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Sodium Valproate Potentiates Staurosporine-Induced Apoptosis in Neuroblastoma Cells Via Akt/Survivin Independently of HDAC Inhibition

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ABSTRACT

Sodium valproate (VPA) has been recently identified as a selective class I histone deacetylase (HDAC) inhibitor and explored for its potential as an anti-cancer agent. The anti-cancer properties of VPA are generally attributed to its HDAC inhibitory activity indicating a clear overlap of these two actions, but the underlying mechanisms of its anti-tumor effects are not clearly elucidated. The present study aimed to delineate the molecular mechanism of VPA in potentiating cytotoxic effects of anti-cancer drugs with focus on inhibition of HDAC activity. Using human neuroblastoma cell lines, SK-N-MC, SH-SY5Y, and SK-N-SH, we show that non-toxic dose (2 mM) of VPA enhanced staurosporine (STS)-induced cell death as assessed by MTT assay, PARP cleavage, hypodiploidy, and caspase 3 activity. Mechanistically, the effect of VPA was mediated by down regulation of survivin, an anti-apoptotic protein crucial in resistance to STS-mediated cytotoxicity, through Akt pathway. Knock down of class I HDAC isoforms remarkably inhibited HDAC activity comparable with that of VPA but had no effect on STS-induced apoptosis. Moreover, MS-275, a structurally distinct class I HDAC inhibitor did not affect STS-mediated apoptosis, nor decrease the levels of survivin and Akt. Valpromide (VPM), an amide analog of VPA that does not inhibit HDAC also potentiated cell death in NB cells associated with decreased survivin and Akt levels suggesting that HDAC inhibition might not be crucial for STS-induced apoptosis. The study provides new information on the possible molecular mechanism of VPA in apoptosis that can be explored in combination therapy in cancer. J. Cell. Biochem. 114: 854–863, 2013.

KEY WORDS: SODIUM VALPROATE (VPA); HDAC; SURVIVIN; APOPTOSIS; STAUROSPORINE; NEUROBLASTOMA

Neuroblastoma is the most common solid tumor in infants and accounts for about 15% of cancer deaths in children [Park et al., 2010]. Though the advances in standard treatments including surgery, radiation, and chemotherapy have shown reasonable success, these approaches have failed to completely eradicate tumor cells [Kushner et al., 1994; Matthay et al., 1999; Schmidt et al., 2000]. Efforts are now being directed towards developing strategies to sensitize tumors to cytoxicity using combinational treatment [Pinkel et al., 1968; Dosik et al., 1978; Campbell et al., 1993].

Histone deacetylases (HDACs) and histone acetyltransferases (HATs) represent two enzyme families that control the level of histone tail acetylation by the removal and addition of an acetyl group from the lysine residues of core nucleosomal histones [Mottet and Castronovo, 2010]. The HATs, via acetylation of histones, allow transcription and gene expression, while the HDACs are usually associated with DNA hypermethylation and gene silencing [Santini et al., 2007]. The superfamily of HDACs comprises of 18 HDACs classified into five main subtypes: classes I, IIa and IIb, IV, and structurally distinct class III. The class I HDACs include HDAC1, 2, 3, and 8. Altered activities of HATs or HDACs are reported in diverse malignancies making them attractive targets for intervention [Marks et al., 2001; Jones and Baylin, 2002]. In vitro and in vivo studies

Abbreviations used: HDAC, histone deacetylase; VPA, sodium valproate; VPM, valpromide; STS, staurosporine; NB, neuroblastoma.

Reecha D. Shah and Jayashree C. Jagtap contributed equally to this work.

Additional supporting information may be found in the online version of this article.

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have demonstrated that HDAC inhibitors suppress tumor growth in a variety of cancer types and are undergoing clinical evaluation as single agents as well as in combination with other agents [Khan and La Thangue, 2012].

Valproic acid (VPA, 2-propylpentanoic acid) is a short branched chain fatty acid widely used in humans as anticonvulsant and a mood stabilizer [Johannessen, 2000]. Recent studies have demonstrated that VPA acts as a selective class I HDAC inhibitor [Tunnicliff, 1999; Johannessen, 2000; Kazantsev and Thompson, 2008]. VPA has been explored for its potential as an anticancer agent in various cancers [Duenas-Gonzalez et al., 2008] as a stand alone cancer therapeutic and in combination with other chemotherapeutic drugs and radiotherapy. While the mechanism of action of VPA is not clearly understood, the majority of studies have linked its HDAC-inhibitory property with anti-cancer activity [Phiel et al., 2001] suggesting a clear overlap of these two actions. The goal of the present study was to understand the molecular mechanism of VPA in potentiating cytotoxic effects of anti-cancer drugs with focus on the role of VPA as an HDAC inhibitor. We show here that VPA can act independent of its HDAC inhibitory properties to sensitize apoptosis induced by staurosporine (STS). We also demonstrate that VPA enhances STS-induced apoptosis by repression of inhibitor of apoptosis protein (IAP)-survivin mediated by down regulation of Akt in human neuroblastoma cells.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Human neuroblastoma cell lines; SK-N-MC, SH-SY-5Y, and SK-N-SH were purchased from American Type Culture Collection (ATCC, Rockville, MD) and maintained in Eagle's Minimum Essential Medium MEM (E) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Carlsbad, CA) and penicillin/streptomycin (Sigma, Saint Louis, MO) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. VPA, STS, MS-275, VPM, and nocodazole were purchased from Sigma.

CELL VIABILITY BY MTT ASSAY

Cell viability was determined by measuring the mitochondrial conversion of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) to a colored product. Cells seeded in 96-well plates for 24 h were treated with serial concentrations of VPA and STS alone or in combination. After 24 h treatment, cells were incubated with 5 mg/ml MTT for 4 h, and subsequently solubilized in dimethyl sulfoxide and absorbance was measured at 570 nm using a microplate reader (Molecular Devices, SPECTRA max 250, USA). Readings in the untreated control cells were considered 100% viable.

FLOWCYTOMETRY ANALYSIS

Cells were treated with VPA, MS-275, or VPM alone or in combination with STS for 24 h and assessed for apoptosis and

