

Sodium valproate affects the expression of p16^{INK4a} and p21^{WAF1/Cip1} cyclin-dependent kinase inhibitors in HeLa cells

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Abstract. p16^{INK4a} and p21^{WAF1/Cip1} are cyclin-dependent kinase inhibitors involved in cell cycle control, which can function as oncogenes or tumor suppressors, depending on the context of various extracellular and intracellular signals, and cell type. In human papillomavirus-induced cervical cancer, p16^{INK4a} shows oncogenic activity and functions as a diagnostic marker of cervical neoplasia, whereas p21^{WAF1/Cip1} acts as a tumor suppressor and its downregulation is associated with the progression of malignant transformation. Several histone deacetylase (HDAC) inhibitors promote the positive and negative regulation of a number of genes, including p16^{INK4a} and p21^{WAF1/Cip1}; however, the effects of sodium valproate (VPA) on these genes and on the proteins they encode remain uncertain in HeLa cervical cancer cells. In the present study, these effects were investigated in HeLa cells treated with 0.5 or 2 mM VPA for 24 h, using reverse transcription-quantitative PCR, confocal microscopy and western blotting. The results revealed a decrease in the mRNA expression levels of p16^{INK4a} and a tendency for p16^{INK4a} protein abundance to decrease in the presence of 2 mM VPA. By contrast, an increase in the protein expression levels of p21^{WAF1/Cip1} was detected in the presence of 0.5 and 2 mM VPA. Furthermore, VPA was confirmed to inhibit HDAC activity and induce global hyperacetylation of histone H3. Notably, VPA was shown to suppress p16^{INK4a}, a biomarker gene of cervical carcinoma, and to increase the abundance of the tumor suppressor protein p21^{WAF1/Cip1}, thus contributing to the basic knowledge regarding the antitumorogenic potential of VPA. Exploration of epigenetic changes associated with the promoters of p16^{INK4a} and p21^{WAF1/Cip1}, such

as histone H3 methylation, may provide further information and improve the understanding of these findings.

Introduction

The proteins p16^{INK4a} and p21^{WAF1/Cip1} inhibit cyclin/cyclin-dependent kinase (CDK) complexes, in which CDKs depend on cyclins. These proteins affect cell cycle progression in the G₁/S phase by directly interfering with CDK activation and inhibiting DNA replication. Thus, p16^{INK4a} and p21^{WAF1/Cip1} potentially act as tumor suppressor genes (1-5). p16^{INK4a} belongs to the INK4a family and regulates the cell cycle by specifically attaching to and inhibiting the expression of CDK4 and CDK6 (6). p21^{WAF1/Cip1} regulates the cell cycle by inhibiting multiple CDKs, including CDK1, CDK2 and CDK4 (3,4,7-9). Aberrant regulation of these proteins, which have a low molecular mass, is characteristic of cervical carcinoma that expresses human papillomavirus (HPV) E6 and E7 oncogenes and their precursor E2 (4,10-12).

Although the p16^{INK4a} tumor suppressor gene is inactivated by mutations or epigenetic changes that lead to excessive cellular proliferation in most tumors, it is expressed at high levels in cervical cancer cells infected with HPV in which the oncoprotein E7 is expressed (4,5,13-19). Consequently, in HPV-transformed cervical cancer, p16^{INK4a} has oncogenic activity through the CDK6-HuR-IL 1A axis and represents a diagnostic marker of cervical neoplasia. Although the p16^{INK4a} gene is highly expressed in this case, it does not exert a negative physiological effect on the cell cycle (10,12).

Transcriptional silencing of p16^{INK4a} results from DNA hypermethylation of the gene promoter in various tumors (20). However, in cervical cancer induced by HPV infection, in which the p16^{INK4a} protein is highly expressed, complete DNA methylation has been reported in the p16^{INK4a} promoter without any influence on its expression, thus indicating no association between this epigenetic marker and reduced expression of p16^{INK4a} (10,21). The increased expression of p16^{INK4a} may also be regulated by histone modifications, such as H3K4me3 (22), which is a histone mark that has been associated with gene activation (23). A reduction in H3K27me3, a transcriptionally repressive epigenetic mark (24), has been reported to occur in the promoter of p16^{INK4a} and involves the participation of the histone demethylase KDM6B (18,25,26).

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Downregulation of $p21^{WAF1/Cip1}$ has been directly associated with cervical cancer compared with normal epithelium; specifically, in HeLa cells, $p21^{WAF1/Cip1}$ is weakly expressed and is associated with the progression of malignant transformation (4,27). Epigenetic alterations in the $p21^{WAF1/Cip1}$ promoter, including DNA hypermethylation and histone H3 hypoacetylation, are key events in the inactivation of this gene (28,29). Histone acetylation is generally associated with chromatin opening and activated gene expression, although inactivation of inducible promoters enriched for H3K14 acetylation has been reported (30).

