Anti-invasive Effects and Proapoptotic Activity Induction by the Rexinoid IIF and Valproic Acid in Combination on Colon Cancer Cell Lines

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Abstract. *In this study, we investigated the antiproliferative and anti-invasive mechanism action of sodium valproate (VPA), an inhibitor of histone deacetylase (HDAC) activity, in combination with the rexinoid 6-OH-11-O-hydroxyphenanthrene (IIF), a ligand of retinoid X receptor (RXR), in the HT-29 and LoVo colon cancer cell lines. VPA inhibited HDAC-1 and increased RXRγ expression. VPA and IIF reduced viability in a dose- and time-dependent manner. The combined use of VPA and IIF enhanced the apoptosis induction. In particular, the BCL2 level decreased, while levels of BAX, cleaved caspase-3 and caspase-9 increased. The same treatment also reduced invasiveness of HT-29 cell line through the inhibition of metalloproteinase-9 (MMP9) expression, and MMP9 and MMP2 activity, with an increase of tissue inhibitors of MMPs TIMP1 and TIMP2. In conclusion, VPA and IIF have strong proapoptotic and anti-invasive effects in the HT-29 colon cancer cell line and their effects are enhanced when used together.*

Transcriptional regulation is a major event in growth arrest, differentiation and/or apoptotic cell death of cancer cells *in vivo* and *in vitro* (1, 2). Acetylation and deacetylation of nucleosome core histones play important roles in the modulation of chromatin structure and the regulation of gene transcription (3, 4). Histone deacetylase (HDAC) inhibitors are emerging as a promising class of antineoplastic agents for the treatment of solid and hematological malignancies (5-7). Valproic acid (VPA) is an eight-carbon branched-chain fatty acid with anticonvulsant properties; it is often the drug of choice in treating some forms of epilepsy. VPA was demonstrated to exert antitumor activity as an HDAC inhibitor (8, 9) and recent studies suggest that combination of retinoids plus HDAC inhibitors may have a greater therapeutic action than treatment with either drug alone (10, 11). Retinoids are known to have effects on the regulation of cell growth, differentiation and apoptosis (12-14). The retinoid signal is mediated through specific nuclear receptors: the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). RXRs can form heterodimers with peroxisome proliferator-activated receptors (PPARs), members of the nuclear receptor superfamily which act as ligand-activated transcription factors with antitumoral properties (15). Many retinoids able to bind RXR, rexinoids, with high affinity have been synthesized, among which is the derivative of all-*trans* retinoic acid (RA), 6-OH-11-*O*-hydroxyphenanthrene (IIF). In previous studies, we demonstrated that this compound had a strong antitumoral effect on several types of carcinoma cells with higher effects with respect to RA, the ligand of RAR (16-19). The rexinoid IIF also has greater antitumor effects when used in combination with ligands of PPARγ *in vitro* (20, 21) and *in vivo* (22). Thus, it is confirmed that the synergistic use of multiple molecules at lower doses might be a good strategy to kill cancer cells without inducing side-effects in patients (23). The purpose of this study was to examine the antiproliferative and anti-invasive effects of the HDAC inhibitor VPA and the rexinoid IIF in human colon cancer cells and to characterize the molecular mechanism of action of these agents, with the hypothesis of a possible additive or synergic effect.

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Materials and Methods

Cell culture and treatment. HT-29 and LoVo human colon carcinoma cells, purchased from the American Type Culture Collection (Rockville, MD, USA), were maintained in RPMI (Sigma, St Louis, MO, 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_2. VPA (Sigma) was dissolved in the culture medium

immediately before use to obtain final concentrations in the range of 0.5-2.0 mM. IIF (pat. WIPO W0 00/17143 of Dr. K.Ammar, Bologna, Italy) was dissolved in propylene glycol (stock solution 7.8×10^{-2} M).

Cell viability. The effect of VPA (0.5-2.0 mM) and IIF (10-30 μM) on cell viability was measured by trypan blue dye exclusion, sulforhodamine B (SRB) assay and by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described (21).

