

## Epigenetic Modifiers as Anticancer Drugs: Effectiveness of Valproic Acid in Neural Crest-derived Tumor Cells

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**Abstract.** Valproic acid (VPA) is an established drug in the long-term therapy of epilepsy. Recently, VPA has demonstrated antitumor activity as a histone deacetylase (HDAC) inhibitor. In this study, the anticancer properties of VPA on neural crest-derived human tumor cell lines G361 melanoma, U87MG glioblastoma and SKNMC Askin tumor cells were investigated. The effect of VPA on cell growth, apoptotic activity and invasive ability were evaluated. Firstly, VPA induced cell growth inhibition and apoptotic activity, as demonstrated by sulforhodamine B protein assay, annexin V assay and by Western blot analysis for Bcl2 and Bax expression levels, in all three cell lines. In addition, VPA led to a decrease of HDAC-1 protein level, as assessed by Western blot analysis. Treatment with VPA caused a decrease in the invasive ability of all three cell lines. Since the invasion process involves a complex system of tightly regulated proteases, matrix metalloproteinases (MMPs) and their tissue-specific inhibitors (TIMPs), the effect of VPA on MMP and TIMP expressions was analysed. Exposure to VPA resulted in a decrease of MMP2 and MMP9 activity and expression level, as assessed by gelatin zymography and Western blot analysis. In addition, exposure to VPA led to enhanced expression of TIMP1, as assessed by Western blot. Taken together, our results, besides providing further evidence that VPA may represent a promising therapeutic strategy in cancer treatment, may help in the design of new protocols geared at the treatment of neural crest-derived tumors.

Among anticancer drugs collectively named 'targeted or molecular therapies', epigenetic drugs are candidates for potent new drugs in cancer therapy. Differently from other agents targeting a single gene product, epigenetic drugs have chromatin as their target. Histone acetyltransferase and histone deacetylase (HDAC) determine the acetylation status of histones and are

therefore considered as key elements in the dynamic regulation of gene expression (1-3). Since the recruitment of HDAC leads to transcriptional repression, inhibitors of this enzymatic activity can reverse aberrant repression in cancer cells and lead to re-expression of epigenetically silenced genes involved in growth and differentiation processes. Therefore, HDAC inhibitors are considered as candidate drugs in cancer therapy (4-6). The effectiveness of these enzyme inhibitors has been established by many *in vitro* and *in vivo* experiments on a variety of tumor types (7-9). We demonstrated previously that valproic acid (VPA) and other short-chain fatty acids, as HDAC inhibitors, can arrest cell growth and induce differentiation in human neuroblastoma cells (10). The effectiveness of HDAC inhibitors, especially VPA, in neuroblastoma cells, prompted us to investigate the anticancer activity of VPA in other neural crest-derived malignancies, such as glioblastoma, melanoma and Askin tumor. The extremely poor prognosis of patients affected by these highly chemoresistant tumors has not significantly improved in recent decades, therefore new approaches in therapeutic regimens are mandatory. In this study, we investigated the effect of VPA on cell proliferation, apoptotic activity and invasive ability in glioblastoma (U87MG), melanoma (G361) and Askin (SKNMC) tumor cells.

### Materials and Methods

**Cell culture and treatment.** U87MG human glioblastoma and G361 human melanoma cells, obtained from Interlab Cell Line Collection (Genoa, Italy), and SKNMC Askin tumor cells, purchased from the American Type Culture Collection (Rockville, USA) were maintained in RPMI (Sigma, USA), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin and grown at 37°C in a humidified air with 5% CO<sub>2</sub>. VPA (Sigma) was dissolved in the culture medium just before use to obtain final concentrations in the range of 0.5-2.0 mM.

**Sulforhodamine (SRB) assay.** The SRB assay is based on the ability of SRB dye to bind protein basic amino acid residues. The amount of dye incorporated by the cells indicates the cell number. Cells were plated in 96-well plates (100 µl/well) and treated with VPA in the range 0.5-2.0 mM for 1, 4 and 6 days. At the end of treatment, cell culture medium was removed and RPMI was added (50 µl/well). Cells were fixed using 25 µl/well 50% aqueous trichloroacetic acid

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for 1 h at 4°C, rinsed with water several times and incubated with 50 µl/well sulforhodamine B solution (0.4%) (Sigma) for 30 min. After rinsing with 1% acetic acid and solubilizing in 10 mM Tris for 5 min, the absorbance of each well was measured in a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. The results were expressed as a percentage of that of the controls (untreated cells).