Histone deacetylase (HDAC) inhibitors (HDACis), such as sodium valproate (VPA), have been reported to induce increased expression of $p21^{WAF1/Cip1}$ in cervical and breast cancer cell lines and chronic lymphocytic leukemia (31-35). It has been suggested that HDACis stimulate $p21^{WAF1/Cip1}$ expression through a selective increase in the degree of histone H3 acetylation (H3Ac) and a decrease in DNA methylation at the gene promoter in rat hippocampus, colon and bladder cancer cell lines, and human lung carcinoma cells (11,36-40). Increased expression of $p21^{WAF1/Cip1}$ has also been reported to be associated with enhanced methylation levels of H3K4me2/me3, and decreased levels of H3K9me2/me3 in rat kidney cells, suggesting a role of methylated H3K in TGF- β 1-mediated $p21$ gene expression and its protective potential in managing chronic renal diseases (41,42).

VPA is an anticonvulsive drug that has been reported to exhibit antitumor effects, either alone or in combination with other drugs, against several cancer types (31,43-51). This drug acts through various mechanisms that involve inhibition of the neurotransmitter γ -aminobutyric acid, and blockage of T-type calcium and voltage-gated sodium channels, and that affect several epigenetic markers and chromatin supraorganization (49,52-54). VPA may also directly interact with isolated DNA and histones H1 and H3 *in vitro*, and affect chromatin at the nucleosome level (55-58).

VPA acts on epigenetic marks by inhibiting class I and II HDACs, often favoring the acetylation of histones H3 and H4 (31,44,59). Moreover, in HeLa cells, a widely used model of cervical cancer, VPA can promote DNA demethylation with the participation of TET and DNMT1 enzymes, and can change the methylation status of different lysine residues in histone H3, in addition to histone acetylation (60-64). Consequently, VPA alters the epigenetic landscape of HeLa cells by modulating the expression of their genes (60).

Considering that VPA promotes cell cycle arrest at the G₁ phase and induces changes in the methylation levels of histones in HeLa cells (63,64), it would be relevant to detect whether this drug induces changes in the expression of genes such as $p21^{WAF1/Cip1}$, which participates negatively in the cell cycle, and $p16^{INK4a}$, which is a biomarker of cervical neoplasia (65). In the present study, the effects of VPA on changes in $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ genes were investigated in HeLa cells to demonstrate whether VPA modulates the expression of a cervical carcinoma biomarker and a tumor suppressor gene. The enzymatic activity of HDAC and the acetylation of histone H3 were also evaluated in this context. This investigation intended to improve understanding of the antitumorigenic effects of VPA, in addition to the alterations in DNA and histone methylation status, and chromatin supraorganization previously reported for these cells (60-64).

Materials and methods

Cell culture and VPA treatments. HeLa cells were acquired at passage 10 from the Emerging Virus Studies Laboratory, University of Campinas (Campinas, Brazil) and were validated at the Technical Division of Support for Teaching, Research, and Innovation, Faculty of Medicine Foundation, University of São Paulo (São Paulo, Brazil). The cells were used at passages 11-45 and were cultured in high-glucose Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% bovine fetal calf serum (FCS; Vitrocell Embriolife), penicillin/streptomycin (100 IU and 100 μ g/ml, respectively; Sigma-Aldrich; Merck KGaA) and 1% sodium pyruvate (Sigma-Aldrich; Merck KGaA) at 37°C in 5% CO₂. For cell treatment, the cells were cultured for 24 h in medium containing 1% FCS and 0.5 or 2.0 mM VPA (Santa Cruz Biotechnology, Inc.), preceded by cell culture for 24 h in the absence of the drug. When HeLa cells were cultured for 24 h in the presence of 1 and 2 mM VPA, cell viability reached values of 94 and 89%, respectively, as detected using the MTT assay (66). Based on previously reported analyses, under 0.5 and 2.0 mM VPA treatment conditions for 24 h, HeLa cells exhibited G₁ phase cell cycle arrest and no induction of apoptotic cell death (67,68). When quantifying DNA fragmentation using the TUNEL assay or calculating cell death ratios in preparations subjected to the Feulgen reaction, the exposure of HeLa cells to 1 mM VPA for 24 h did not result in an increase of apoptosis (61). In the present study, control cells were cultured in the absence of VPA. For immunofluorescence assays, the cells were seeded onto round coverslips in 24-well plates at a concentration of 5x10⁴ cells/ml and 100 μ l/well. For western blotting (WB) and HDAC activity assays, the cells were cultured in 6-well plates at a concentration of 1.0x10⁵ cells/ml and 4 ml/plate. For reverse transcription-quantitative PCR (RT-qPCR), the cells were cultured in 25-cm² culture flasks at a concentration of 6x10⁴ cells/ml and 5 ml/flask.