Detection of apoptosis. Apoptosis was assessed qualitatively after Hoechst 33342 staining to characterize nuclear morphology. Briefly, after treatment, cells were stained with 5 μg/ml of Hoechst 33342 for 10 min at 37˚C. Cells were examined using a fluorescence microscope (Nikon Eclipse TE2000; Nikon, Tokyo, Japan). Normal cells have a smooth, round nucleus characterized by faint staining. Apoptotic cells were identified by the presence of brightly labelled pyknotic nuclei or by chromatin condensation and nuclear fragmentation. To detect caspase-3, cells were grown on coverslips and after treatment they were fixed with 4% paraformaldehyde and treated with Triton X-100 (0.1%) in phosphate buffer saline (PBS) and saturated in PBS-bovine serum albumin (BSA) 4% for 30 min. Cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-cleaved caspase-3 (Sigma) for 1 h at room temperature. After washing, slides were mounted in glycerol-PBS 1× medium containing 30 mg/ml 1,4-diazabicyclo(2,2,2)octane (Sigma). Evaluation of antibody specificity was also carried out by omitting the primary antibody.

Zymography. Cells were seeded and after 18 h were placed in serum-free medium (RPMI) with VPA (1 mM) or IIF (20 μM) for 24 h. MMP2 and MMP9 activity was determined by gelatin zymography as previously described (20). 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 protein levels, the cells were plated and treated with VPA and IIF as described above for zymography. Cell lysates were prepared, run, and blotted as as previously described (20). The membranes were probed with specific antibodies: mouse monoclonal anti-HDAC1 (Epigentek, Farmingdale, NY, USA); anti-PPARγ and anti-RXRγ (Cell Signaling, Danvers, MA, USA) and rabbit polyclonal anti-MMP2, anti-MMP9, anti-TIMP1, anti-TIMP2, anti-caspase-9 (all Santa Cruz Biotechnology, Santa Cruz CA, USA); anti-Bax, anti-Bcl2 (Sigma). Primary antibodies were dissolved in Transfer Buffer Saline (TBS)-5% milk diluted 1:500 overnight at 4˚C, and after washing, with the peroxidase-conjugated antibody. The proteins were detected by luminol (GE Healthcare, Milan, Italy). Bands were quantified by using a densitometric image 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, Milan, Italy) as previously described (20). Cells were treated or not with VPA (1.0 mM) and IIF (20 μM) for 24 h and seeded into the upper part of each chamber for 8 h. The invasion activity was evaluated by counting cells in five random fields using microscopy at ×100 magnification.

Table I. *Combination index. The combination index (CI) for viability assay of sodium valproate (VPA) plus 6-OH-11-O-hydroxyphenanthrene (IIF) after 120 h of treatment was calculated to determine the synergistic (CI<1), additive (CI=1), or antagonistic (CI>1) effects of the combinations using the Loewe method.*

	IIF (μM)		
	10	20	30
LoVo			
VPA 0.5 mM	>1	>1	>1
VPA 1.0 mM	>1	<1	>1
VPA 2.0 mM	<1	<1	>1
HT-29			
VPA 0.5 mM	>1	>1	>1
VPA 1.0 mM	<1	<1	<1
VPA 2.0 mM	<1	<1	<1

Isobologram analysis. The combination index (CI) was calculated with Calcusyn 2.1 software (Biosoft, Cambridge, UK) for experimental treatment combinations to determine the synergistic, additive, or antagonistic effects of the combinations using the Loewe method (24). When the CI is 1, the equation represents the conservation isobologram and indicates additive effects. CI values of <1.0 indicate synergistic effects and >1.0 indicate antagonistic effects.

Statistical analysis. Data are expressed as the mean (±SD). Differences were analyzed by Student's *t*-test and considered statistically significant at $p<0.05$ comparing the control and experimental samples.

Results

We started to investigate the effect on cell viability of VPA, an HDAC inhibitor, and IIF, a rexinoid, on two colon cancer cell lines characterized by a high degree of tumorigenicity: HT-29 and LoVo. We studied if the anticancer efficacy of the two compounds could be enhanced by using them together. Figure 1 shows the effect on viability of various doses of VPA (0.5-2.0 mM) and IIF (10-30 μM) on LoVo (Figure 1A) and HT-29 (Figure 1B) cell lines assessed by the SRB assay after 24, 72 and 120 h. Figure 2 shows the impact of combined treatment with VPA and IIF on viability of the two cell lines. Both compounds reduced cell viability in a dose-dependent manner (Figure 1) but the effect quite dramatically was higher and earlier when the compounds were used in combination (Figure 2). These data were confirmed also by MTT assay (not shown). Isobologram analysis revealed a synergistic effect (CI<1.0) for the combination in both cell lines when VPA and IIF were used at 1.0 mM and 20 μM respectively, in particular in the HT-29 cell line (Table I). For this reason, the subsequent experiments were conducted under this experimental condition and only for the HT-29 cell line.