**MTT assay.** The effect of VPA on cell viability was also evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (11).

**Annexin V apoptosis detection assay.** Experiments were performed on cells seeded in 6-well plates with slides, at a density of  $8 \times 10^4$  cells/well, treated for one day with VPA in the range 0.5-1.0 mM. Samples were prepared using 0.5 µg of Annexin V FITC (Bender MedSystems, Austria) for 15 min at the dark. After incubation, the cells were fixed with paraformaldehyde at 4°C for 30 min and marked with 10 µl (1 µg/ml) of propidium iodide per 100 µl assay buffer. After washing with phosphate-buffered saline (PBS) and water, samples were observed under fluorescence microscope using a dual filter set for FITC and rhodamine. Apoptosis was evaluated by counting FITC-labelled cells in five random fields and expressed as percentage of total cell number in comparison to untreated cells.

**Zymography.** MMP2 and MMP9 activity was determined by gelatin zymography (12). Cells were seeded and after 18 h were placed in serum-free medium (RPMI) with VPA at 1 or 2 mM, or not (control) for 24 h. After centrifugation ( $300 \times g$  for 10 min), the supernatant was separated and protein concentration determined using 10 µg per lane, with sample buffer (Tris-HCl 1 M, pH 6.8, sodium dodecyl sulphate (SDS) 2%, glycerol 10%, applied to 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin (Sigma). After electrophoresis, SDS was removed from the gel by washing twice with 2.5% Triton X-100 for 1 h. After a brief rinse, the gel was incubated at 37°C for 18 h in buffer at pH 7.6, containing 100 mM Tris-HCl, 10 mM  $\text{CaCl}_2$ , 20 mM NaCl. The gel was stained with 1% Coomassie Brilliant Blue R250 for 2 h and then treated with destaining solution (40% methanol, 10% acetic acid, 50% distilled water). The MMP activities, indicated by clear bands of gelatin digestion on a blue background, were quantified by using densitometric image analysis software (Image Master VDS; Pharmacia Biotech, Uppsala, Sweden).

**Western blot analysis.** To determine HDAC-1, MMP2, MMP9, TIMP1, TIMP2, Bax and Bcl2 protein levels, the cells were plated and treated with VPA as described above for zymography. The cells were detached and were collected by centrifugation at  $300 \times g$  for 10 min and pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5% Triton X-100, 5 µM  $\text{Na}_3\text{VO}_4$ ) and sonicated on ice in the presence of protease inhibitors. Protein concentration was determined by the method of Lowry (13). Cell lysates (50 µg of protein per lane) were size fractionated in 10% SDS-polyacrylamide gel, prior to transfer to Hybond TM-C Extra membranes (GE Healthcare, UK) by standard protocols. Membranes were blocked for 2 h with 5% milk in transfer buffer saline (TBS) at RT. The membranes were incubated overnight at 4°C with anti-HDAC-1 (Epigentek, USA), anti-MMP2, anti-MMP9, anti-TIMP1, anti-TIMP2 (all Santa Cruz Biotechnology, USA), anti-Bax or anti-Bcl2 (Sigma) primary antibody dissolved in TBS-5% milk. The membranes were washed twice with TBS-5% milk and were incubated for 1 h with the respective peroxidase-conjugated antibodies. The primary antibodies were diluted 1:500 and the antirabbit

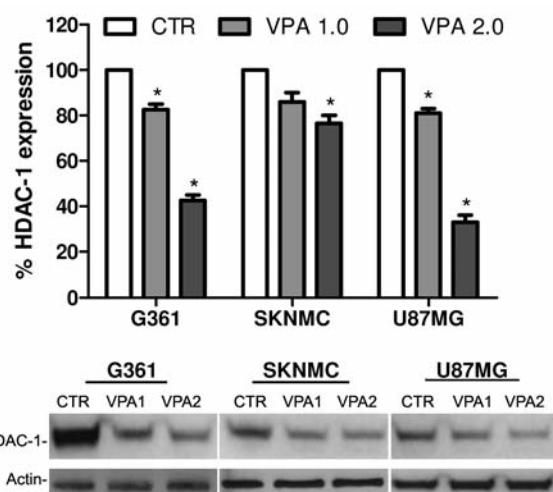


Figure 1. Effect of VPA (mM) on HDAC-1 protein levels, as assessed by Western blot in cell lysates of G361, SKNMC and U87MG cells after 24 h of treatment. Densitometric data are expressed as the percentage of treated samples with respect to control (CTR). Each bar represents the mean ( $\pm$ SEM) of three independent experiments; \* $p < 0.05$  compared to control.