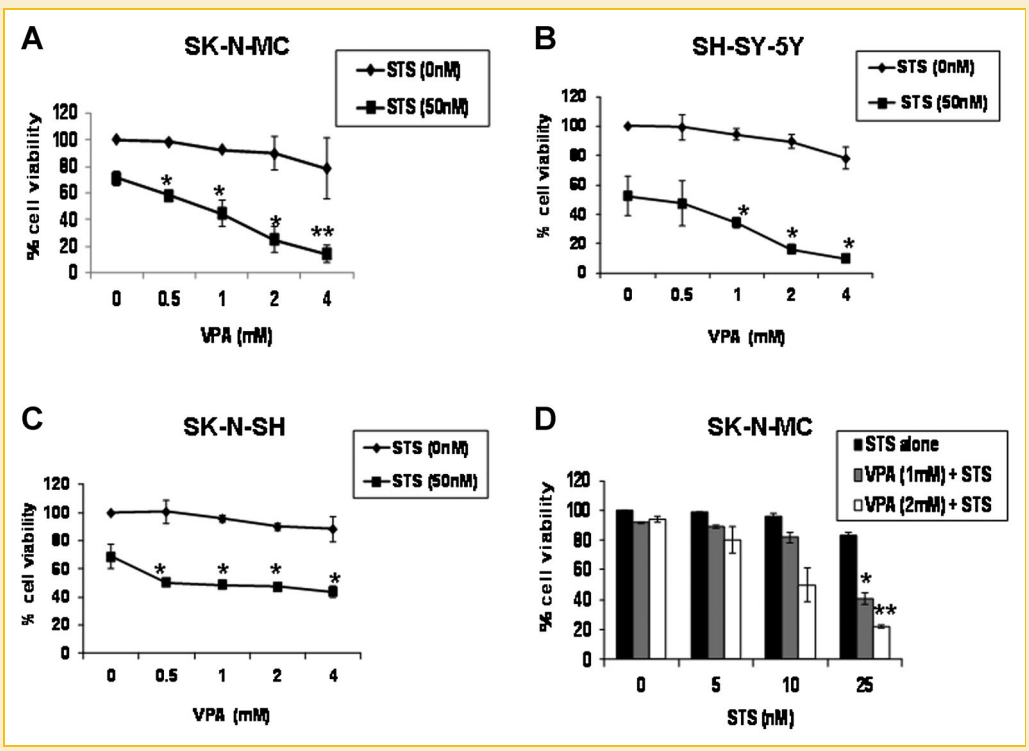

Fig. 1. Dose-dependent effect of VPA alone and in combination with STS in NB cell lines. NB cell lines (A) SK-N-MC, (B) SH-SY-5Y, and (C) SK-N-SH were treated with STS (50 nM) alone and in combination with VPA (1–4 mM) for 24 h, and cell viability was measured by MTT assay (D) SK-N-MC cells were treated with STS (5–25 nM) alone or in combination with VPA (1 and 2 mM) for 24 h and cell viability was measured by MTT assay. Data represented is mean ±SD of three independent experiments done in triplicates. $^{*}P < 0.05$, $^{**}P < 0.01$, STS treatment alone versus STS in combination with VPA.

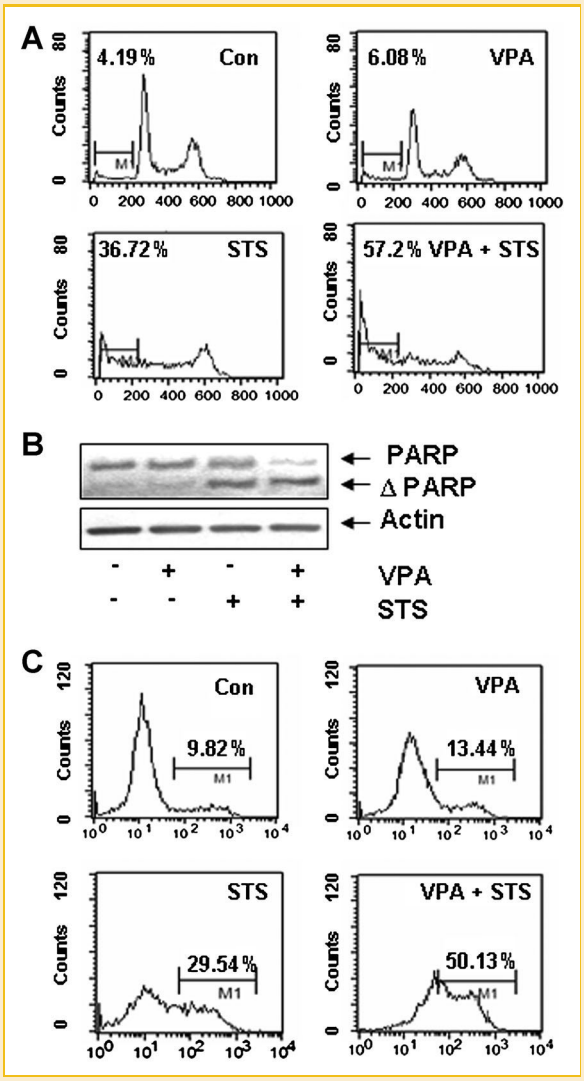

Fig. 2. VPA enhances STS-induced apoptosis in SK-N-MC cells. Cells were treated with STS (50 nM) and VPA (2 mM) alone or in combination for 24 h and analyzed for apoptosis (A) hypodiploid population by flowcytometry (B) PARP cleavage by immunoblotting and (C) caspase 3 activity by flowcytometry.

cell cycle profiles. Control and treated cells were harvested, washed in cold PBS, and fixed in 70% ethanol. The cells were washed in PBS, centrifuged, and the pellet was incubated with RNase A (5 mg/ml; Amersham Biosciences, USA) for 15 min. and further incubated with propidium iodide (50 μg/ml) for 2 h at 37°C. DNA content was determined on the FL-2A channel with a flow cytometer equipped with a 488 nm argon laser (FACS Vantage, Becton Dickinson, San Jose, CA). Ten thousand events were scored for each sample and data were analyzed using CellQuest Pro software.

WESTERN BLOTTING

Cells were harvested and washed twice with PBS and lysed in RIPA buffer (Millipore, USA) (0.5 M Tris–HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA) and protease inhibitor cocktail (Roche Diagnostics, Meyland, France) followed by centrifugation at 12,000 rpm for 30 min at 4°C. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of total protein or the nuclear extract were subjected to SDS–PAGE followed by immunoblotting using the following antibodies: rabbit anti-PARP, rabbit anti-survivin, goat anti-Akt (1:1,000, Santa Cruz, CA), mouse anti-actin. (1:10,000, MP Biomedicals, USA) and rabbit anti-histone 3 lysine residue 9 (H3K9; 1:1,000, Millipore). The secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit IgG, goat-anti-mouse IgG or donkey anti-goat IgG (Bio-Rad). Immunoreactive bands were visualized using an enhanced chemiluminescence system (Pierce Thermo Scientific, USA) according to the manufacturer's instructions. The expression of the protein was normalized with respect to actin. Protein bands were then quantified by densitometry using IMAGE J 3.0 software.

MEASUREMENT OF CASPASE-3 ACTIVITY

Caspase 3 activity was assayed using QIA91 Caspase 3 detection kit (Calbiochem, EMD Chemicals, USA) according to the manufacturer's protocol. The samples were analyzed by flowcytometry using FL-1 channel.

IMMUNOFLUORESCENCE MICROSCOPY

For immunofluorescence staining, cells grown on coverslips were washed three times with PBS, fixed in 3.7% paraformaldehyde solution at RT for 10 min and permeabilized in 0.2% Triton X-100 for 5 min. Cells were washed with PBS three times at each interval. Coverslips were incubated with 3% BSA at RT for 1 h. Incubation with primary antibodies was done for 2 h at RT: rabbit anti-survivin (1:100, Santa Cruz) or rabbit anti-H3K9(1:200, Millipore), washed three times with PBS and then incubated with goat anti-rabbit Cy3 (1:250, Molecular Probes, Invitrogen) secondary antibody at RT for 1 h. Finally, nuclei were stained with DAPI (Sigma) and cover slips were mounted using anti-fade mounting reagent. The expression of survivin and H3K9 was observed by a confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

RT-PCR AND QUANTITATIVE RT-PCR

Total RNA was isolated from SK-N-MC cells using the TRIzol method (Invitrogen, Carlsbad, CA). cDNA was prepared from 5 μg of total RNA by reverse transcription with Moloney murine leukemia virus reverse transcriptase (Invitrogen) at 37°C for 60 min. The class I HDAC, that is, HDAC1, 2, 3, and 8 transcripts of 200 bp were amplified from the cDNA using recombinant Taq polymerase (Invitrogen). β-Actin was used as an internal control. The following primers were used for RT-PCR: Forward 5′ GGAAATCTATCGCCCTCACA 3′ and Reverse 5′ AACAGGCCATCGAATACTGG 3′ for HDAC1, Forward 5′ GTCCCTTCGTTAGGGGGATA 3′ and Reverse 5′ CCAAAATCCCACAAATCACC 3′ for HDAC2, Forward 5′ GAGGGATGAACGGGTAGACA 3′ and Reverse 5′ TCCTCCCCACACTTGAAAAC 3′ for HDAC3, Forward 5′ CGACGGAAATTTGAGCGTAT 3′ and Reverse 5′ CCAACATCAGACACGTCACC 3′ for HDAC8, Forward 5′ GTGGGGCGCCCCAGGCACCA 3′ and Reverse 5′ CTCCTTAATGTCACGCACGATTTC 3′ for β-Actin.