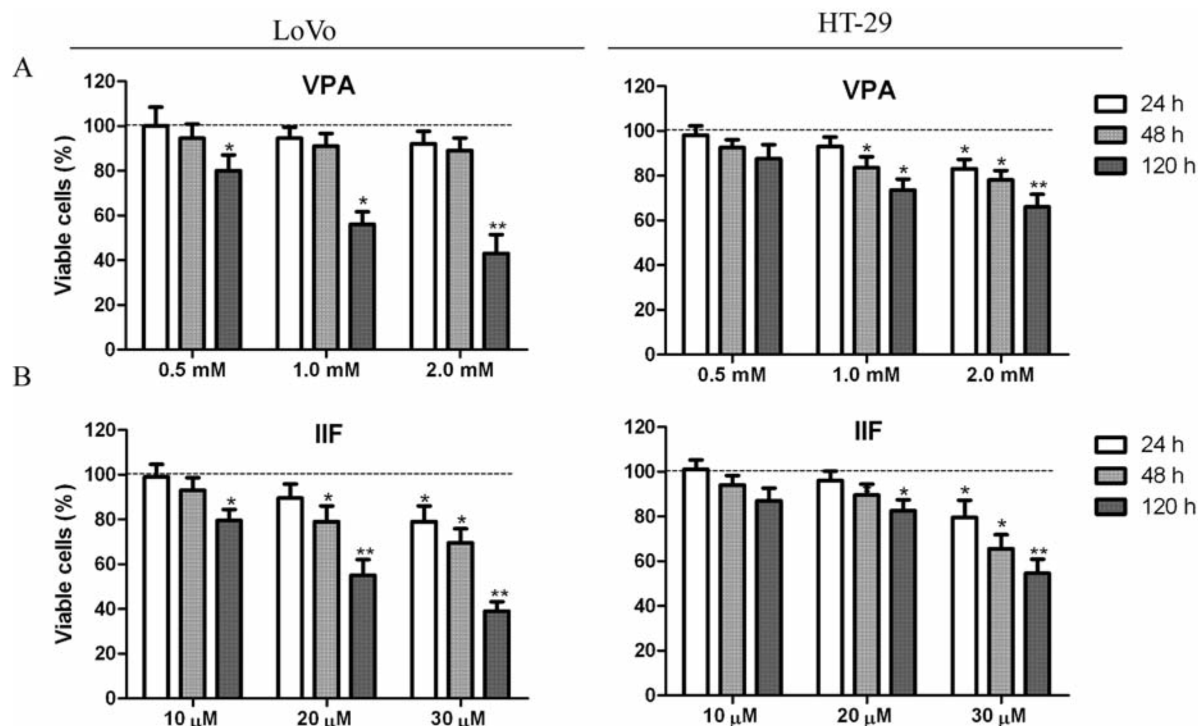

Figure 1. *Effect of different doses of sodium valproate (VPA) (A) and 6-OH-11-O-hydroxyphenanthrene (IIF) (B) on cell growth as assessed by the sulforhodamine B (SRB) assay with respect to the control (100%) of HT-29 and LoVo colon cancer cell lines. Each bar represents the mean (±SD) of six replicate cultures from three independent experiments. *p<0.05; **p<0.01.*

