**VALPROIC ACID: GROWTH INHIBITION OF HEAD AND NECK CANCER BY INDUCTION OF TERMINAL DIFFERENTIATION AND SENESCENCE**

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**Abstract:** Background. There are limited studies on the effects of drugs that modulate epigenetic regulation for head and neck squamous cell carcinoma (HNSCC). This study determined the effect of valproic acid (VPA) on HNSCC.

Methods. Growth inhibition effects of VPA alone or in combination with 5-aza-2′-deoxycytidine (5-aza-dC) or all-trans retinoic acid (ATRA) was evaluated with MTT and clonogenic assays on 5 HNSCC cell lines. The mechanism of growth inhibition was investigated by looking at markers of terminal differentiation and senescence.

Results. Growth inhibition profiles of HNSCC cell lines varied in response to VPA. Inhibition of clonogenic survival in response to VPA was associated with an upregulation of p21, expression of terminal differentiation markers, and cellular senescence. Notably, a combination treatment of 5-Aza-dC-VPA-ATRA enhanced growth inhibition in cells resistant to VPA.

Conclusion. VPA is a potent inhibitor of proliferation in some HNSCC cell lines, and may be used to treat HNSCC.

**Keywords:** histone deacetylase inhibitor; valproic acid; head and neck cancer; terminal differentiation; senescence

**Head and neck cancer** is 1 of the 6 most common cancers in the world.¹ Despite advances in cancer diagnosis and treatment, head and neck squamous cell carcinoma (HNSCC) remains a disease associated with high morbidity and mortality.² Additionally, many patients with HNSCC develop second primary tumors and/or recurrent disease which complicates patient management.³,⁴ The morbidity and mortality directly associated with HNSCC underscores the need to develop more effective therapeutic regimes to control the disease more efficiently.

The development of promising cancer therapeutics is based on the understanding that cancer results from a plethora of genetic and epigenetic alterations in key regulatory pathways that collectively deregulate major control of cell growth. For example, histone acetyl transferases and histone deacetylases (HDACs) both control the level of acetylation and hence transcription activity within a cell.⁵ Deregulation and overexpression of these enzymes have been reported in several cancers including HNSCC,⁶⁻⁹ and HDAC inhibitors (HDACIs) can be used to inhibit the growth of cancers in vitro and in vivo with relatively little toxicity to normal cells.¹⁰⁻¹⁶ Furthermore, HDACIs have also been shown to enhance the effects of chemotherapy and improve radiosensitivity in cancer cells.¹⁷⁻¹⁹

Although the mechanism by which HDACIs inhibit cancer growth is not entirely clear, they have been shown to cause cell-cycle arrest in G1 and/or G2 phase, induce apoptosis, and/or differentiation in vitro and block angiogenesis in vivo.¹⁹⁻²⁵ The ability of HDACIs to control cancer growth, and its relative nontoxicity in normal untransformed cells,²⁶,²⁷ have propelled them into phase I and phase II clinical trials.²⁶⁻³²

One of these compounds currently in phase II clinical trials to treat solid tumors is valproic acid (VPA).²⁶,³³⁻³⁶ VPA is a short chain fatty acid widely used in humans as an anticonvulsant and mood stabilizer, particularly in the long-term treatment of epilepsy.²⁷⁻³⁹ In vitro, VPA has been shown to inhibit proliferation and induce differentiation in both solid and hematological cancers.²²,⁴⁰⁻⁴² Although VPA is not...
as potent as some of the other HDACIs, VPA has numerous advantages over other HDACIs. First, VPA has HDACI activity at physiologically achievable concentrations that are associated with minimal side effects; second, it is easily administered; third, it has long biological half-life; and fourth, VPA can act synergistically with other chemotherapeutic drugs.

In the current study, we investigated the growth inhibition effects of VPA in a panel of HNSCC cell lines. In addition, we investigated the molecular mechanism underlying the effects of VPA. Furthermore, we also determined if VPA could enhance the efficacy of other drugs including DNA methyl transferase inhibitor 5-aza-2’-deoxycytidine (5-aza-dC) and all-trans retinoic acid (ATRA) in the inhibition of HNSCC growth.