peroxidase-conjugated antibody was diluted 1:1000 with 5% milk in TBS-1% Tween. The proteins were detected by luminol (GE Healthcare, UK). Bands were quantified by using a densitometric images analysis software (Image Master VDS). The amount of protein in each lane was the same, as confirmed by loading with actin (Sigma).

**Invasion assay.** Invasion of cells into Matrigel was determined using Boyden chambers (NTG, Italy) with polycarbonate membrane, 8.0-µm pore size (NTG). The filter was coated with 12.5 µg of Matrigel (Sigma). After 2 h, cells treated or not with VPA (1.0-2.0 mM) for 24 h, were seeded into the upper part of each chamber ( $4 \times 10^5$  cell/well in 800 µl in serum-free RPMI). In the lower part, 200 µl RPMI with 10% FBS were added as chemoattractant. After incubation for 8 h at 37°C, non-migratory cells on the upper surface of the filter were wiped with a cotton swab, while cells migrated on the lower surface of the filter were fixed and stained with toluidine blue. The invasion activity was evaluated by counting cells in five random fields using microscopy at  $\times 100$  magnification.

**Statistical analysis.** Data were expressed as the mean ( $\pm$ SEM). Differences were analyzed by Student's *t*-test and considered statically significant at  $p < 0.05$  between the control and experimental samples.

## Results

Western blot analysis revealed that treatment with VPA led to a decrease in HDAC-1 expression level: this decrease was dose dependent and occurred in all treated cell lines (Figure 1). The effect of VPA on G361, SKNMC and U87MG cell growth, as measured by SRB assay, is shown in Figure 2. VPA dramatically inhibited cell proliferation in all three cell lines. These data were confirmed by MTT assay (data not reported). Growth inhibition was accompanied by changes indicating that cells underwent apoptosis: Annexin V staining showed an

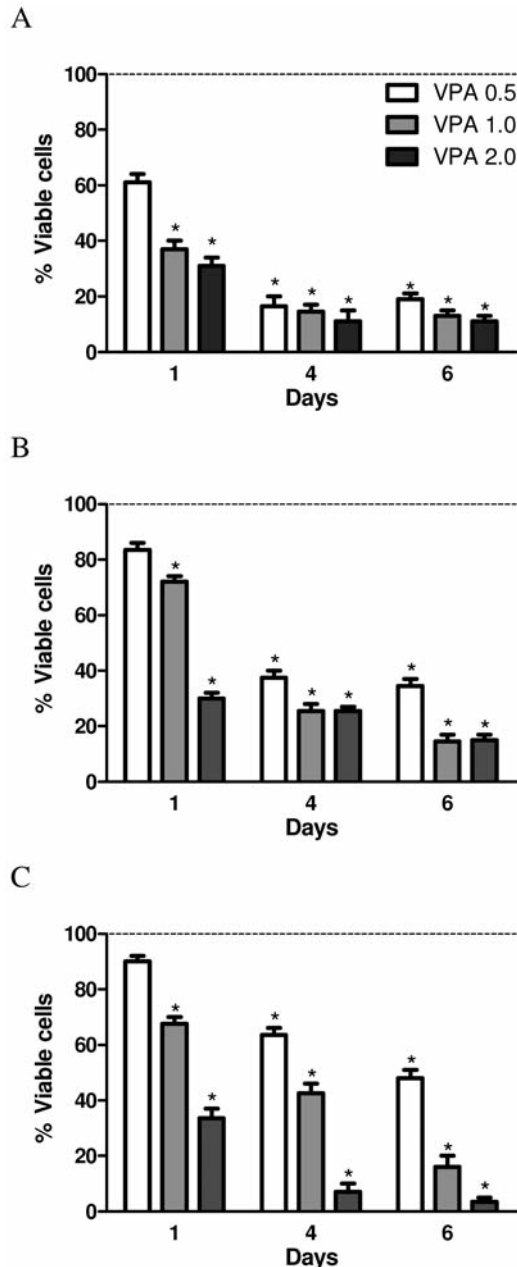


Figure 2. Effect of different doses (mM) of VPA on G361 (A), SKNMC (B) and U87MG (C) cell growth as assessed by SRB assay with respect to the control (100%). Each bar represents the mean ( $\pm$ SEM) of six replicate cultures from three independent experiments; \* $p$ <0.05 compared to control.

