E10, BD Pharmingen) p27 (clone HBB6, Calbiochem San Diego CA, USA) β-actin (clone Ac-15, Abcam)] and with specific horseradish peroxidase-conjugated secondary antibodies in TBS-T. Protein bands were visualized using a chemiluminescent detection system (Amersham biosciences, Piscataway, NJ, USA).

RNA extraction and reverse transcription. RNA from frozen cells was isolated using Tri reagent (SIGMA-Aldrich) according to the manufacterer's instructions, with slight modifications. In brief, the cells were homogenized in Trizol and chloroform was added. After incubation and centrifuge, the supernatant (water phase) was removed to fresh tubes. RNA was precipitated from supernatant by adding an equal volume of isopropyl alcohol. The samples were centrifuged the supernatant was removed, and the RNA pellet was washed twice in 75% ethanol. The pellet was air-dried at room temperature and dissolved in sterile water. The concentration purity, and amounts of total RNA were determined by UV (SmartSpec™, BIO-RAD) and O.D. 260/280 nm ratios >1,98 were obtained for all samples. For cDNA synthesis, 1 μg total RNA of each samples was reverse transcribed using AMV Reverse Transcriptase and as primers oligo-p(dT) (1st Strand cDNA synthesis kit for RT-PCR (AMV)-Roche Applied Science).

Quantitative Real-Time RT-PCR. Real-Time quantitative RT-PCR was carried out using the Mx3005P™ (Strategene®) which detects the signal from the fluorigenic SYBR green during PCR. The volume of each reaction was 25 μl containing 2,5 μl of the cDNA sample, 5 pmol each primer *p16* [S/5'- AAG CCA TTG CGA GAA CTT-3', AS/5'-CAG AGG GCA GAAAGA AAA-3'] and for β*2-Microglobulin* [S/5'-GAGTATGCCTGCCGTGTG-3', AS/5'ATCCAAATGCGGCATCT-3'] 12.5 μl of SYBR®Green PCR Master Mix (PE Applied Biosystems). The thermal cycling conditions comprised 10 min at 95°C. Thermal cycling consisted of 40 cycles at 95°C for 30 s, 55°C for 45 s, 72°C for 1min and 72°C for 7 min. Each assay include: a standard curve (from 1 μg to 125 ng of cDNA from lymphocytes) in duplicate, a no-template control (water), 500 ng of calibrator cDNA in triplicate (from lymphocytes). All samples with a coefficient of variation (CV) higher than 10% were rested.

The amount of target, normalized to the endogenous reference (β2-microglobulin) and relative to the calibrator, was performed by $2^{-\Delta\Delta CT}$ method. The relative expression was calculated with the "delta-delta Ct method" ($\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$). The Ct (threshold cycle) values were normalised against the endogenous reference gene β2-microglobulin ($\Delta Ct = Ct_{p16} - \Delta Ct_{\beta 2\text{-microglobulin}}$) and were compared with the calibrator. Considering that ΔΔCt