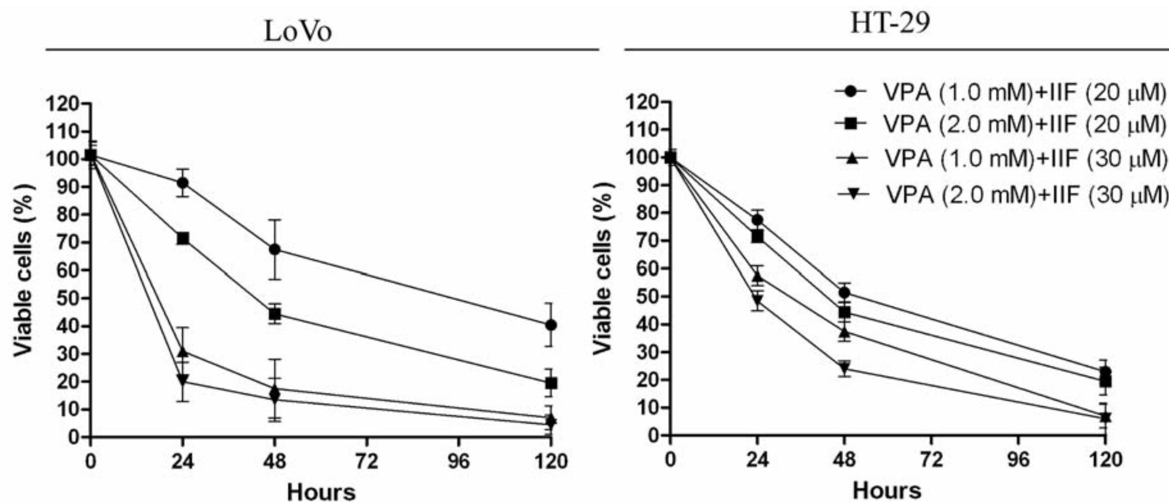

Figure 2. *Effect of different doses of sodium valproate (VPA) and 6-OH-11-O-hydroxyphenanthrene (IIF) in combination on LoVo and HT-29 cell growth as assessed by the sulforhodamine B (SRB) assay with respect to the control (100%). Each bar represents the mean (± SD) of six replicate cultures from three independent experiments. *p<0.05; **p<0.01.*

Western blot analysis revealed that treatment with VPA, alone or in combination with IIF, led to a decrease in HDAC1 expression level (Figure 3). Interestingly, this treatment also increased expression of RXRγ, the main ligand of IIF (20) and of PPARγ, a possible heterodimer of RXRγ (Figure 3). Growth inhibition was accompanied by apoptosis induction: Hoechst assay demonstrated an increased rate of apoptosis after exposure to VPA, and to IIF, and particularly after

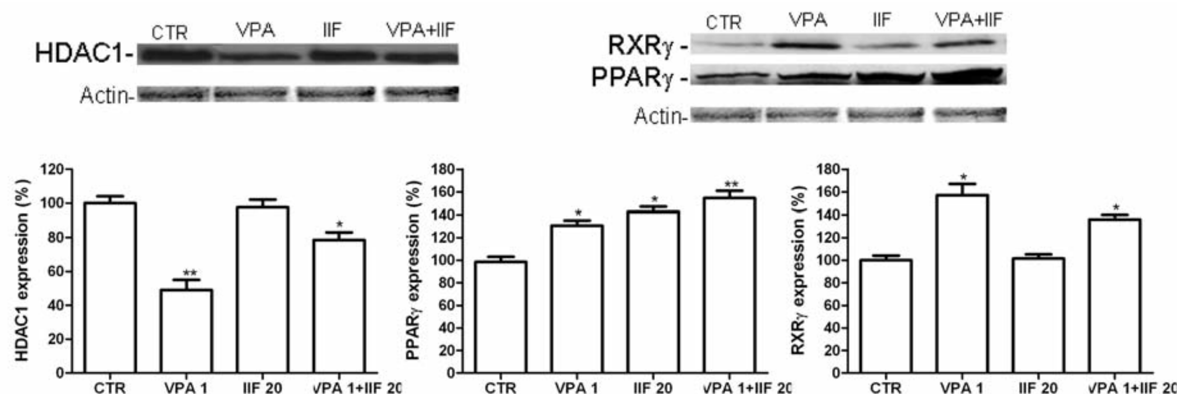

Figure 3. *Effect of sodium valproate (VPA) (1 mM) and 6-OH-11-O-hydroxyphenanthrene (IIF) (20 μM) alone and in combination on histone deacetilase-1 (HDAC-1), retinoid x receptor-γ (RXRγ) and peroxisome proliferator-activated receptor-γ (PPARγ) expression levels, as assessed by western blot in cell lysates of HT-29 cells, after 24 h of treatment. Densitometric data are expressed as a percentage of treated samples with respect to the control (CTR). Each bar represents the mean (±SD) of three independent experiments.* $^*p<0.05$; $^{**}p<0.01$.