For quantitative RT-PCR, amplification was carried out in a total volume of 20 μl containing 0.25 μM of each primer (Forward 5′

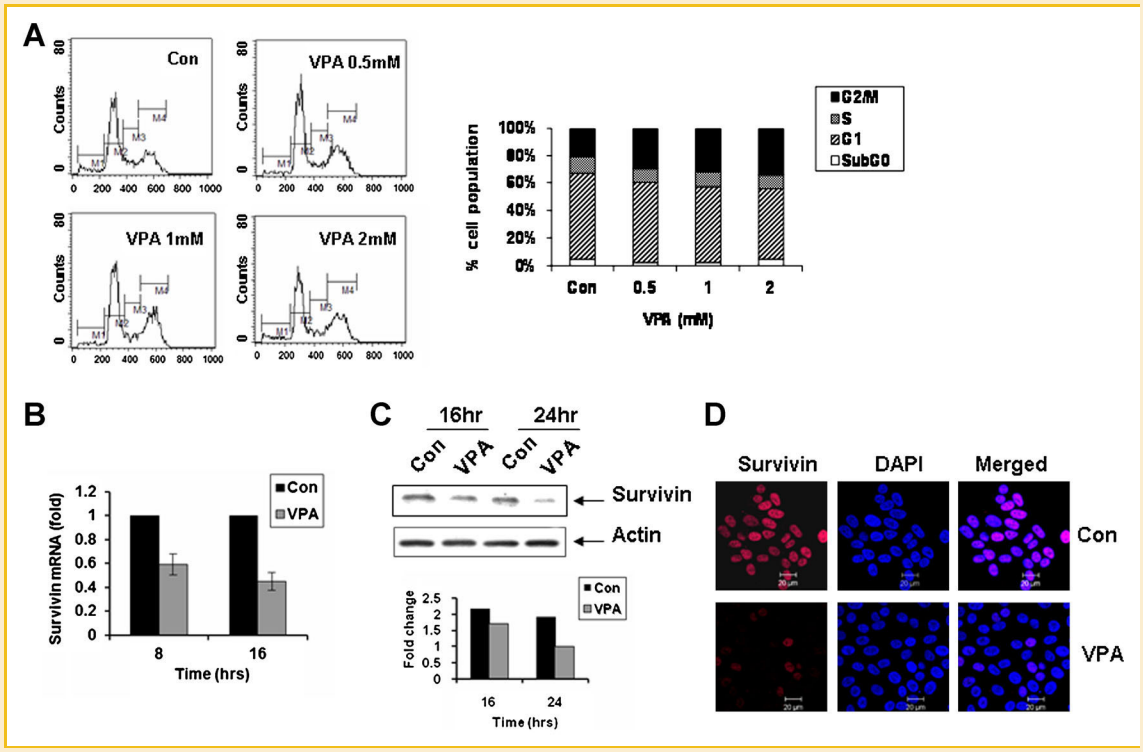

Fig. 3. VPA arrests cells in G2/M phase and down regulates expression of survivin. A: SK-N-MC cells were treated with VPA (0.5–2 mM) for 16 h and cell cycle profile was determined by flowcytometry. The stacked bar graph shows the population of cells in different phases of cell cycle. B: Cells were treated with VPA (2 mM) for 8 and 16 h and assessed for transcript levels of survivin by quantitative RT-PCR. Fold change was calculated with respect to actin (C) Cells were treated with VPA (2 mM) for 16 and 24 h and assessed for expression of survivin by immunoblotting. Actin was loading control (D) Expression of survivin on treatment with VPA (2 mM) for 24 h analyzed by immunofluorescence. The panel shows survivin (red), nucleus (blue) and merged images with dual staining.

ACCTGAAAGCTTCCTCGACA 3′ and Reverse 5′ TAACCTGCCATTGGAACCTC 3′ for survivin; Forward 5′ TCTATGGCGCTGAGATTGTG 3′ and Reverse 5′ CTTAATGTGCCCGTCCTTGT 3′ for Akt-1; Forward 5′ CAGCTAGGACTCGTTTGGTTG 3′ and Reverse 5′ CACACTACGAGACCTGCATCA 3′ for Akt-2), 10 μl of SYBR Green Supermix (Bio-Rad) and 600 ng of cDNA. PCR reactions were performed in duplicate and heated at 95°C for 1 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 45 s. The specific mRNA was determined in samples by relative quantification with respect to actin using delta C_T method.

TRANSFECTIONS WITH ANTISENSE OLIGONUCLEOTIDE (ASO)

Cells were transfected with phosphorothioate antisense oligonucleotides (Integrated DNA Technologies, Coralville, IA) against class I HDACs with LipofectAMINE 2000 (Invitrogen) as per the manufacturers' instructions. Briefly, 0.5×10^6 cells were transfected with 200 nM each of HDAC1 antisense 5′ CTCATCGCCACTCTCCTCAG 3′, HDAC2 antisense 5′ TCTCCTCCATCCACTACTCC 3′, HDAC3 antisense 5′CACAGCATCCCAAGCCACTCT 3′, HDAC8 antisense 5′GATCAGTACCCTCTCTCCCA 3′ using LipofectAMINE 2000 for 16 h, and the expression of H3K9, total Akt1/2 and survivin was assessed by Western blotting analysis. Transfected cells were also assessed for HDAC activity assay as described below. Cells transfected with scrambled sequence 5′ ACCCTTGCGTCCGCTGCG 3′ served as controls.

TRANSFECTION WITH SIRNA

Cells were cultured for 24 h and transfected with total Akt siRNA oligonucleotides (150 nM) using LipofectAMINE 2000 according to the manufacturer's instructions (Cell Signaling Technology, USA). After 48 h, medium was replaced and transfected cells were treated with STS for 24 h and processed for cell viability assay as described above.

HDAC ACTIVITY

Nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic extraction reagents kit (Pierce Thermo Scientific) according to the manufacturer's protocol. Total HDAC activity was measured using the EpiQuik HDAC Activity/Inhibition Assay Kit (Epigentek, USA). Briefly, the nuclear extracts were incubated with specific substrate for 1 h at 37°C, followed by capture antibody for 60 min and then detection antibody for 30 min at room temperature. Absorbance was determined at 450 nm using a microplate spectrophotometer. HDAC activity was calculated according to the manufacturer's instructions.

STATISTICAL ANALYSIS

Data are expressed as mean ± SD. Treated cells and the corresponding controls were compared using Student's *t*-test. *P* values <0.05 were considered significant.

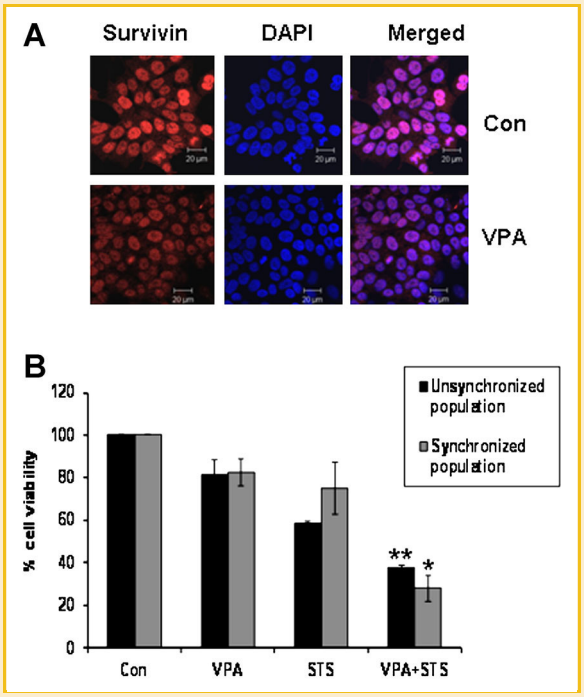

Fig. 4. VPA down regulates survivin in G2/M synchronized cells and sensitizes cells to STS-triggered apoptosis. A: SK-N-MC cells were synchronized in G2/M phase by treatment with nocodozole (30 nM) for 12 h and treated with VPA (2 mM) for 24 h, cells were stained with survivin (red) and DAPI (blue) for nucleus. B: Unsynchronized and G2/M arrested cells were exposed to VPA (2 mM) and STS (50 nM) alone and in combination for 24 h and cell viability was measured by MTT assay. Data in (B) represents mean ± SD of three independent experiments done in triplicates. $^{*}P < 0.05$, $^{**}P < 0.01$, STS treatment alone versus STS in combination with VPA.