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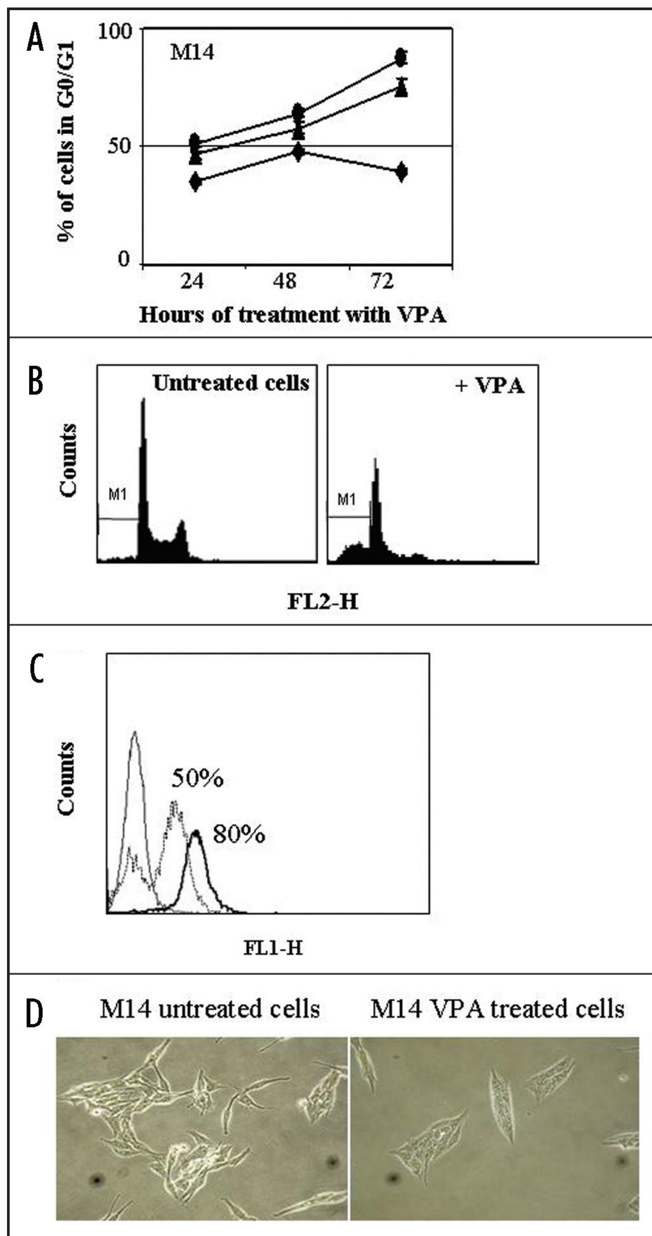

Figure 3. The effect of VPA on cell cycle distribution (A and B), apoptosis evaluated by tunel assay (C) and morphology (D) of M14 human melanoma cells. A, B) M14 cells in parallel wells were trypsinized 24, 48 and 72 hours after treatment with VPA, fixed in ethanol, stained with PI, and analyzed by flow cytometry for DNA content. (♦, untreated cells; ▲, 2.5 mM VPA; and ●, 5 mM VPA). Note the predominant of sub-G_1 DNA peak (M1) representing cells undergone in apoptosis after 72 h of exposure to VPA (right panel). C) Effect of VPA on the induction of apoptosis in M14 cells. Apoptotic cells were detected by TUNEL assay and flow cytometry after 72 h exposure to 2.5 mM and 5 mM dose of VPA, gray-pale line untreated cells, gray line 2.5 mM VPA and black line 5 mM VPA.The percentage of FITC-positive cells in M14-treated samples is reported in the panel. (D) Rounded-up cells remained after 72 hours of VPA treated dishes (right) compared to untreated cells which showed long dendritic processes (left).

validation required equal efficiencies of target genes and reference amplification, standard curve assays were obtained for target gene and reference by cDNA sample diluted at two fold intervals and at each dilution, cycle threshold were plotted against the Log value of the input cDNA concentration, then quantitative PCR efficiencies