Immunofluorescence. Cells were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 10 min at 25°C, rinsed in PBS, permeabilized with 0.2% Triton X-100 (MilliporeSigma) for 10 min at 25°C and blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) for 30 min at 25°C. The cells were then incubated overnight with mouse anti-p16^{INK4a} (1:100 dilution; cat. no. sc-56330), mouse anti-p21^{WAF1/Cip1} (1:100 dilution; cat. no. sc-6246) (both from Santa Cruz Biotechnology, Inc.) and rabbit anti-H3Ac (1:1,000 dilution; cat. no. 06-599; Sigma-Aldrich; Merck KGaA) primary antibodies in 1% BSA blocking solution at 4°C in the dark, followed by extensive PBS washes. To detect p16^{INK4a} and p21^{WAF1/Cip1}, the cells were incubated with a FITC-conjugated goat anti-mouse antibody (1:50 dilution; cat. no. F0257; Sigma-Aldrich; Merck KGaA) for 1 h at 4°C in the dark, followed by nuclear counterstaining with TO-PRO-3 (1:1,000 dilution; Thermo Fisher Scientific, Inc.) for 1 h at 4°C. To detect H3Ac, an Alexa-Fluor 488-conjugated goat anti-rabbit secondary antibody (1:1,000; cat. no. A-11008; Thermo Fisher Scientific, Inc.) was used to incubate the cells for 1 h at 4°C in the dark, followed by counterstaining with DAPI for 5-10 min at 25°C. The preparations were then rinsed in PBS and mounted using VECTASHIELD (Vector Laboratories, Inc.). The images were captured using a Leica

TCS SP5 II confocal microscope (Leica Microsystems GmbH) at the Central Laboratory of High-Performance Technology in Life Science (University of Campinas).

WB. The p16^{INK4a}, p21^{WAF1/Cip1} and H3Ac proteins were examined after total proteins were extracted from HeLa cells using RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 0.5 mM EGTA, and 1 mM PMSF] for ≥30 min on ice. The Bradford assay (Sigma-Aldrich; Merck KGaA) was used to detect protein concentrations, using BSA as a standard. Absorbance values were quantified after all samples were incubated for 1 h at room temperature at 595 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Inc.). Protein samples (60 µg) were then incubated in heated sample buffer [0.06 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.025% Bromophenol Blue] for 5 min and were separated by SDS-PAGE on 15% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes (Thermo Fisher Scientific, Inc.), which were blocked in 4% BSA for 2 h, at 25°C and separately incubated with mouse anti-p16^{INK4a} (1:150; cat. no. MA5-17054; Thermo Fisher Scientific, Inc.) mouse anti-p21^{WAF1/Cip1} (1:100; cat. no. 1026-MSM11-P1; Thermo Fisher Scientific, Inc.) and rabbit anti-H3Ac (1:4,000; cat. no. PA5-114693; Thermo Fisher Scientific, Inc.) primary antibodies overnight in 1X Tris-buffered saline -0.1% Tween 20 (TBST; cat. no. 91414; Sigma-Aldrich; Merck KGaA) blocking solution at 4°C. After extensive washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse (1:2,000; cat. no. 1706516; Bio-Rad Laboratories, Inc.) and anti-rabbit (cat. no. 31460; Invitrogen; Thermo Fisher Scientific, Inc.) secondary antibodies to detect p16^{INK4a}, or p21^{WAF1/Cip1} and H3Ac, respectively; for detection of p21^{WAF1/Cip1} a dilution of 1:2,000 was used, whereas for H3Ac a dilution of 1:4,000 was used. In all cases, incubation was performed in 1% BSA blocking solution for 2 h at 25°C. Protein blots were imaged using an ECL Western Blotting Detection System (Amersham; Cytiva) and were visualized by chemiluminescence using a ChemiDoc Imaging System (Bio-Rad Laboratories, Inc.) at the Laboratory of Tissue Biology of the University of Campinas. As a control for differences in protein loading, the membranes were incubated overnight at 4°C with rabbit anti-β-actin primary antibody (1:1,000 dilution; cat. no. 4970; Cell Signaling Technology, Inc.), followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:4,000 dilution; cat. no. 31460; Invitrogen Thermo Fisher Scientific, Inc.) for 1 h at 4°C. ImageJ version II 1.46r software (National Institutes of Health) was used to estimate p16^{INK4a}/β-actin, p21^{WAF1/Cip1}/β-actin and H3Ac/β-actin ratios. The assays were repeated five times.

HDAC assay. The enzymatic activity of HDAC in VPA-treated HeLa cells, expressed relative to untreated controls, was detected using an HDAC assay kit (cat. no. CS1010; Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. Cells were lysed in RIPA buffer and incubated in 96-well plates with the reaction substrate (peptide with acetylated lysine and a fluorescent group attached) for 30 min at 30°C. The revealing reaction solution was then added,

promoting the breakage of the deacetylated substrate by the HDACs present in the samples and the liberation of the fluorescent group. Subsequently, the solution was incubated for 10 min at room temperature. Fluorescence was measured at 360 nm (test wavelength) and 460 nm (reference wavelength) using a Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Inc.).

RT-qPCR. Total RNA was isolated using the PureLink RNA Mini Kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. RNA integrity number was evaluated using a Nano-Vue spectrophotometer (Cytiva). RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The PCR primers were obtained from data reported in the literature (Table I) (32,69,70). Subsequently, 1 µl cDNA (4 ng/µl) was amplified using the Real Q Plus 2X Master Mix Green, High ROX kit (cat. no. A323402; Ampliqon A/S) and 400 nM of each primer in a final volume of 20 µl. The cycling conditions were as follows: 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 1 min. Expression levels were detected using Bio-Rad CFX Maestro (Bio-Rad Laboratories, Inc.). The dissociation curve was evaluated to confirm specific amplification. The data were normalized using the Q-Gene program version 4.3 (71,72). Cycle threshold values were calculated from experiments performed in triplicate and normalized with respect to the housekeeping gene *GAPDH*. Relative quantification was achieved using the comparative 2^{-ΔΔC_q} method (73).