increased rate of apoptosis in cells treated with VPA, even at low concentration (0.5 mM), in all three cell lines with respect to the controls (Figure 3). Since Bcl2 overexpression and Bax underexpression have been demonstrated in many cancer cells, where their modulation is indicative of apoptosis, we evaluated the effect of VPA on these factors. Western blotting analysis demonstrated that exposure to VPA modified the basal level of

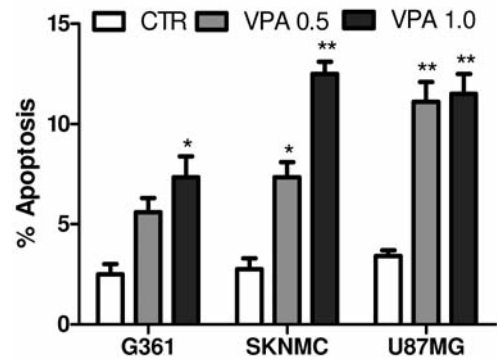


Figure 3. Apoptosis induction after 24 h exposure to VPA (mM) in G361, SKNMC and U87MG cells as assessed by annexin V assay. Apoptosis was evaluated by counting FITC-labeled cells in at least five random fields expressed as percentage of total cells. CTR: Untreated cells. Each bar represents the mean ( $\pm$ SEM) of four experiments, performed in duplicate; \* $p$ <0.05, \*\* $p$ <0.01 compared to control.

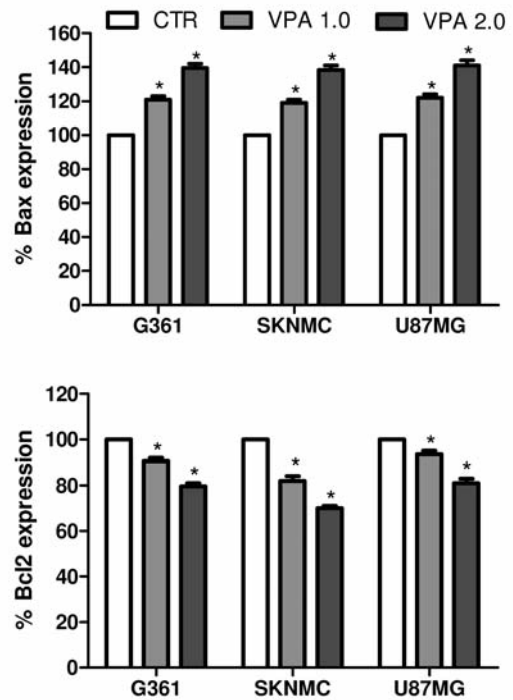


Figure 4. Western blot analysis of Bcl2 and Bax protein level in G361, SKNMC and U87MG cells after 24 h exposure to VPA (mM). Densitometric data are expressed as the percentage of treated samples with respect to the control (CTR). Each bar represents the mean ( $\pm$ SEM) of three independent experiments; \* $p$ <0.05 compared to control.