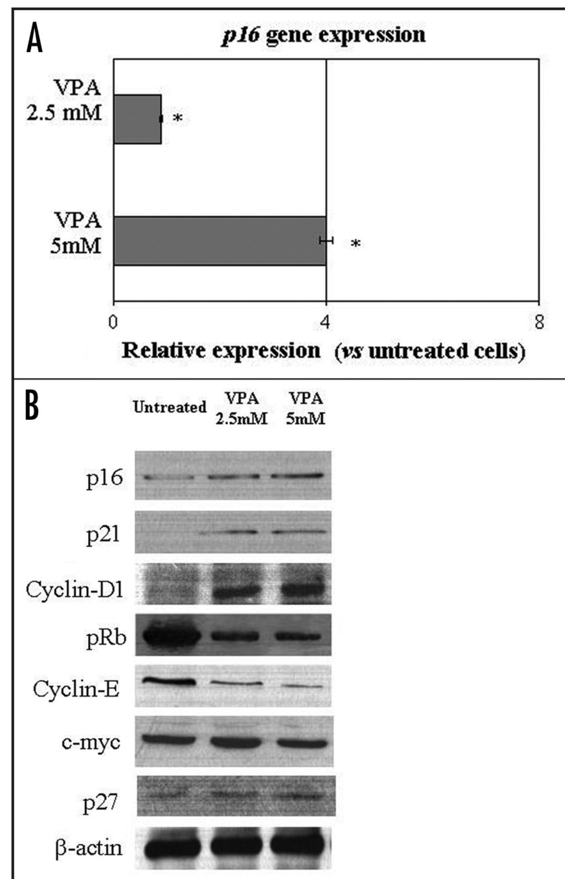

Figure 4. (A) *p16* expression gene increased in the M14 cells exposed to VPA for 48 h. Target mRNA levels were evaluated by quantitative RT-PCR and standardized to β2-microglobulin mRNA levels (ΔΔCT method). Expression levels in the control group were designated as 1.0, and relative expression levels in the VPA 2.5 mM (upper gray) and VPA 5 mM (lower gray) are presented. Data are represented as the mean ± SD from three independent experiments $^*p < 0.05$. (B) Representative Western blotting illustrating the expression of cell cycle-related proteins in M14 cells after 48 h of exposure to VPA. β-actin was used as a control for sample loading.

(E) were calculated from the slopes ($E = 10^{[-1/slope]} - 1$).

Statistical analysis. Results are expressed as means ± SD for at least three distinct experiments. Demonstration of significant differences among means was performed by Student's t analysis considering 0.05 the threshold value of p.

RESULTS

Biological effect on M14 cell induced by VPA treatment as consequence of HDAC activity inhibition. First, we tested the ability of valproic acid in inhibiting the HDAC activity in our in vitro melanoma model, indeed we observed that a dose-dependent inhibition of HDAC activity occurred when VPA was administered for 48 h. Hence using 1 mM of valproic acid we observed 30% of HDAC inhibition activity, and after treatment with the highest dose of 5 mM a 50% of HDAC inhibition was estimated (Fig. 1A). In addition acetylation of tubulin increased when melanoma cells were exposed to valproic acid at the concentration of 2.5 mM and 5 mM after 48 h (Fig. 1B).

Thus, the cytotoxic activity of valproic acid was studied in M14 human melanoma cell line, a colorimetric assay (MTS) was assessed after 72 h of continuous exposure to different doses of VPA. As

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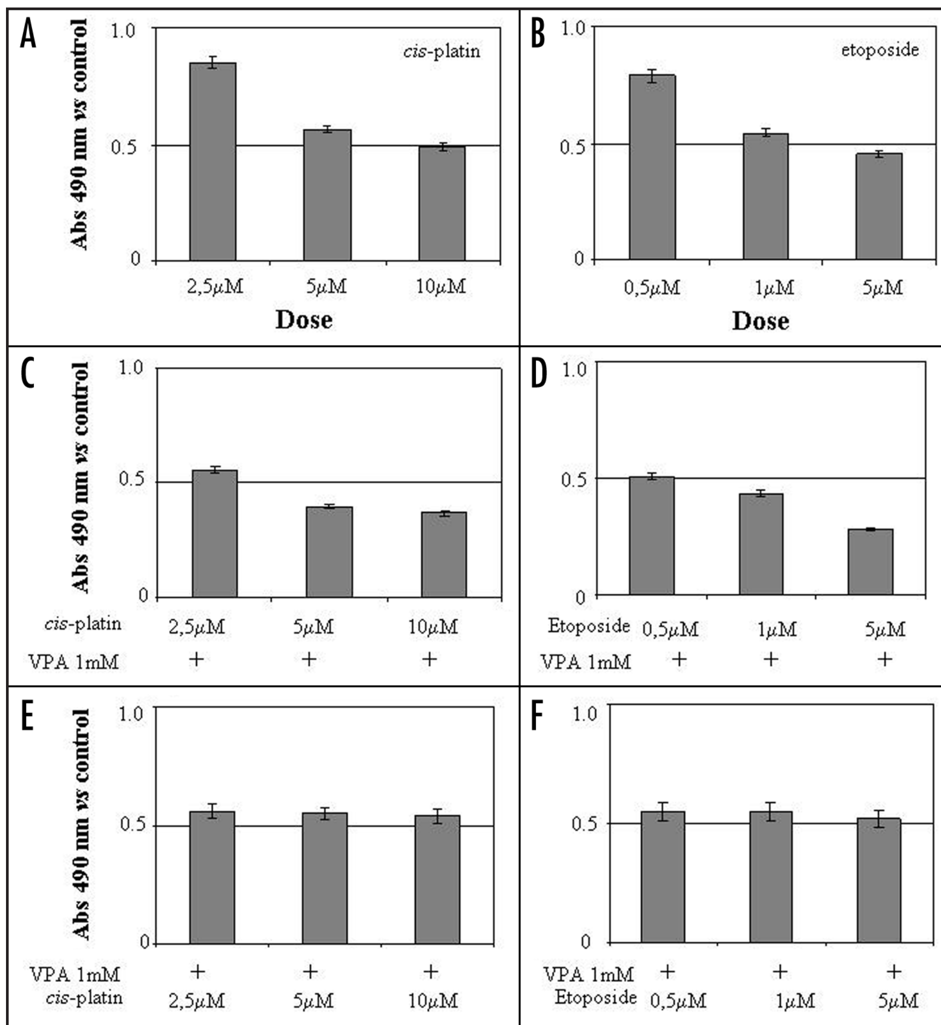