Statistical analysis. GraphPad Prism version 9.5.0 (525) (Dotmatics) was used for statistical analysis. For comparisons between more than two groups, one-way ANOVA followed by Dunnett's test was used for WB data, and Kruskal-Wallis followed by Dunn's test was used for fluorescence intensity (FI) and RT-qPCR data. Mann-Whitney U test was applied to compare H3Ac FI data between two groups. To compare HDAC activity between more than two groups, one-way ANOVA and Dunnett's post hoc test were used. P<0.05 was considered to indicate a statistically significant difference.

Results

VPA affects p16^{INK4a} protein and gene expression in HeLa cells. HeLa cells cultured in the presence of 2 mM VPA exhibited a significant average decrease of ~11% in the nuclear protein abundance of p16^{INK4a}, and an average decrease of ~45% in the mRNA expression levels of p16^{INK4a} in comparison to untreated controls, based on the immunofluorescence data and RT-qPCR results (Fig. 1A-D). Although the results obtained by WB did not indicate a statistically significant difference in the protein expression levels of p16^{INK4a}, there was a tendency for p16^{INK4a} expression to decrease in response to VPA treatment (Fig. 1E).

VPA affects p21^{WAF1/Cip1} protein and gene expression in HeLa cells. Immunofluorescence and WB analyses revealed that VPA treatment increased p21^{WAF1/Cip1} protein abundance in a dose-dependent manner (Fig. 2A-C and E). When considering the immunofluorescence data, average increases of ~42 and

Table I. Primers used for reverse transcription-quantitative PCR.

Genes	Sequences	(Refs.)
<i>p16</i>	F: CAACGCACCGAATAGTTACGG	(69)
	R: GCGCAGTTGGGCTCCG	(69)
<i>p21</i>	F: TGATGCGCTAATGGCGGGCT	(32)
	R: TGCTGGTCTGCCGCCGTTT	(32)
<i>GADPH</i>	F: GAATGGGCAGCCGTTAGGAA	(70)
	R: ATCACCCGGAGGAGAAATCG	(70)

F, forward; R, reverse.

148% were detected after treatment with 0.5 and 2 mM VPA, respectively. When considering the WB data, average increases of ~62 and 88% were detected after treatment with 0.5 and 2 mM VPA, respectively. However, the results of RT-qPCR indicated that the mRNA expression levels of *p21^{WAF1/Cip1}* were reduced by an average of 37% when cells were treated with 2 mM VPA (Fig. 2D).

Reduced HDAC activity concomitant with increased H3Ac is induced in VPA-treated HeLa cells. Immunofluorescence signals for H3Ac intensified ~58% when HeLa cells were cultured in the presence of 2 mM VPA (Fig. 3A-C). Notably, no effect on H3Ac nuclear signals was detected in HeLa cells cultured in the presence of 0.5 mM VPA (data not shown). WB results demonstrated an increase in H3Ac abundance in a dose-dependent manner in response to VPA (Fig. 3D), whereas HDAC activity was significantly inhibited, with an average decrease of 20% following 2 mM VPA treatment (Fig. 3E).

Discussion

The present results indicated that, in addition to VPA affecting epigenetic markers by inducing histone acetylation, DNA demethylation, and histone methylation or demethylation in HeLa cells (60-64), it may induce suppression of a gene that acts on oncogenic activity (*p16^{INK4a}*) and increase the protein abundance of a tumor suppressor gene (*p21^{WAF1/Cip1}*) in these cells, thus contributing to evidence of the pharmacological potential of VPA.

The present results detected decreased expression levels of *p16^{INK4a}* in response to VPA treatment. Although *p16^{INK4a}* is often considered a tumor suppressor gene (4,6), it has been reported to participate in the oncogenic activity of cervical cancer (5,12,13,65). It has been demonstrated that silencing *p16^{INK4a}* with small interfering RNA can inhibit the proliferation of cervical tumor cells, causing apoptosis and cell cycle arrest at the G₁ phase (12,18). In human fibroblasts, *p16^{INK4a}* levels have been reported to diminish following exposure to relatively high concentrations of HDACis, such as Trichostatin A and sodium butyrate (74).

Further experiments are required to confirm the effects of VPA on *p16^{INK4a}* protein expression levels, since, in the present study, they were shown to decrease in response to VPA; however, this finding was not statistically significant.

If the present results are not verifiable, and significantly decreased protein expression levels of *p16^{INK4a}* are not demonstrated under the same conditions as those reported in the present study, or in response to >24 h treatments or >2 mM VPA concentrations, this may be due to ineffective *p16^{INK4a}* protein degradation. Such an event could result, for instance, from proteasome ineffectiveness, thus impairing protein degradation. Although proteasomes are abundant in HeLa cells (75), ubiquitin/proteasome pathway impairments are currently under focus in the literature in other cell types, such as U2OS human bone osteosarcoma cells and 293 cells, and in bacteria (*Mycobacterium tuberculosis*) (76,77). A recent study demonstrated that valproate treatment (5 mM) for 36 h may mediate proteasome dysfunction, resulting in the accumulation of abnormal ubiquitinated proteins in Cos-7 and A549 cell lines (78).