RESULTS

VPA ALONE DOES NOT AFFECT VIABILTY BUT ENHANCES STS-INDUCED CELL DEATH

The effect of VPA on cell viability was measured by MTT assay in three human NB cell lines SK-N-MC, SH-SY-5Y, and SK-N-SH by exposing them to 0.5–4 mM of VPA for 24 h. As shown in Figure 1A–C, VPA (0.5–4 mM) had no effect on cell viability in human NB cell lines—SK-N-MC, SH-SY-5Y, and SK-N-SH but enhanced cell death in a dose dependent manner in SK-N-MC and SH-SY-5Y cell lines exposed to STS (50 nM). Interestingly, 25 nM STS in the presence of VPA (1 mM) induced significant increase in cell death (>50%) and 2 mM VPA rendered the cells sensitive to non-toxic dose of STS (10 nM; Fig. 1D). Induction of apoptosis was analyzed in cells treated with VPA (2 mM) and STS (50 nM) alone and in combination. VPA sensitized the cells to STS-mediated apoptosis with a significant increase (57.2%) in hypodiploid population as determined by flowcytometry analysis (Fig. 2A). Apoptosis was also confirmed by cleavage of PARP (Fig. 2B) and increase in caspase 3 activity (50.13%; Fig. 2C). Taken together, these results suggest that VPA enhanced susceptibility of the cells to STS-induced apoptosis in NB cells.

VPA ARRESTS CELLS IN G2/M PHASE AND DOWNREGULATES EXPRESSION OF SURVIVIN

Our previous studies indicated that SK-N-MC cells synchronized in G2/M were resistant to STS compared to G1 cells [Chandele et al., 2004]. We therefore addressed whether cell death enhanced by VPA could be due to accumulation of cells in G1 phase of the cell cycle. Surprisingly, we found that exposure of cells to different doses of VPA resulted in a decrease in G1 population with concomitant increase in G2/M cells in comparison with controls (Fig. 3A). In line with our earlier findings that upregulation of survivin conferred resistance in G2/M population in SK-N-MC cells, we explored whether the sensitizing effect of VPA involved regulation of survivin. As shown in Figure 3B, the level of survivin mRNA was significantly reduced following 8 and 16 h of treatment with VPA. Next, we examined whether VPA affected survivin at the protein level. As shown in Figure 3C, a marginal decline in the expression of survivin protein was observed on 16 h and the level was significantly decreased with 24 h of treatment. Figure 3D represents a decrease in the total number of survivin positive cells on treatment with VPA for 24 h as seen by immunofluorescence. These results together suggest that downregulation of survivin may be associated with the potentiating effect of VPA in STS-induced apoptosis. Based on our earlier finding that survivin plays a crucial role in STS-mediated apoptosis in SK-N-MC cells [Chandele et al., 2004], we postulated that if down regulation of survivin contributes to STS-triggered apoptosis, VPA should render G2/M cells that are resistant to this insult because of high levels of survivin sensitive to STS-mediated apoptosis. For this purpose, SK-N-MC cells were synchronized in G2/M phase using nocodazole for 12 h and treated with VPA for 24 h. Figure 4A represents the synchronized population of G2/M cells expressing survivin and VPA remarkably decreased survivin expression in such population. Importantly, combination of STS and VPA resulted in significant increase in cell death in G2/M cells (>60%) compared with STS alone (approximately 20%; Fig. 4B). Taken together, these findings suggested that VPA arrests the cells in G2/M and sensitizes cells to STS-induced cell death by down regulating expression of survivin.

EXPRESSION OF SURVIVIN MODULATED BY VPA IS UNDER THE REGULATION OF AKT

One of the mechanisms by which the expression of survivin is regulated is via the phosphatidylinositol 3-kinase/Akt pathway [Zhao et al., 2010]. We therefore examined the effect of VPA on Akt expression. The levels of Akt1 and Akt2 mRNA were reduced by approximately 80% in cells treated with VPA for 16 h (Fig. 5A). Western blot analysis revealed that cells treated with VPA exhibited a significant decrease in Akt protein at 16 and 24 h (Fig. 5B). To validate the role of Akt in downregulation of survivin, the expression of survivin was determined in SK-N-MC cells treated with Akt inhibitor. Analysis by immunofluorescence demonstrated that treatment with Akt II inhibitor (5 μM) for 16 h resulted in reduced number of survivin positive cells compared with control group (Fig. 5C). Furthermore, cells transfected with siRNA to Akt showed a significant decrease in the level of survivin (Fig. 5D) and these cells were also sensitized to STS-induced cell death (Fig. 5E). These findings suggested that the expression of survivin was under the

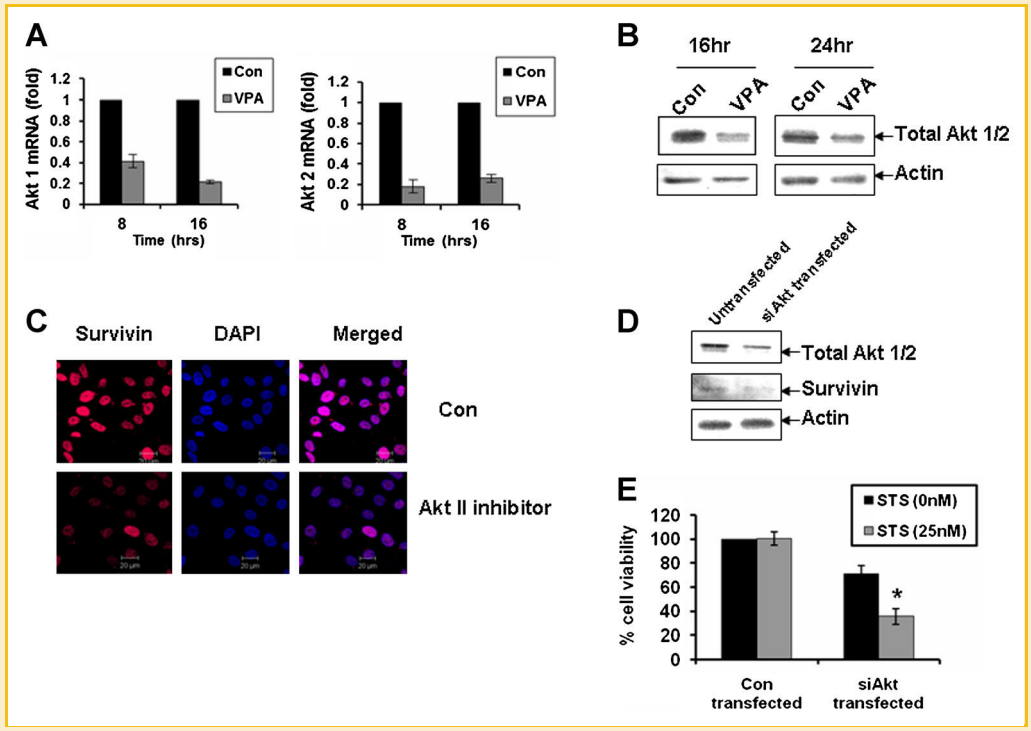

Fig. 5. Downregulation of survivin by VPA is regulated via Akt. A: SK-N-MC cells were treated with VPA (2 mM) for 8 and 16 h and assessed for transcript levels of Akt-1 and Akt-2 by quantitative RT-PCR. Fold change was calculated with respect to actin. B: Cells were treated with VPA (2 mM) for 16 and 24 h and Akt level was determined by immunoblotting. C: Cells were exposed to 5 μM Akt II inhibitor for 16 h and stained for survivin (red) and DAPI (blue) for nucleus. D: Cells were transfected with siRNA directed to total Akt1/2 and assessed for the expression of survivin by immunoblotting. Actin was loading control. E: Cells transfected with siAkt were treated with STS (25 nM) for 24 h and cell viability was measured by MTT assay. Data are mean ±SD of three independent experiments done in triplicates. $^{*}P < 0.01$, untreated versus STS treated groups.

regulation of Akt and that knockdown of Akt was crucial in sensitizing cells to STS-induced cytotoxicity.