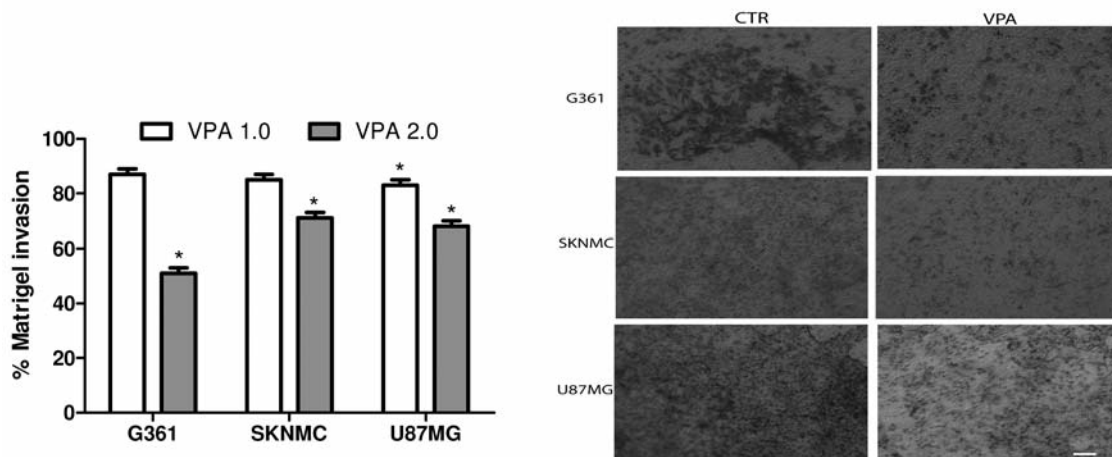


Figure 5. Matrigel invasion assay on G361, SKNMC and U87MG cell lines after 24 h treatment with VPA (mM). Cells migrated through the matrigel-coated membranes were fixed, stained, photographed and counted under light microscopy; bar: 200  $\mu$ m. Densitometric data are expressed as the percentage of treated samples with respect to control (100%). Each bar represents the mean ( $\pm$ SEM) of three experiments, performed in duplicate; \* $p$ <0.05 compared to control.

these proteins: a significative dose-dependent increase of Bax expression and a concurrent significant dose-dependent decrease of Bcl2 occurred after VPA treatment in G361, SKNMC and U87MG cells (Figure 4). Invasion Matrigel assay was performed in order to determine the ability of VPA to inhibit tissue invasion (Figure 5). Our results showed that treatment with VPA was followed by a decrease in the invasive activity in all the three cell lines, especially in G361 melanoma cells where 2.0 mM VPA caused a 50% reduction in the migrating cells with respect to the controls. As invasiveness is mediated by the effect of MMP, and in particular by MMP2 and MMP9, we measured the activity of these two gelatinases. As detected by gelatin zymography, a decrease in gelatinolytic activity of MMP2 and MMP9 was observed after exposure to VPA in all three cell lines (Figure 6). Furthermore, the expressions of MMP2 and MMP9 in VPA-treated cells were reduced with respect to controls, as shown by Western blot analysis (Figure 7). In order to determine whether the exerted effect of VPA on MMPs was accompanied by an effect on TIMPs, we analysed TIMP1 and TIMP2 expression level by Western blotting. As shown in Figure 8, treatment with VPA produced an increase of TIMP1 expression level in all three cell lines, while it did not modify the basal level of TIMP2 (data not shown).

## Discussion

Epigenetic therapy represents a novel and alternative therapeutic approach in the treatment of cancer. Among epigenetic drugs, inhibitors of HDAC have shown biological activity in several studies on different tumors (4, 7, 8). HDAC inhibitors can suppress the activity of multiple HDACs, leading to an increase in histone acetylation. Histone acetylation can induce an

enhancement of gene expression that elicits extensive cellular morphological and metabolic changes, such as growth arrest and differentiation in cancer cells, both *in vivo* and *in vitro* (14-16). We reported previously that short-chain fatty acid HDAC inhibitors, such as valproic acid, tributyrin and sodium butyrate, induced differentiation and growth inhibition in neuroblastoma cells, involving the cyclin-dependent kinase inhibitor p21<sup>WAF1/CIP1</sup> in their mechanism of action (10). These results prompted us to investigate the anticancer activity of VPA in other neural crest-derived human malignancies. In the current investigation, we firstly evaluated the effect of VPA in inducing growth arrest in G361 melanoma, U87MG glioblastoma and SKNMC Askin tumor cells. In all three cell lines, treatment with VPA caused a pronounced dose dependent growth inhibition. This effect was accompanied by a proapoptotic action. During apoptosis, the exposure of phosphatidylserine (PS) residues at the outer plasma membrane has been demonstrated to occur early in the apoptotic process, preceding the loss of plasma membrane integrity and DNA fragmentation (17). Treatment with VPA increased PS exposure, as detected by Annexin V assay, in all three cell lines. The induction of apoptosis was confirmed by modulation of pro- and anti-apoptotic factors, Bax and Bcl2 respectively. In particular, we found that exposure to VPA enhanced the expression level of Bax, whereas it induced down-regulation of Bcl2. Our observation that VPA induced proapoptotic activity and modulated apoptosis-related proteins is consistent with a previously demonstrated role for VPA and other HDAC inhibitors in affecting apoptotic pathways in cancer cells (18-20). Invasion of tumor cells into normal tissue involves the interaction of tumor cells with extracellular matrix and surrounding cells, and their ability to secrete matrix-degrading