Although VPA is known to affect DNA methylation in HeLa cells (61), previous studies have identified no association between *p16^{INK4a}* DNA methylation and expression of *p16^{INK4a}* protein (10,21). The decreased expression of *p16^{INK4a}* in response to VPA treatment appears to be more concerned with changes in the methylation levels of H3K4 and H3K27 (25,26). If H3Ac, which was found to be increased in VPA-treated HeLa cells, is also involved in this epigenetic modulation, further experiments involving chromatin immunoprecipitation (ChIP) assays are required for a better understanding of such an event. Histone acetylation is generally associated with chromatin opening and activated gene expression, although an exception relating inactivation of inducible promoters enriched for H3K14 acetylation has been reported (30).

The low *p21^{WAF1/Cip1}* protein levels in untreated HeLa cells, as detected by immunofluorescence, were supported by a previous report on the same cell line (27). These levels were increased following treatment with VPA for 24 h, as revealed using immunofluorescence and WB; this finding is consistent with published results obtained in several tumor cell lines, including HeLa cells, cultured under different experimental conditions (treatment with 1.2, 4 and 5 mM VPA for 72 h) (31-34). However, the discordant results between *p21^{WAF1/Cip1}* gene and protein expression detected in triplicate assays were unexpected, and the mechanism underlying this difference remains unclear. It may be the case that treatment for >24 h with >2 mM VPA is required for the attainment of the expected increase in *p21^{WAF1/Cip1}* gene expression associated with the increased protein abundance. Upregulation of *p21* has been detected in HeLa cells treated with >2 mM VPA for 48 and 72 h (68). It may be hypothesized that, if *p21^{WAF1/Cip1}* expression decreases were sustained in further experiments under the same experimental conditions as those described in the present study, the drug treatment initially triggered a decrease in *p21^{WAF1/Cip1}* mRNA expression and that, due to post-transcriptional regulation, reduced protein degradation or enhanced protein stabilization influenced by proteasome dysfunction may have resulted in the accumulation of the *p21^{WAF1/Cip1}* protein, thus causing the discrepancy between RT-qPCR, and WB and immunofluorescence data (78). Further experiments to provide a deeper understanding of the present results are thus required.

The *p21^{WAF1/Cip1}* gene, which is responsible for translation of a CDK inhibitor (CKI) that inhibits cyclin-CDK complexes, is crucial for the control of cell proliferation mediated by