Figure 5. Cytotoxic effect of *cis*-platin (A), etoposide (B), combination contemporaneous exposure to valproic acid and *cis*-platin (C) or etoposide (D) and combination of VPA followed by *cis*-platin (E) or etoposide (F) on M14 melanoma cells, evaluated by MTS assay as described in section materials and methods. Data were shown by mean ± SD (vertical bar) of triplicates experiments.

shown in Figure 2A is evident a dose-dependent effect of valproic acid on M14 cells. In particular we observed that IC_{50} dose was achieved at the concentration of 2.99 mM (±1,10). Hence, M14 cell growth was inhibited of about 25% already after 24 h of exposure to VPA at the dose of 1 mM and after 72 h at the same dose, 60% of cell growth inhibition occurred (Fig. 2B). Around the IC_{50} dose (2.5 mM) we observed an inhibition of cell growth of about 55% after 48 h of treatment. When cultured in presence of VPA 5 mM the number of viable cells clearly decreased respect to initial seeded cells suggesting a prevalence of cell mortality.

Cell cycle perturbation and apoptosis induced by VPA treatment. Cytofluorimetric analysis showed that melanoma cells treated with VPA were progressively blocked in the G_0/G_1 phase of cell cycle (Fig. 3a). After 48h of exposure to 2.5 mM of valproic acid 58% of M14 cells were blocked in G_0/G_1 phase. At the dose of 5 mM the percentage of cells in G_0/G_1 cell cycle compartment, achieved the 64%. Besides, G_0/G_1 cell cycle phase accumulation of about 75% after 72 h of treatment with 2.5 mM VPA was observed, and 87% of cells after 5 mM of VPA exposure.

As evident in Figure 3B, the sub-G_1 peak (M1) was visible during the analysis of cell cycle, 72 h after treatment with 2.5 mM VPA, suggesting clearly an induction of apoptosis due to valproic acid treatment. Indeed by TUNEL assay we evaluated the induction of programmed cell death (Fig. 3C) compared to untreated cells, and we detected a 50% of apoptosis in M14 cells treated with 2.5 mM of VPA and of about 80% in cells treated at the 5 mM dose.

In addition what we observed was that fewer attached cells remained after 72 hours of VPA (2.5 mM) treatment and that cells switched into rounded-up phenotype (Fig. 3D right panel) losing long dendritic processes (Fig. 3D left panel).

p16 upregulation and molecular G_1 pathway modulation after valproic acid exposure. To explain biological effect due to VPA treatment, associated to its ability as HDAC inhibitor, we analysed which molecular modulation could occurred in M14 cells. We first studied the effect of valproic acid on *p16* gene, quantifying the expression by QRT-PCR; it is known in fact that in this cell line p16 is down-regulated since M14 cells have been already characterized for the expression of the p16 and cyclin-D1.[5]

As reported in Figure 4A, we observed 1.6-fold upregulation of *p16* gene expression in M14 treated cells with 2.5 mM of VPA and 4-fold with 5 mM after 48 h of exposure to drug. We confirmed this upregulation by Western blotting too, checking that as well gene expression, the protein level was changed. Indeed we observed an upregulation of p16 protein associated to the cyclin-D1 and p21 upregulation after treatment with both dose 2.5 mM and 5 mM of VPA, in addition Rb protein was found ipo-phosphorylated after valproic acid administration. On the other hand, since the cells were blocked in G_1 phase, unsurprisingly the cyclin-E was found downregulated after treatment with VPA, while the expression of c-myc and p27 did not change even at the concentration of 5 mM.