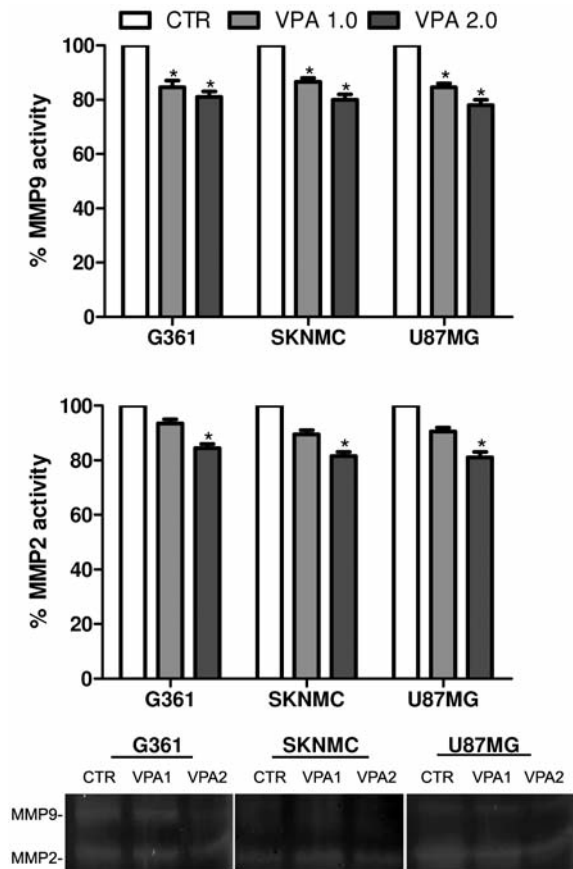


Figure 6. Effect of VPA (mM) on MMP2 and MMP9 activity of G361, SKNMC and U87MG cell, as assessed by zymography after 24 h of treatment. Gelatin zymogram showed the MMP9 (91 kDa) and MMP2 (72 kDa) activity in serum-free conditioned media. Densitometric data are expressed as the percentage that of treated samples with respect to control (CTR). Each bar represents the mean ( $\pm$ SEM) of three independent experiments; \* $p$ <0.05 compared to control.

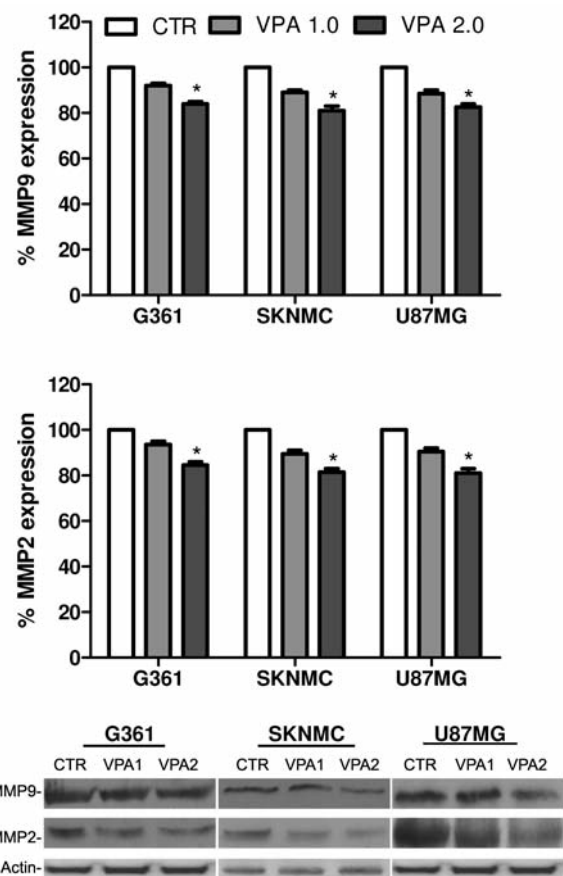


Figure 7. Effect of VPA (mM) on MMP9 and MMP2 protein levels as assessed by Western blot of cell lysates of G361, SKNMC and U87MG cells, after 24 h of treatment. Densitometric data are expressed as the percentage that of treated samples with respect to control (CTR). Each bar represents the mean ( $\pm$ SEM) of three independent experiments; \* $p$ <0.05 compared to control.