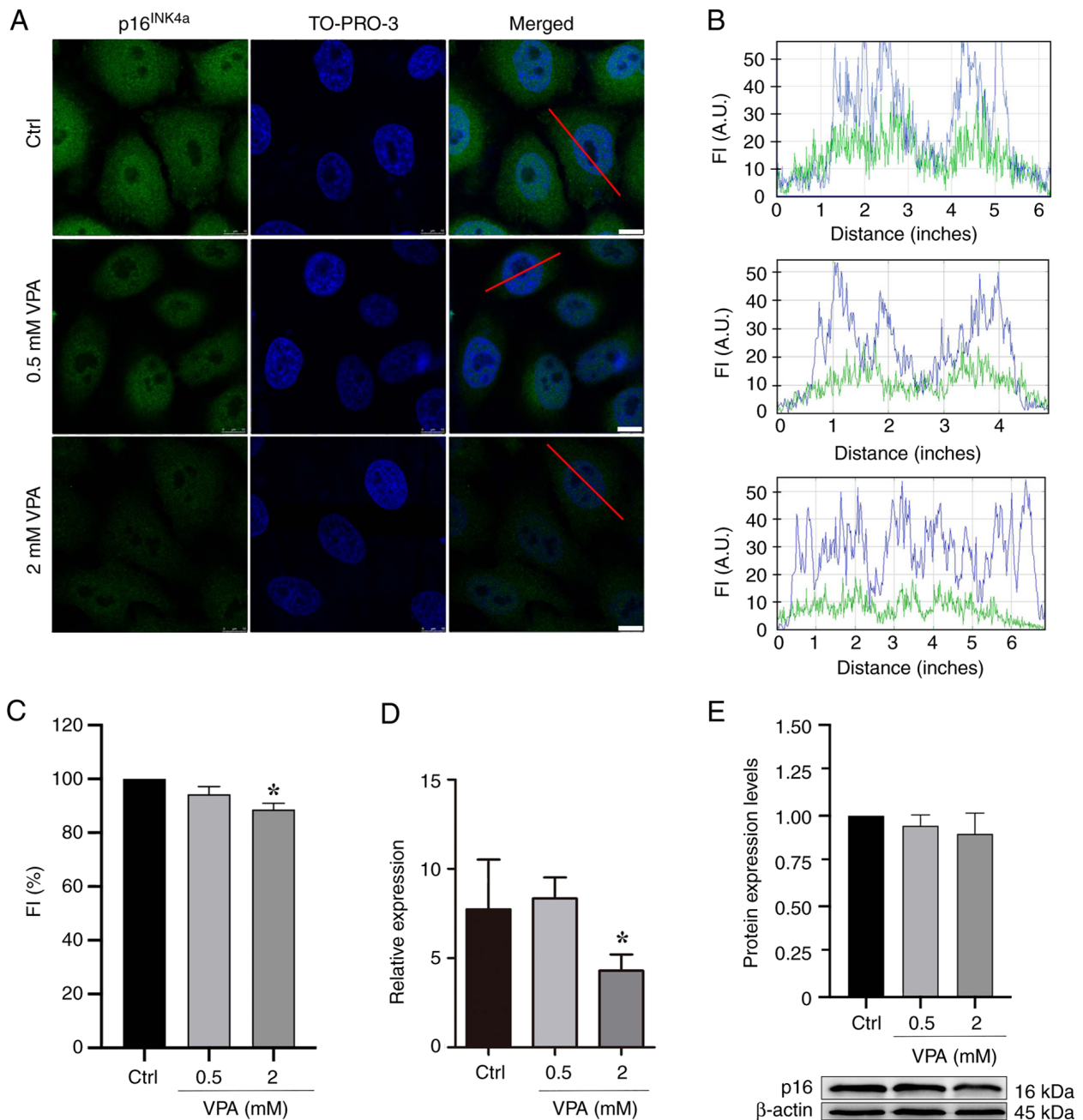


Figure 1. p16^{INK4a} protein abundance and gene expression levels in VPA-treated HeLa cells as assessed using confocal microscopy, RT-qPCR and WB. (A) Confocal microscopy images. Images are representative of three independent experiments, comprising the analysis of 60 nuclei. Scale bars, 10 μm. (B) Graphs representing FI profiles along the red line drawn in the merged image of selected nuclear images to identify the immunofluorescence signals for p16^{INK4a} (green) and the TO-PRO-3-stained DNA (blue). (C) Fluorescence intensity of p16^{INK4a} signals decreased in response to 2 mM VPA treatment relative to untreated control. (D) mRNA expression levels of the *p16^{INK4a}* gene analyzed using RT-qPCR and normalized to endogenous GAPDH control decreased significantly after cell treatment with 2 mM VPA. (E) WB and respective densitometry of five independent experiments indicated no statistically significant changes in p16^{INK4a} protein abundance following VPA treatment, although a trend toward decreased values was observed. β-actin was used as a loading control. Data are presented as the mean ± standard error of the mean. *P<0.05. A.U., arbitrary units; Ctrl, control; FI, fluorescence intensity; RT-qPCR, reverse transcription-quantitative PCR; VPA, valproate; WB, western blotting.

HDACs, which are enzymes that attach to the gene promoter and negatively regulate its expression (79). When HDAC abundance diminishes in human hepatocellular carcinoma, the expression of the CKI p21 can induce cell cycle blockage at G₁ phase (80). VPA-inhibited HDAC activity in HeLa cells is well known (60,61). HDACs, such as VPA, are a class of promising antitumor agents that, through epigenetic modulation, can regulate the expression of tumor suppressor genes and genes that participate in the oncogenic process (12,38,81-84).

Decreased HDAC activity concomitant with increased H3Ac was observed in the present study. However, because upregulation of *p21^{WAF1/Cip1}* gene expression could not be detected under the present experimental conditions, although *p21^{WAF1/Cip1}* protein abundance was shown to be increased in response to VPA treatment, and previous reports have indicated a G₁ phase arrest and no acceleration of apoptosis under treatment with this drug (61,67,68), participation of VPA-induced HDAC inhibition in the decreased expression