KNOCKDOWN OF CLASS I HDACS DOES NOT ENHANCE STS-INDUCED APOPTOSIS OR AFFECT EXPRESSION OF AKT AND SURVIVIN

HDACs are considered to be promising targets for cancer therapy and hence there is a growing interest in developing inhibitors to HDACs. Recent studies suggest that VPA is an effective inhibitor of class I HDACs [Tunnicliff, 1999; Johannessen, 2000; Duenas-Gonzalez et al., 2008; Kazantsev and Thompson, 2008]. It was therefore of interest to investigate whether the effect of VPA was related to its function as an HDAC inhibitor. For this purpose, we first determined the expression of class I HDACs in SK-N-MC cells. As illustrated in Figure 6A, SK-N-MC cells expressed high levels of HDAC-1, 2, 3, and 8 transcripts. Analysis by immunofluorescence and immunoblotting using antibody to acetylated H3K9 showed that treatment with VPA caused significant inhibition of HDACs (Fig. 6B). To establish whether the inhibition of HDACs by VPA was crucial in STS-induced apoptosis, we tested the effect of STS in SK-N-MC cells transfected with ASO designed against class I HDAC isoforms 1, 2, 3, and 8. The efficacy of transfection was determined by increased acetylation of H3K9 (Fig. 6C and Fig. S1). Furthermore, HDAC activity was decreased in ASO transfected cells and was comparable with that in VPA-treated cells (Fig. 6D). Though inhibition of HDACs resulted in a marginal reduction in viability, there was no significant difference in cell death induced by STS in control versus ASO transfected cells (Fig. 6E). Interestingly, knockdown of class I HDACs did not affect the expression of Akt and survivin (Fig. 6F,G). These findings suggest that the sensitizing effect of VPA may not involve its function as an HDAC inhibitor.

HDAC INHIBITOR-MS-275 HAD NO EFFECT ON STS-INDUCED APOPTOSIS

To further rule out the possibility of the role of HDAC inhibition in VPA-induced effect, we used MS-275, a potent HDAC inhibitor with micromolar affinity for class I HDACs in our experiments. As shown in Figure 7A, MS-275 was highly effective in inhibiting HDACs. We next tested the effect of MS-275 on the cell cycle profile. We found that exposure of cells to different doses of MS-275 for 16 h had no effect on the cell cycle profiles (Fig. 7B). To further examined whether MS-275 could modulate STS-induced cell death, induction of apoptosis was analyzed in SK-N-MC cells treated with MS-275 (500 nM) and STS (25 nM) alone and in combination. There was no significant difference in the hypodiploid population in cells treated with STS alone and in combination with MS-275 (Fig. 7C). Interestingly, MS-275 did not decrease the levels of Akt and survivin (Fig. 7D).

VPM, AN AMIDE ANALOGUE OF VPA, ENHANCED STS-INDUCED APOPTOSIS

As a control drug to exclude pharmacological actions unrelated to the HDAC inhibitory activity of VPA, we used valpromide (VPM), an

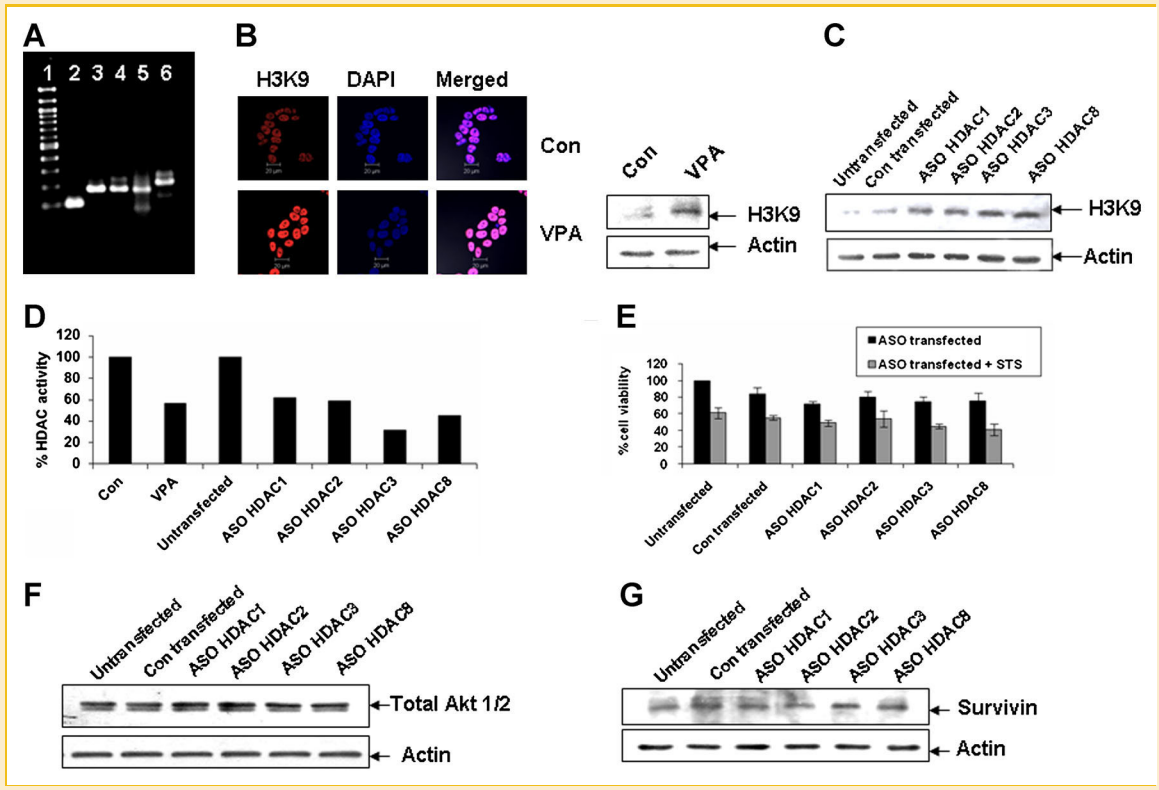

Fig. 6. Down regulation of class I HDACs did not affect STS-induced apoptosis and expression of Akt and survivin. A: SK-N-MC cells cultured for 24 h were analyzed by RT-PCR for transcript levels of HDAC 1, 2, 3, and 8 as shown in lanes 3,4,5,6. Mol. wt markers (100 kb) and actin are shown in lanes 1 and 2, respectively. B: Cells treated with VPA (2 mM) for 16 h were probed with antibody to acetylated H3K9 and analyzed by immunoflorescence and immunoblottting. C: Cells were transfected with control or HDAC ASO and analyzed for acetylated H3K9 by immunoblotting. Actin was loading control. D: Nuclear extracts of the cells treated either with VPA (2 mM) for 16 h or transfected with HDAC ASOs were assessed for HDAC activity. The data represent % HDAC activity as compared to the control untreated cells or the control transfected cells. E: Control and HDAC ASO transfected cells were treated with STS (50 nM) for 24 h and cell viability was determined by MTT assay. Data in (E) represent mean ±SD of three independent experiments done in triplicates. Cells were transfected with control and HDAC ASOs and immunoblotting was performed for (F) Akt and (G) survivin. Actin was loading control.