combined treatment with VPA plus IIF (Figure 4A). The apoptotic pathway involved a reduction of BCL2 and induction of BAX (Figure 4B) expression, with caspase-9 activation (Figure 4C). Caspase-3 is an effector caspase downstream from both intrinsic and extrinsic apoptotic pathway mediators and it has been documented to be a reliable index of apoptotic response in several tissues, including the intestine (25). Exposure to VPA and IIF resulted in a pronounced increase in caspase-3 activation and –once again- the greatest increase was in response to VPA and IIF in combination (Figure 4D). In order to test the effect of the two compounds on the metastatic potential of the cell lines, we have examined the capacity of cells to migrate by using Matrigel invasion. In Figure 5, it can be seen that treatment with the two compounds in association reduced the migration of cells to a higher degree than using each compound alone. As invasiveness is mediated by MMPs, and in particular by MMP2 and MMP9, and their respective TIMPs, TIMP1 and TIMP2, we measured their expression after treatment with VPA and IIF. Figure 6 shows that the inhibitory effect on MMP9 expression exerted by IIF used alone was enhanced when the compound was added to VPA in the incubation medium. In contrast, the combination of VPA and IIF resulted in an almost 30% increase of TIMP2 and TIMP1 protein levels. Finally, the inhibitory effect on MMP2 and MMP9 activity exerted by VPA and IIF, as measured by zymography, is shown in Figure 7.

Discussion

It is now clear that to fight cancer, it is necessary use multiple drugs that have multiple targets which reduce growth and proliferation of cancer cells (23). Recently, it has been demonstrated that epigenetic therapy represents a novel and alternative therapeutic approach in the treatment of cancer. Among epigenetic drugs, the HDAC inhibitors leading to an increase in histone acetylation and, in consequence, to an enhancement of gene expression, can induce growth arrest and differentiation in colon cancer cells, both *in vivo* and *in vitro* (26). 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 (27). 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 (28). Retinoids are derivatives of vitamin A with important physiological function and are known to induce growth arrest and differentiation in cancer cells (19, 29). Among retinoids, different ligands of RARs, such as RA, are used in the clinical treatment of tumors, but therapy can induce side-effects and induction of multidrug resistance (11). At the same time, rexinoids can have the same antitumor effects as ligands of RARs, but less side-effects, probably because of their different action on gene expression (19, 22, 30). In this study for the first time the antitumor action of VPA in combination with the rexinoid IIF on colon cancer cells is shown. We analyzed the effect of VPA on HDAC protein levels by western blot assay: we found that VPA inhibited class I HDAC, a result which is in agreement with a previous study (9). IIF increased the expression of its ligand RXRγ, as verified in other colon cancer cell lines (21). Moreover, we showed that both IIF and VPA can modulate the expression of PPARγ, a possible heterodimer of RXRs. These data confirm previous research on the effects of HDAC inhibitors on PPARs (31). We showed a strong effect on viability, in particular when the two drugs were used in combination and we demonstrated that if used at low concentrations, there was a synergic effect (CI<1). These