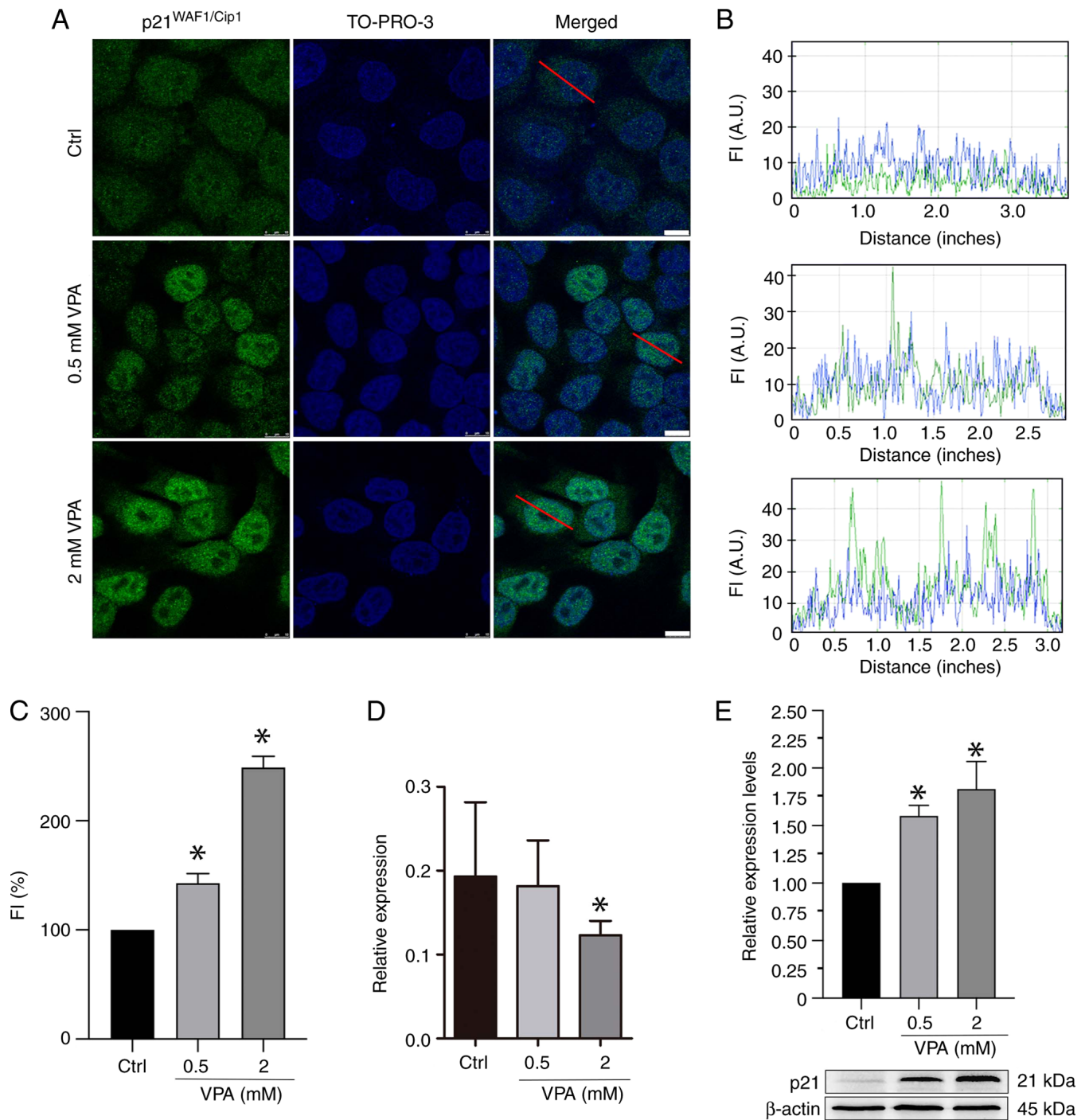


Figure 2. p21^{WAF1/Cip1} protein abundance and gene expression levels in VPA-treated HeLa cells as assessed using confocal microscopy, RT-qPCR and WB. (A) Confocal microscopy images. Images are representative of three independent experiments, comprising the analysis of 80 nuclei. Scale bars, 10 μm. (B) Graphs representing FI profiles along the red line drawn in the merged image of selected nuclear images to identify the immunofluorescence signals for p21^{WAF1/Cip1} (green) and TO-PRO-3-stained DNA (blue). (C) Fluorescence intensity of p21^{WAF1/Cip1} signals increased in response to 0.5 and 2 mM VPA treatment. (D) mRNA expression levels of the p21^{WAF1/Cip1} gene analyzed using RT-qPCR and normalized to endogenous GAPDH control decreased significantly after cell treatment with 2 mM VPA. (E) WB and respective densitometry of five independent experiments indicated significantly increased abundance of the p21^{WAF1/Cip1} protein following VPA treatment. β-actin was used as a loading control. Data are presented as the mean ± standard error of the mean. *P<0.05. A.U., arbitrary units; Ctrl, control; FI, fluorescence intensity; RT-qPCR, reverse transcription-quantitative PCR; VPA, valproate; WB, western blotting.

of the p21^{WAF1/Cip1} gene could not be considered. Therefore, based only on the results detected in the present study, it could not be concluded that VPA-induced global acetylation of histone H3 exerted a direct effect on the expression of the p21^{WAF1/Cip1} gene under the present experimental conditions.

In conclusion, the present study makes a significant contribution to the indication that VPA can act as a multitarget drug. In addition to the well-known effects of VPA inducing decreased HDAC activity, increased histone acetylation, and changes in DNA and histone methylation status (60-64), the present study indicated that this drug may suppress the p16^{INK4a} gene, which