proteases. Several models have been used to study migration and invasiveness in cancer cells, the most widely used being the Matrigel invasion assay. By using this test, we found that VPA led to a dose-dependent decrease in cell migration in all three cell lines, confirming the effectiveness of HDAC inhibitors at reducing the invasive capacity (21). Since cell invasion involves a complex system of tightly regulated proteases, the MMPs, and their specific inhibitors, TIMPs, we analyzed the effect of VPA on expression and activity of these proteins. In all three cell lines, we found that exposure to VPA resulted in a decrease of MMP2 and MMP9 expression and lytic activity, as demonstrated by Western blotting and zymography. In addition, exposure to VPA led to enhanced expression of TIMP1. These results are consistent with a previously demonstrated role for other HDAC inhibitors in inhibiting the *in vitro* migration of cancer cells by concomitantly down-regulating protein level and activity of MMPs and up-regulation of TIMP protein levels

(22). Furthermore, as HDAC inhibitors function by inhibiting histone deacetylases, we analyzed the effect of VPA on these protein levels by Western blot assay: we found that VPA inhibited class I HDAC, a result in agreement with a previous study (16). Several HDAC inhibitors are currently in phase I and phase II clinical trials as cancer therapeutics, however, the use of some of the established HDAC inhibitors is limited by their toxicity (23, 24). In contrast with other HDAC inhibitors, VPA is a clinically well-characterized and well-tolerated drug, and its toxicological and pharmacological profiles have been well studied both in adults and in children (25). Collectively, our findings demonstrate the effectiveness of VPA in down-modulating the malignant phenotype of neural crest-derived cancer cells in different ways, including the inhibition of growth, the induction of apoptosis and a reduction in the invasive and metastatic potential, suggesting a role for VPA in the treatment of neural crest-derived tumors.

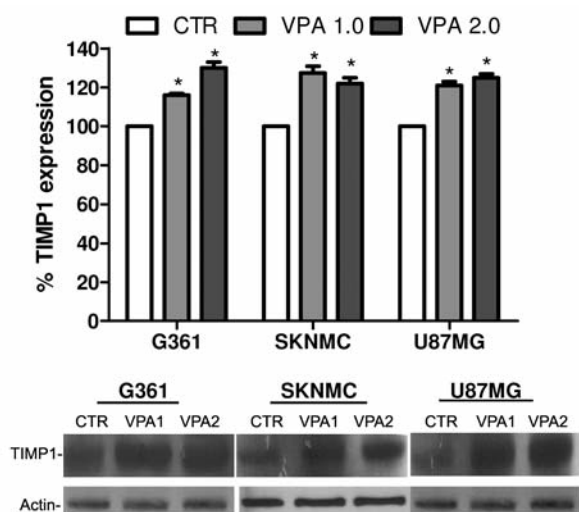


Figure 8. Effect of VPA (mM) on TIMP1 protein levels as assessed by Western blot of cell lysates of G361, SKNMC and U87MG cells after 24 h of treatment. Densitometric data are expressed as the percentage that of treated samples with respect to control (CTR). Each bar represents the mean ( $\pm$ SEM) of three independent experiments; \* $p < 0.05$  compared to control.