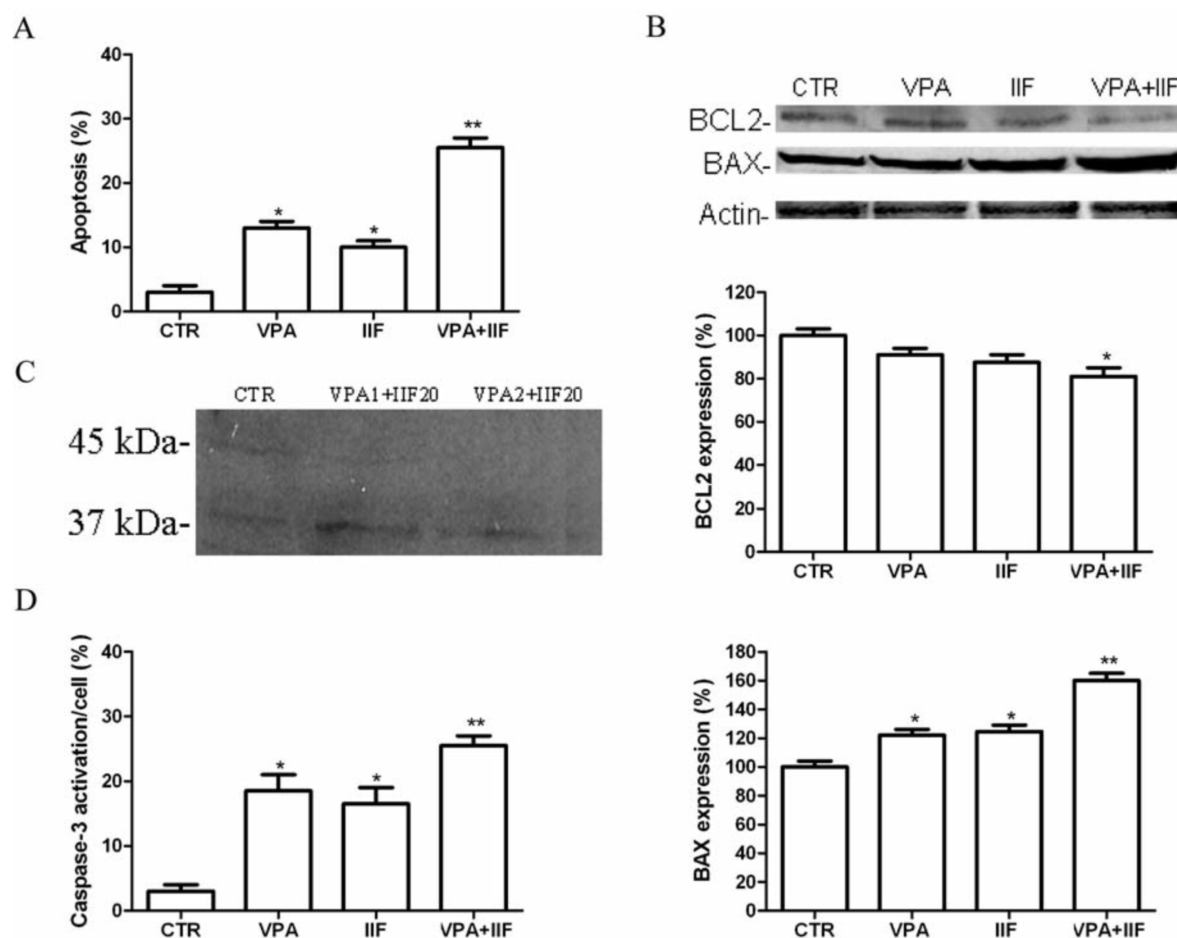

Figure 4. *Apoptosis induction after 24 h exposure to sodium valproate (VPA) (1 mM) and 6-OH-11-O-hydroxyphenanthrene (IIF) (20 μM) alone and in combination on HT-29 cells. A: Apoptosis was evaluated by counting Hoechst labeled cells with fragmented nuclei in at least five random fields expressed as a percentage of the total cells. CTR: untreated cells. Western blot analysis for BAX and BCL2 (B) and caspase-9 (active form: 37 kDa) (C) proteins in HT-29 cells. Densitometric data are expressed as a percentage of treated samples with respect to the control (CTR). D: Cleaved caspase-3-fluorescein isothiocyanate (FITC)-conjugated cells were counted in at least five random fields, expressed as a percentage of the total cells. Each bar represents the mean (±SD) of three experiments. *p<0.05; **p<0.01.*