Effect of cytotoxic drugs in combination with cis-platin or etoposide and valproic acid in M14 cells. To evaluate a possible role of VPA in sensitising to chemioterapic agents, after G_1 cell cycle pathway restoring, we exposed M14 cells to contemporaneous administration of *cis*-platin or etoposide and valproic acid. We observed a dose dependent inhibition effect on cell proliferation after both treatment: VPA + *cis*-platin and VPA + etoposide, respectively. Thus the contemporary administration of VPA 1 mM and *cis*-platin 2.5 μM determined about 50% of cell proliferation inhibition. The same effect with a 50% of cytotoxic activity was observed when 1 mM VPA and 0.5 μM etoposide were administered. These data indicate a synergic effect, since the combination index was found <1 (0.833) for both treatment: VPA + *cis*-platin and VPA + etoposide (Figs. 5c,d). Furthermore changing the administration: first Valproic acid (1 mM) for 24 h followed by etoposide or *cis*-platin exposure for 72 h, the dose-dependent effect was loosen, although this scheduled treatment determined 50% of cell proliferation inhibition at the

lowest concentration dose of etoposide and *cis*-platin (Fig. 5E and F).

Treatment of M14 cells with *cis*-platin or etoposide did not affect the expression of p16 (data not shown) and additionally did not induce apoptosis (Fig. 6). When valproic acid was administrated in association with chemotherapy agents, the induction of apoptosis was again evident (sub G_1 peak-M1). After 48 h of exposure to VPA 1 mM and *cis*-platin or etoposide we observed of about 20% apoptosis compared to treatments with chemotherapy in single administration. These data suggest a central role of VPA in inducing apoptosis in melanoma cells and the usefully valproic acid combination therapy.

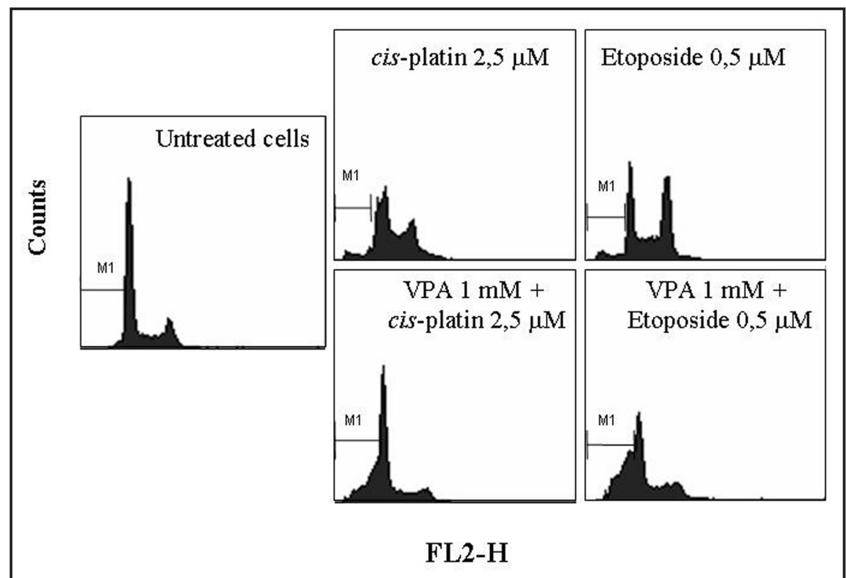

Figure 6. Flow cytometry analysis of sub-G_1 DNA peak (M1) representing cells undergone in apoptosis after 72 h of exposure to VPA and *cis*-platin or etoposide.

DISCUSSION

Melanoma is the leading cause of death from skin tumors worldwide, the molecular mechanisms involved in melanoma pathogenesis are beginning to be un-revealed. Most melanoma cells were found to present G_1 checkpoint genetic alterations affecting cyclin-D1 and $p16^{INK4A}$ with deletion, ipermethylation or frameshift mutation of this gene. Increased acetylation of the lysine residues on histone is thought to result in the relaxing of chromatin, allowing access of transcription factors to DNA and therefore an increase in transcription.[20] To restore the expression of gene aberrantly silenced in tumour could be a reliable approach in cancer therapy.