amide analogue of VPA lacking HDAC inhibitory activity. As expected, there was no difference in the expression of H3K9 determined by immunofluorescence (Fig. 8A) or in HDAC activity (Fig. 8B) between control cells and VPM-treated cells. To further test whether VPM could modulate STS-induced cell death, induction of apoptosis was analyzed in SK-N-MC cells treated with VPM (4 mM) and STS (25 nM) alone and in combination. Flowcytometry analysis revealed that VPM sensitized the cells to STS-mediated apoptosis with a significant increase (45.69%) in hypodiploid population compared to STS-treated cells (28.07%; Fig. 8C). A significant decrease in the expression of total and cleaved fragment of PARP was seen with combination treatment in comparison with cells treated with STS alone (Fig. 8D). Also, VPM significantly down-regulated Akt and survivin. These findings with VPM and VPA led us to conclude that inhibition of HDAC activity by VPA may not be crucial for potentiating STS-triggered apoptosis.

DISCUSSION

In this study, we demonstrated that non-toxic doses of VPA sensitized NB cells to STS-mediated cytotoxicity by down regulation of survivin via Akt pathway. VPA has been demonstrated to induce growth inhibition in malignant and benign neuroblastoma cell lines—UKF-NB-3, SK-N-AS, UKF-NB-4 with IC50 values ranging from 1.0 to 2.8 mM [Hřebačková et al., 2009]. In our study, VPA was found to be non-toxic up to 2 mM in human NB cell lines. Importantly, while 1 mM VPA was effective in enhancing cell death induced by STS at cytotoxic concentration, 2 mM VPA rendered NB cells sensitive to nontoxic doses of STS. Considering the significance of the emerging concept of dose-response to reduce exposure to toxic agents, our study strongly suggests that VPA can be successfully used for reducing the doses of anticancer drugs in combinational chemotherapy.

Based on our earlier study [Chandele et al., 2004], we speculated that VPA might sensitize cells to STS by accumulating cells in G1 phase, however, we found an increase in the proportion of cells in G2/M phase that was concomitant with decrease in G1 population. This finding is in accordance with a recent report that VPA arrested NB cells in the G2/M phase of cell cycle [Condorelli et al., 2008]. Other studies reported VPA-induced G0/G1 arrest in melanoma cells [Valentini et al., 2007] and in cell lines and primary cultures of myeloma [Kaiser et al., 2006]. Survivin, the smallest member of the inhibitors of apoptosis (IAP) family, is abundantly expressed in

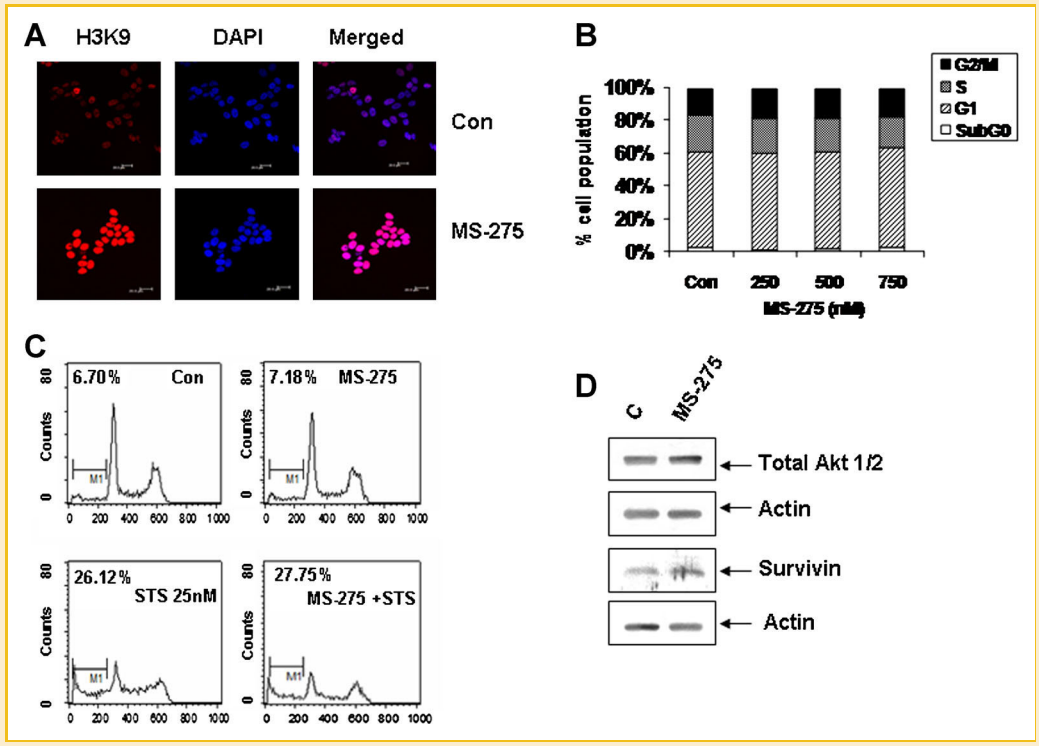

Fig. 7. HDAC inhibitor-MS-275 does not enhance STS-induced apoptosis in SK-N-MC cells. A: Cells were treated with MS-275 (500 nM) for 16 h and inhibition of HDAC was assessed by immunofluorescence using acetylated H3K9 antibody. B: Cells were treated with MS-275 (250–750 nM) for 16 h and cell cycle profile was determined by flowcytometry. The stacked bar graph shows the population of cells in different phases of cell cycle. C: Cells were treated with STS (25 nM) and MS-275 (500 nM) alone or in combination for 24 h and hypodiploid population was measured for apoptosis. D: Cells were treated with MS-275 (500 nM) for 16 h and Akt and survivin levels were determined by immunoblotting. Actin was loading control.

many cancers. The expression is associated with enhanced growth in tumors, poor prognosis, and response to chemotherapy [Lladser et al., 2011; Park et al., 2012]. In neuroblastomas, survivin expression is associated with high risk, unfavorable prognosis and resistance to drug-mediated cytotoxicity [Azuhata et al., 2001]. On these lines, we explored the possibility that VPA regulated survivin to sensitize the cells to STS-mediated cytotoxicity. As expected, VPA decreased the expression of survivin in SK-N-MC cells and sensitized G2/M cells to STS-induced apoptosis.

Though mechanisms involved in regulation of survivin are not clearly understood, recent studies have suggested the role of p53 [Mirza et al., 2002] and RB/E2F pathway [Jiang et al., 2004] *in* transcriptional regulation of survivin. Radioresistance in breast cancer cell lines involves activation of survivin regulated by NF-kappaB and c-myc [Papanikolaou et al., 2010]. Consistent with the report that HDACi such as valproic acid and butyrate impede Akt expression resulting in Akt deactivation and apoptotic cell death [Chen et al., 2006], we observed that exposure of SK-N-MC cells to VPA decreased expression of Akt at the transcript and protein level. Furthermore, down regulation of Akt using small interference RNA or treatment with Akt inhibitor-Akt inhibitor II, significantly suppressed the expression of survivin suggesting that it was under the regulation of Akt. Similar findings have been reported in human multiple myeloma cells [Hideshima et al., 2007] and breast cancer cell line MCF-7 [Huang et al., 2012]. Additionally, knockdown of Akt sensitized SK-N-MC cells to STS-induced apoptosis. Based on these current findings and our earlier studies that survivin is crucial for STS-induced apoptosis, it is logical to conclude that the apoptosis enhancing effect of VPA involves Akt/survivin pathway.