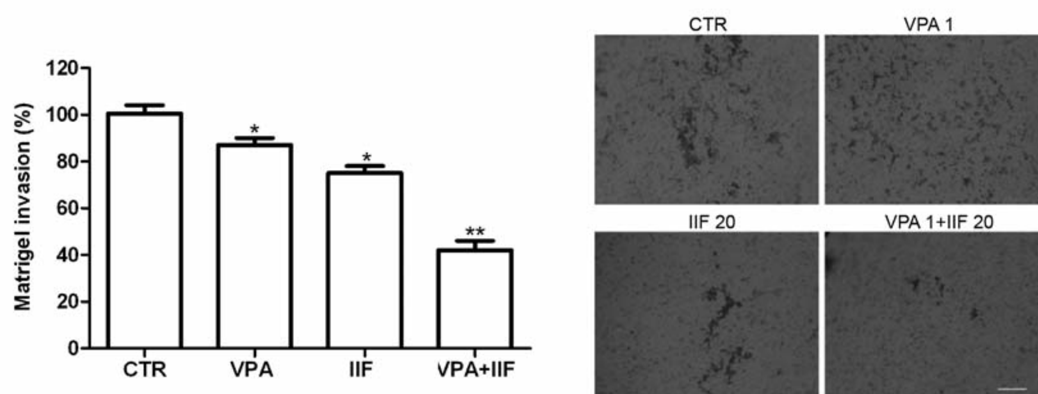

Figure 5. *Matrigel invasion assay on HT-29 cells after 24 h treatment with sodium valproate (VPA) (1.0 mM) and 6-OH-11-O-hydroxyphenanthrene (IIF) (20 μM). Cells migrated through the matrigel-coated membranes were fixed, stained, photographed and counted under light microscopy; bar: 1000 μm. Densitometric data are expressed as the percentage of treated samples with respect to the control (100%). Each bar represents the mean (±SD) of three experiments. *p<0.05 compared to control.*