Valproic acid has been shown to have HDAC inhibitor activity in vitro and to induce differentiation of carcinoma cells and leukaemia blasts from acute myeloid leukaemia patients. In addition it was also effective in reducing tumour growth and metastasis formation in animal studies.[9] At higher doses, these class of drugs are selectively cytotoxic, killing a wide range of tumour and transformed cell lines but not normal cell lines.[21,22]

Numerous studies have provided evidence that HDACis induce cell cycle arrest mediated at least in part, by substantial increase in the expression of the CDKN1A gene, which encodes the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$. Other cell cycle inhibitor that participate in the proliferative arrest elicited by HDACis are three of the four members of the INK4 family inhibitors of cyclin-D-dependent kinase cdk4/6, namely, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$.[23]

Since deregulation of melanoma cell growth has been mainly associated with alterations in the p16-cyclin D1/cdk4-pRb pathway, we studied the effect of VPA on p16 modulation, first by evaluating the gene expression by QRT-PCR and consequently by western blotting. Both techniques revealed an upregulation of p16 associated with increased expression of others protein involved in G_0/G_1 pathway as p21 and cyclin-D1 and related to ipo-phosphorilation of pRb. No change in c-myc and p27 expression was found, suggesting that the block in G_1 due to the treatment of VPA, occurred specifically through p16 and p21 cdk4/6-cyclinD1 inhibitors. Interesting similar data about p27 was obtained by Klisovic et al. in cutaneous melanoma cell lines following treatment with TSA.[24]

It has been reported that cells, which reexpressed $p16^{INK4A}$, were found to have undergone senescent-like changes.[25,26] Indeed what we observed, reported in Figure 3D, is an altered phenotype of M14 cell after treatment with VPA: cells loosed the long dendritic processes transforming into rounded-up phenotype. Thus the results suggest that the binding of the reexpressed $p16^{INK4A}$ to cdk4/6 prevented the association of cdk4/6 with cyclin-D1, inhibited the catalytitc activity of cdk4 and the phosphorilation of pRb, blocked cell cycle progression, and lead to senescent like changes. In a similar study, combined VPA and ATRA treatment of acute myeloid leukaemia (AML) blasts resulted in both reduced cell survival and a shift towards a more mature and post-mitotic phenotype.[27] Biological effect of valproic acid on M14 cells were correlated with the ability of VPA in inhibiting HDAC activity and afterward in acetylated tubulin.

Even though it has been reported that inhibition of cell cycle progression at the G_1-S boundary through overexpression of the cyclin-dependent kinase inhibitor p16, abrogated cell death mediated by HDACis.[19] Here we report that VPA was able, in the order of IC50 dose (2,99 mM), to induce apoptosis in M14 cells as evident by TUNEL assay and sub-G_1 peak, observed during cell cycle analysis.

The well characterized pharmacokinetics of valproic acid and its limited toxicity makes this agent an attractive candidate for combination regimens including HDAC inhibitors. We hypothesized affect cells escaping from G_1 VPA-induced arrest, by comination treatment with VPA and chemiotherapic agents. We treated M14 cells in combination: VPA and *cis*-platin or etoposide, detecting cytotoxicity of combination therapy and apoptosis induction. We observed that the contemporaneous exposure of cells to *cis*-platin or etoposide and VPA at the lowest concentration determined a synergic effect of combined therapy. Furthermore chemioterapic single therapy (*cis*-platin or etoposide alone, respectively) did not induce apoptosis in M14 melanoma cells, only when VPA was administered in combination with *cis*-platin or etoposide, apoptotic process occurred. Furthermore the scheduled therapy administering VPA followed etoposide or *cis*-platin indicated a saturation in cell proliferation inhibition since the dose dependent effect observed in the previous contemporaneous administration was loosen.

This demonstrates that valproic acid is able to sensitize M14 cells to chemotherapy treatment, and that HDACis as VPA are potentially

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useful class of compounds for elucidating cell signaling pathways and for developing new approaches to the treatment of melanoma. The exact molecular mechanisms involved in melanoma cell cycle arrest and apoptosis induced by HDAC inhibitors is not completely clear, more studies are required and principally combination with other agents has to be developed in to successfully treat malignant melanoma. Combination anticancer therapies including VPA with other drugs, especially nontoxic drugs, may offer a substantial advantage over VPA monotherapy in a clinical setting. Our data suggest that combination therapies of VPA may enhance the efficacy of other antitumoral drugs to which the targeted tumour cells have an inherent or acquired resistance.

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