HDACs are aberrantly expressed in tumors [Lindemann and Johnstone, 2004] and several compounds with HDAC inhibitory property have shown promising results as anticancer agents [Rasheed et al., 2007]. Since VPA is a known class I inhibitor, we examined whether the effect of VPA could be attributed to its function as an HDAC inhibitor. Interestingly, down regulation of individual class I HDACs had no effect on STS-induced cell death in SK-N-MC cells. Though it may be argued that down regulation of individual HDAC may not mimic the effect by VPA, it should be noted that in the present study, knockdown of each of the HDAC isoforms resulted in decrease of total HDAC activity comparable with that of VPA. In this context, a recent study by Dejligbjerg et al. [2008] suggested that the transcriptional response to enzymatic inhibition by VPA or belinostat is different from that induced by knockdown of individual class I HDAC isoforms. In another study, contrary to our findings, knockdown of HDAC2 completely mimicked the effects of VPA on survivin and cell migration, and over-expression of survivin could also rescue the effects of HDAC2 knockdown on cell migration [Zhang et al., 2012]. The discrepancy may be due to difference in cell types.

Though HDAC inhibitors, VPA and butyrate have been demonstrated to abrogate Akt1 and Akt2 expression, the precise

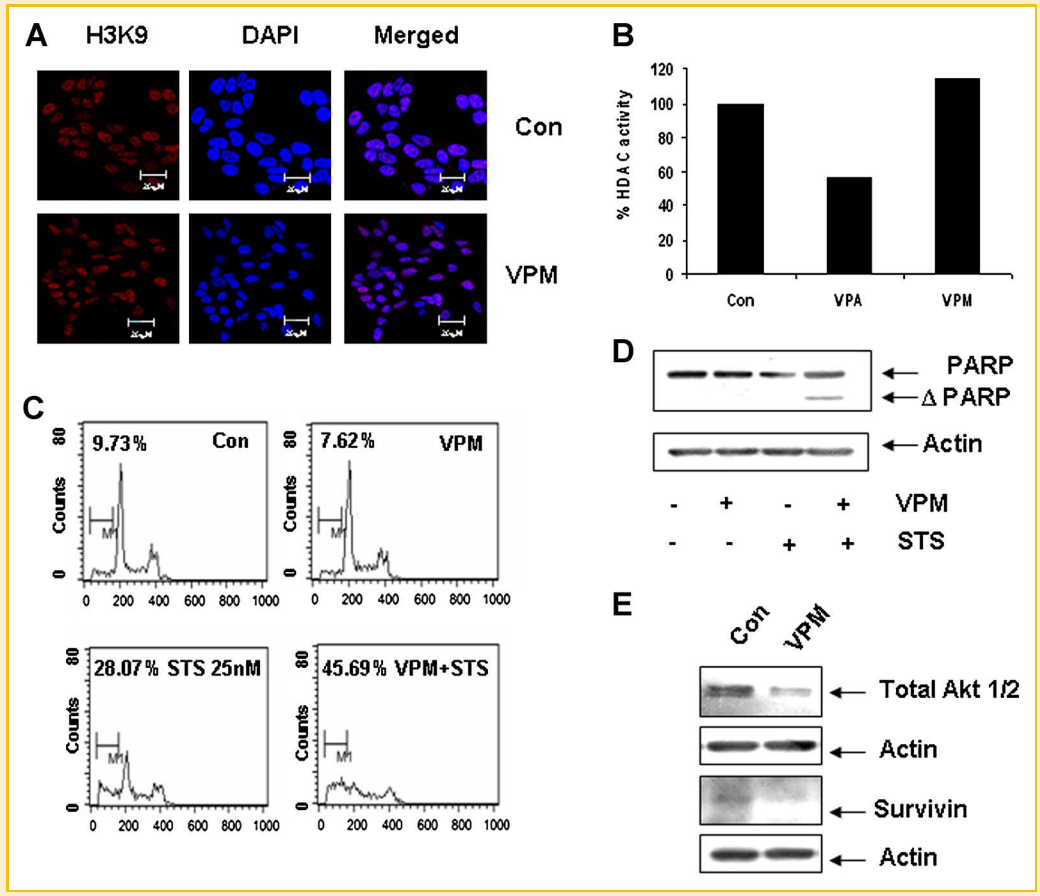

Fig. 8. VPM—an analogue of VPA enhances STS-induced apoptosis in SK-N-MC cells. Cells were treated with VPM (4 mM) for 16 h and inhibition of HDAC was assessed by (A) immunofluorescence using acetylated H3K9 antibody and (B) HDAC activity assay. The data in (B) represent % HDAC activity in comparison with control untreated cells. C: Cells were treated with STS (25 nM) and VPM (4 mM) alone or in combination for 24 h and analyzed for apoptosis by flowcytometry and (D) PARP cleavage by immunoblotting. E: Cells were treated with VPM for 16 h and Akt and survivin levels were determined by immunoblotting. Actin was loading control.

role of HDAC inhibition in these studies was not defined [Chen et al., 2006]. It is important to note that in our experimental system, inhibition of class I HDAC activity had no effect on Akt or survivin expression. Furthermore, experiments conducted with MS-275, a synthetic benzamide derivative and a class I isoform selective HDAC inhibitor [Khan et al., 2008] also confirmed that HDAC inhibition alone was not adequate to enhance STS-induced cell death or decrease the levels of Akt and survivin. Finally, to delineate the role of VPA as HDAC inhibitor and as anticancer agent, we performed studies with VPM, a structurally related analogue of VPA, but is not an inhibitor of HDACs [Göttlicher et al., 2001]. We found that VPM down-regulated the expression of Akt and survivin and effectively enhanced STS-induced apoptosis in SK-N-MC cells. This finding is in line with a recent study that there was no correlation between the effects of VPA on HDAC inhibition and changes in the degree of Erk1/2 phosphorylation, cell growth, or motility [Gotfryd et al., 2010].

In all, our study concludes that VPA-induced down regulation of survivin was due to decreased Akt levels and this effect was not dependent on HDAC activity. However, we do not rule out the possibility of other survival/apoptotic targets like XIAP, Bcl-xL, Bid etc that might be altered and contribute to the enhanced anticancer activity of VPA [Mühlethaler-Mottet et al., 2008]. Additional studies are warranted to investigate such molecules and their relevance to mechanism of action of specific drugs for combination therapy.

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REFERENCES

Azuhata T, Scott D, Takamizawa S, Wen J, Davidoff A, Fukuzawa M, Sandler A. 2001. The inhibitor of apoptosis protein survivin is associated with high-risk behavior of neuroblastoma. J Pediatr Surg 36:1785–1791.

Campbell LA, Seeger RC, Harris RE, Villablanca JG, Matthay KK. 1993. Escalating dose of continuous infusion combination chemotherapy for refractory neuroblastoma. J Clin Oncol 11:623–629.

Chandele A, Prasad V, Jagtap JC, Shukla R, Shastry PR. 2004. Upregulation of survivin in G2/M cells and inhibition of caspase 9 activity enhances resistance in staurosporine-induced apoptosis. Neoplasia 6:29–40.

Chen J, Ghazawi FM, Bakkar W, Li Q. 2006. Valproic acid and butyrate induce apoptosis in human cancer cells through inhibition of gene expression of Akt/protein kinase B. Mol Cancer 5:71.

Condorelli F, Gnemmi I, Vallario A, Genazzani AA, Canonico PL. 2008 Inhibitors of histone deacetylase (HDAC) restore the p53 pathway in neuroblastoma cells. Br J Pharmacol 153:657–668.