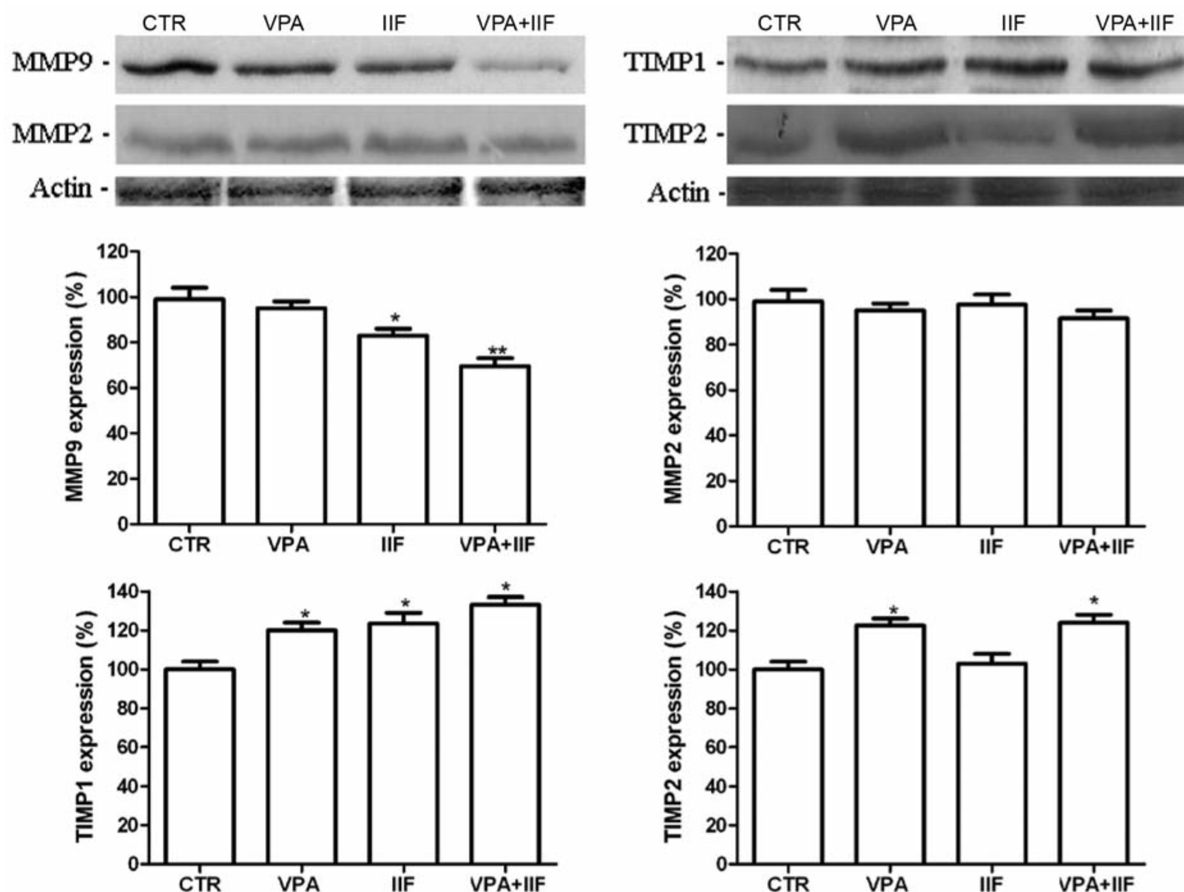

Figure 6. *Western blot analysis on metalloproteinase-2 (MMP2), metalloproteinase-9 (MMP9), tissue inhibitors of MMPs-1 (TIMP1) and tissue inhibitors of MMPs-2 (TIMP2) protein level, after 24 h exposure to sodium valproate (VPA) (1.0 mM) and 6-OH-11-O-hydroxyphenanthrene (IIF) (20 μM) alone and in combination on HT-29 cells. Densitometric data are expressed as the percentage of treated samples with respect to the control (CTR). Each bar represents the mean (±SD) of three independent experiments.*p<0.05; **p<0.01.*

results are very interesting because they indicate a possible application *in vivo*. Effects on viability were due to the induction of apoptosis by modulation of the pro- and anti-apoptotic factors, BAX and BCL2, respectively. In particular, we found that exposure to VPA and IIF enhanced the expression of BAX, whereas it induced down-regulation of BCL2. We demonstrated the activation of the mitochondrial pathway, by activation of caspase-9 and expression of cleaved caspase-3, in particular when the drugs were used in combination. This confirmed the pro-apoptotic effects of VPA (32-34) and IIF (21) on colon cancer cells. 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 proteases. The Matrigel invasion assay confirmed the effectiveness of VPA and IIF in reducing the invasive capacity of cancer cells (21, 35). Since cell invasion involves a complex system of tightly regulated proteases, in particular the MMPs and their specific inhibitors (TIMPs), we analyzed the effects of drugs on expression and activity of these proteins. We found that exposure to VPA and IIF resulted in a decrease of MMP2 and MMP9 lytic activity, but only of MMP9 expression, as demonstrated by zymography and western blotting. In addition, exposure to VPA and IIF led to enhanced expression of TIMP1 and TIMP2. In conclusion, our findings demonstrate the effectiveness of VPA and IIF in the inhibition of growth by induction of apoptosis through the mitochondrial pathway and a reduction in the invasive and metastatic potential, suggesting a role of the two drugs in the treatment of colon cancer, in particular when used in combination.

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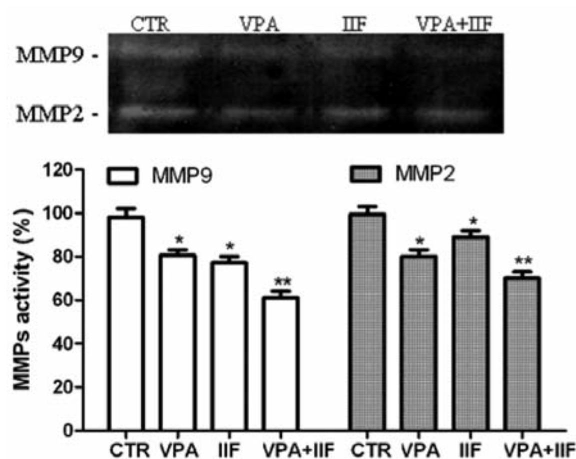

Figure 7. *Effect of sodium valproate (VPA) (1.0 mM) and 6-OH-11-O-hydroxyphenanthrene (IIF) (20 μM) on metalloproteinase-2 (MMP2) and metalloproteinase-9 (MMP9) activity of HT-29 cell line, assessed by zymography after 24 h of treatment. Gelatin zymogram revealed the MMP9 (91 kDa) and MMP2 (72 kDa) activity in serum-free conditioned media. Densitometric data are expressed as the percentage that of treated samples with respect to the control (CTR). Each bar represents the mean (±SD) of three independent experiments. *$p<0.05$; **$p<0.01$.*

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