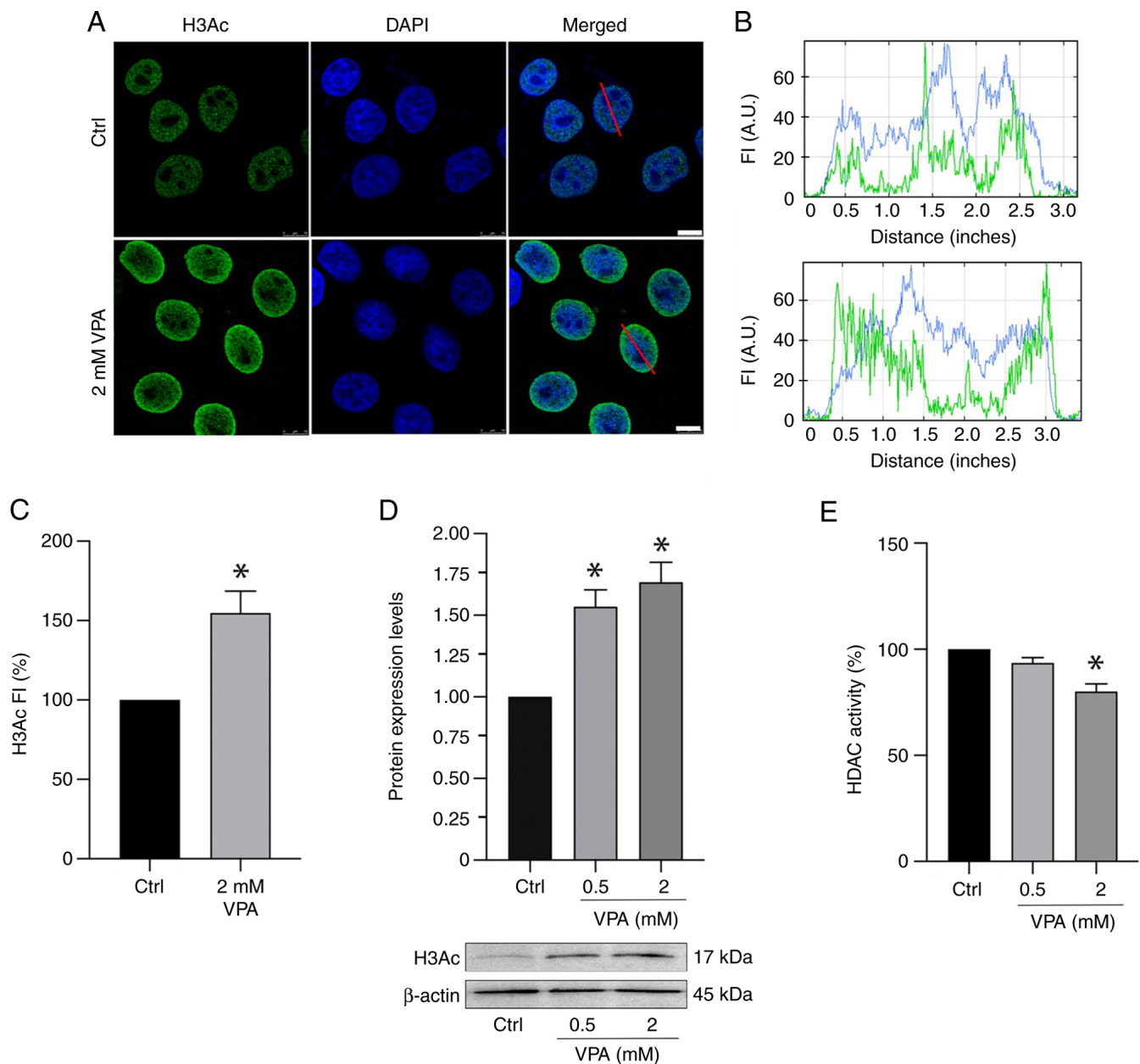


Figure 3. H3Ac and HDAC activity in VPA-treated HeLa cells. (A) Confocal microscopy images. Images are representative of three independent experiments, comprising the analysis of 80 nuclei. Scale bars, 10 μ m. (B) Graphs representing FI profiles along the red line drawn in the merged image of selected nuclear images to identify the immunofluorescence signals for H3Ac (green) and TO-PRO-3-stained DNA (blue). (C) Fluorescence intensity of H3Ac signals increased following treatment with 2 mM VPA. (D) Western blotting and respective densitometry of five independent experiments indicated increased abundance of the H3Ac protein following VPA treatment. β -actin was used as a loading control. (E) HDAC enzymatic activity was reduced after cell treatment with 2 mM VPA, evaluated from five independent experiments. Data are presented as the mean \pm standard error of the mean. * $P < 0.05$. A.U., arbitrary units; Ctrl, control; FI, fluorescence intensity; H3Ac, histone H3 acetylation; HDAC, histone deacetylase; VPA, valproate.

acts on oncogenic activity, and increase the abundance of the $p21^{WAF1/Cip1}$ protein, which is a product of a tumor-suppressing gene in HeLa cells. Given that these findings provide novel data on the activity of VPA, and that HDACs have emerged as promising agents in cervical cancer therapy (84), the present study is relevant, and may contribute to the fields of cell, molecular and cervical cancer biology. Since the expression of $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ may be regulated in HeLa cells by HDACs, which are known to affect epigenetic marks, including histones and non-histone proteins, an investigation into the effects of VPA directly on the promoters of these genes would be relevant. Studies involving H3K4me2/me3, H3K9me2/me3, H3K27me3

and H3Ac levels at the $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ promoters, as determined using ChIP assays, may contribute additional important information to complement the present results.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MAR and ALC conceived, designed, and performed the experiments, and confirm the authenticity of all the raw data. MAR, ALC, CM and MLSM analyzed the data. MLSM and CM contributed the reagents/materials/analysis tools. MLSM and MAR wrote the original draft of the manuscript. MLSM revised the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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