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## References

- Marson CM: Histone deacetylase inhibitors: design, structure-activity relationship and therapeutic implications for cancer. *Anticancer Agents Med Chem* 9: 661-692, 2009.
- Glass CK and Rosenfeld MG: The co-regulator exchange in transcriptional functions of nuclear receptors. *Genes Dev* 14: 121-141, 2000.
- Kouzarides T: Acetylation: a regulatory modification to rival phosphorylation? *EMBO J* 19: 1176-1179, 2000.
- Bolden JE, Peart MJ and Johnstone RW: Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discov* 5: 769-784, 2006.
- Santini V, Gozzini A and Ferrari G: Histone deacetylase inhibitors: molecular and biological activity as a premise to clinical application. *Curr Drug Metab* 8: 383-393, 2007.
- Kramer OH, Gottlicher M and Heinzel T: Histone deacetylase as a therapeutic target. *Trends Endocrinol Metab* 12: 294-300, 2001.
- Marks PA and Xu WS: Histone deacetylase inhibitors: Potential in cancer therapy. *J Cell Biochem* 107: 600-608, 2009.
- Marks PA, Miller T and Richon VM: Histone deacetylases. *Curr Opin Pharmacol* 3: 344-351, 2003.
- Kramer OH, Knauer SK, Zimmermann D, Stauber RH and Heinzel T: Histone deacetylase inhibitors and hydroxyurea modulate the cell cycle and cooperatively induce apoptosis. *Oncogene* 27: 732-740, 2008.
- Rocchi P, Tonelli R, Camerin C, Purgato S, Fronza R, Bianucci F, Guerra F, Pession A and Ferreri AM: p21<sup>Waf1/Cip1</sup> is a common target induced by short-chain fatty acid HDAC inhibitors (valproic acid, tritutyryn and sodium butyrate) in neuroblastoma cells. *Oncology Rep* 13: 1139-1144, 2005.
- Pauwels R, Balzarini J, Baba M, Snoeck R and Schols D: Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J Virol Methods* 20: 390-391, 1988.
- Leber TM and Balkwill FR: Zymography: a single-step staining method for quantitation of proteolytic activity on substrate gels. *Anal Biochem* 249: 24-28, 1997.
- Lowry DH, Rosebrough NJ, Farr AL and Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275, 1951.
- Milutinovic S, D'Alessio AC, Detich N and Szyf M: Valproate induces widespread epigenetic reprogramming which involves demethylation of specific genes. *Carcinogenesis* 28: 560-571, 2007.
- Gottlicher M, Minucci S, Zhu J, Kramer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG and Heinzel T: Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J* 20: 6969-6978, 2001.
- Gurvich N, Tsygankoval OM, Meinkoth JL and Klein PS: Histone deacetylase is a target of valproic acid-mediated cellular differentiation. *Cancer Res* 64: 1079-1086, 2004.
- Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST and Van Oers MH: Annexin V for flow cytometric of phosphatidylserine expression on B-cells undergoing apoptosis. *Blood* 84: 1415-1420, 1994.
- Valentini A, Gravina P, Federici G and Bernardini S: Valproic acid induces apoptosis, p16<sup>INK4A</sup> up-regulation and sensitization to chemotherapy in human melanoma cells. *Cancer Biol Ther* 6: 185-191, 2007.
- Furchert SE, Lanvers-Kaminsky C, Jurgens H, Jung M, Loidi A and Fruhwald MC: Inhibitors of histone deacetylases as potential therapeutic tools for high-risk embryonal tumors of the nervous system of childhood. *Int J Cancer* 120: 1787-1794, 2007.
- Facchetti F, Previdi S, Ballarini M, Minucci S, Perego P, La Porta CA: Modulation of pro- and anti-apoptotic factors in human melanoma cells exposed to histone deacetylase inhibitors. *Apoptosis* 9: 573-582, 2004.
- Mottet D and Castronovo V: Histone deacetylases: target enzymes for cancer therapy. *Clin Exp Metastasis* 25: 183-189, 2008.
- Klisovic DD, Klisovic MI, Efron D, Liu S, Marcucci G and Katz SE: Depsipeptide inhibits migration of primary and metastatic uveal melanoma cell lines *in vitro*: a potential strategy for uveal melanoma. *Melanoma Res* 15: 147-153, 2005.
- Sandor V, Bakke S, Robey RW, Kang MH, Blagosklonny MV, Bender J, Brooks R, Piekarsz RL, Tucker E, Figg WD, Chan KK, Goldspiel B, Fojo AT, Balcerzak SP and Bates SE: Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176), in patients with refractory neoplasms. *Clin Cancer Res* 8: 718-728, 2002.
- Bruserud O, Stapnes C, Ersvaer E, Gjertsen BT and Rynningen A: Histone deacetylase inhibitors in cancer treatment: a review of the clinical toxicity and the modulation of gene expression in cancer cells. *Curr Pharm Biotechnol* 8: 388-400, 2007.
- Baheta RA, Nau H, Michaelis M and Cinatl J Jr: Valproate and valproate-analogues: potent tools to fight against cancer. *Curr Med Chem* 9: 1417-1433, 2002.

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