Dejligbjerg M, Grauslund M, Litman T, Collins L, Qian X, Jeffers M, Lichenstein H, Jensen PB, Sehested M. 2008. Differential effects of class I isoform histone deacetylase depletion and enzymatic inhibition by belinostat or valproic acid in HeLa cells. Mol Cancer 7:70.

Dosik GM, Rodriguez V, Benjamin RS, Bodey GP. 1978. Neuroblastoma in the adult: Effective combination chemotherapy. Cancer 41:56–63.

Duenas-Gonzalez A, Candelaria M, Perez-Plascencia C, Perez-Cardenas E, de la Cruz-Hernandez E, Herrera LA. 2008. Valproic acid as epigenetic cancer drug: Preclinical, clinical and transcriptional effects on solid tumors. Cancer Treat Rev 34:206–222.

Gotfryd K, Skladchikova G, Lepekhin EA, Berezin V, Bock E, Walmod PS. 2010. Cell type-specific anti-cancer properties of valproic acid: Independent effects on HDAC activity and Erk1/2 phosphorylation. BMC Cancer 10:383.

Göttlicher M, Minucci S, Zhu P, Krämer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG, Heinzel T. 2001. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 20:6969–6978.

Hideshima T, Catley L, Raje N, Chauhan D, Podar K, Mitsiades C, Tai YT, Vallet S, Kiziltepe T, Ocio E, Ikeda H, Okawa Y, Hideshima H, Munshi NC, Yasui H, Richardson PG, Anderson KC. 2007. Inhibition of Akt induces significant downregulation of survivin and cytotoxicity in human multiple myeloma cells. Br J Haematol 138:783–791.

Hřebačková J, Poljaková J, Eckschlager T, Hraběta J, Procházka P, Smutný S, Stiborová M. 2009. Histone deacetylase inhibitors valproate and trichostatin A are toxic to neuroblastoma cells and modulate cytochrome P450 1A1, 1B1 and 3A4 expression in these cells. Interdisc Toxicol 2(3):205–210.

Huang KF, Zhang GD, Huang YQ, Diao Y. 2012. Wogonin induces apoptosis and down-regulates survivin in human breast cancer MCF-7 cells by modulating PI3K-AKT pathway. Int Immunopharmacol 12(2):334–341.

Jiang Y, Saavedra HI, Holloway MP, Leone G, Altura RA. 2004. Abberant regulation of survivin by the RB/E2F family of proteins. J Biol Chem 279:40511–40520.

Johannessen CU. 2000. Mechanisms of action of valproate: A commentatory. Neurochem Int 37:103–110.

Jones PA, Baylin SB. 2002. The fundamental role of epigenetic events in cancer. Nat Rev Genet 3:415–428.

Kaiser M, Zavrski I, Sterz J, Jakob C, Fleissner C, Kloetzel PM, Sezer O, Heider U. 2006. The effects of the histone deacetylase inhibitor valproic acid on cell cycle, growth suppression and apoptosis in multiple myeloma. Haematologica 91:248–251.

Kazantsev AG, Thompson LM. 2008. Therapeutic application of histone deacetylase inhibitors for central nervous system disorders. Nat Rev Drug Discov 7:854–868.

Khan O, La Thangue NB. 2012. HDAC inhibitors in cancer biology: Emerging mechanisms and clinical applications. Immunol Cell Biol 90:85–94.

Khan N, Jeffers M, Kumar S, Hackett C, Boldog F, Khramtsov N, Qian X, Mills E, Berghs SC, Carey N, Finn PW, Collins LS, Tumber A, Ritchie JW, Jensen PB, Lichenstein HS, Sehested M. 2008. Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors. Biochem J 409: 581–589.

Kushner BH, LaQuaglia MP, Bonilla MA, Lindsley K, Rosenfield N, Yeh S, Eddy J, Gerald WL, Heller G, Cheung NK. 1994. Highly effective induction therapy for stage 4 neuroblastoma in children over 1 year of age. J Clin Oncol 12:2607–2613.

Lindemann R, Johnstone R. 2004. Histone deacetylase inhibitors: Promising candidates for chemotherapeutic drugs. Gene Ther Mol Biol 8:61–74.

Lladser A, Sanhueza C, Kiessling R, Quest AF. 2011. Is survivin the potential Achilles' heel of cancer? Adv Cancer Res 111:1–37.

Marks P, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. 2001. Histone deacetylases and cancer: Causes and therapies. Nat Rev Cancer 1:194–202.

Matthay KK, Villablanca JG, Seeger RC, Stram DO, Harris RE, Ramsay NK, Swift P, Shimada H, Black CT, Brodeur GM, Gerbing RB, Reynolds CP. 1999. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. N Engl J Med 341:1165–1173.

Mirza A, McGuirk M, Hockenberry TN, Wu Q, Ashar H, Black S, Wen SF, Wang L, Kirschmeier P, Bishop WR, Nielsen LL, Pickett CB, Liu S. 2002. Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. Oncogene 21:2613–2622.

Mottet D, Castronovo V. 2010. Histone deacetylases: Target enzymes for cancer therapy. Clin Exp Metastasis 25:183–189.

Mühlethaler-Mottet A, Meier R, Flahaut M, Bourloud KB, Nardou K, Joseph JM, Gross N. 2008. Complex molecular mechanisms cooperate to mediate histone deacetylase inhibitors anti-tumour activity in neuroblastoma cells. Mol Cancer 7:55.

Papanikolaou V, Iliopoulos D, Dimou I, Dubos S, Kappas C, Kitsiou-Tzeli S, Tsezou A. 2010. Survivin regulation by HER2 through NF-kappaB and c-myc in irradiated breast cancer cells. J Cell Mol Med 15(7):1542–1550.

Park JR, Eggert A, Caron H. 2010. Neuroblastoma: Biology, prognosis, and treatment. Hematol Oncol Clin North Am 24:65–86.

Park DS, Hwang KE, Shim H, Kim BR, Choi KH, Park SH, Park SD, Jeong ET, Kim HR. 2012. Elevated survivin is associated with a poor response to chemotherapy and reduced survival in lung cancer with malignant pleural effusions. Clin Exp Metastasis 29(2):83–89.

Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS. 2001. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J Biol Chem 276:36734–36741.

Pinkel D, Pratt C, Holton C, James D, Jr., Wrenn E, Jr., Hustu HO. 1968. Survival of children with neuroblastoma treated with combination chemotherapy. J Pediatr 73:928–931.

Rasheed WK, Johnstone RW, Prince HM. 2007. Histone deacetylase inhibitors in cancer therapy. Expert Opin Investig Drugs 16:659–678.

Santini V, Gozzini A, Ferrari G. 2007. Histone deacetylase inhibitors: Molecular and biological activity as a premise to clinical application. Curr Drug Metab 8:383–393.

Schmidt ML, Lukens JN, Seeger RC, Brodeur GM, Shimada H, Gerbing RB, Stram DO, Perez C, Haase GM, Matthay KK. 2000. Biologic factors determine prognosis in infants with stage IV neuroblastoma: A prospective Children's Cancer Group study. J Clin Oncol 18:1260–1268.

Tunnicliff G. 1999. Actions of sodium valproate on the central nervous system. J Physiol Pharmacol 50:347–365.

Valentini A, Gravina P, Federici G, Bernardini S. 2007. Valproic acid induces apoptosis,p16INK4A upregulation and sensitization to chemotherapy in human melanoma cells. Cancer Biol Ther 6:185–191.

Zhang L, Wang G, Wang L, Song C, Leng Y, Wang X, Kang J. 2012. VPA inhibits breast cancer cell migration by specifically targeting HDAC2 and down-regulating Survivin. Mol Cell Biochem 361(1–2):39–45.

Zhao P, Meng Q, Liu LZ, You YP, Liu N, Jiang BH. 2010. Regulation of survivin by PI3K/Akt/p70S6K1 pathway. Biochem Biophy Res Commun 395:219–224.