MATERIALS AND METHODS

Cell Lines and Reagents. The HNSCC cell line SCC-15 was obtained from the American Type Culture Collection, whereas ORL-48, ORL-156, ORL-166, ORL-215, ORL-295N, and ORL-310N were established in our laboratory. All HNSCC cell lines and normal keratinocyte primary cultures (ORL-295N and ORL-310N) were cultured and confirmed to be of keratinocyte in origin by cytokeratin staining as described previously. To ensure that our observations were not due to genetic changes induced by tissue culture conditions, cells lines that were established in our laboratory were used at passage 20 or below. VPA (Sigma–Aldrich, St. Louis, MO) and 5-aza-2’-deoxycytidine (5-Aza-dC; Sigma–Aldrich) was prepared at 300 mM and 1 mM respectively, in serum free DMEM/F12. ATRA (Sigma–Aldrich) was prepared at 100 mg/mL in dimethyl sulfoxide.

Cell Viability Assay. The acute growth inhibitory effect of VPA was assessed using the MTT dye reduction assay as described by others. HNSCC cell lines and normal oral keratinocytes were seeded at 5000 cells per well in 96-well tissue culture plates and incubated for 24 hours at 37°C in a 5% CO2 incubator. Cells were treated with 0.25 to 3 mM VPA for 48, 72, 96, or 120 hours before performing the assay. Due to the limited number of cells for normal oral keratinocyte primary cultures, the growth inhibition effect of VPA was only evaluated at 96 hours posttreatment for ORL-295N and ORL-310N.

For drug combination treatments, growth inhibitory effects on SCC-15 and ORL-48 cell lines were also accessed using MTT assay, after treatment with either 1 of the following: VPA (5d); 5-Aza-dC (5d); ATRA (5d); VPA (3d) + 5-Aza-dC (2d); VPA (3d) + ATRA (2d); 5-Aza-dC (3d) + ATRA (2d); 5-Aza-dC (2d) + VPA (2d) + ATRA (1d); or VPA (2d) + 5-Aza-dC (2d) + ATRA (1d). From here on, VPA was used at 1 mM as this is a physiologically achievable dose, whereas 5-Aza-dC and ATRA were used at 0.5 μM and 1 μM, respectively. Results from these experiments were confirmed using the real-time cell analyzer (RTCA; ACEA Biosciences, San Diego, CA). Cells were monitored continuously and recorded as a cell index every 30 minutes for approximately 200 hours as reported previously.

Clonogenic Assay. The chronic growth inhibitory effects of VPA was investigated using clonogenic assay. Cells were plated into 60 mm2 cell culture dishes and incubated for 24 hours at 37°C in a 5% CO2 incubator. After treatment with 0.25 to 3 mM VPA for 96 hours, the cells were tryspinized and plated at 200 cells per well in a 6-well tissue culture plate. Cells were then further incubated for 10 to 14 days to allow for colony formation. The cells were fixed with 0.2% crystal violet in 10% formalin in phosphate-buffered saline (PBS), the number of grossly visible colonies was counted, and colony formation efficiency was calculated with reference to untreated cells. The average plating efficiency of the cells was approximately 10%.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blots. SCC-15, ORL-48, ORL-156, and ORL-166 cells were seeded at 5E4 cells/mL in 100 mm2 tissue culture dishes for 24 hours, and then treated with 1 mM VPA for 4, 8, 24, or 48 hours. Total protein was extracted, resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membrane as previously described. Membranes were immunoblotted with antibodies for acetylated histone 3 (AcH3; 0.05 μg/mL; Upstate Biotechnology, Lake Placid, NY), p21 (2 μg/mL; Calbiochem–Novabiochem International, La Jolla, CA), or p53 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with the respective horseradish peroxidase-conjugated secondary antibodies. Proteins bands were detected using enhanced chemiluminescence method (Alpha Innotech Corporation, San Leandro, CA) and membranes were re-probed with monoclonal actin antibody (1:1000; Chemicon, Temecula, CA) to control for loading variations.

RNA Isolation and cDNA Synthesis. After treatment with VPA for 8, 24, 48, and 72 hours, total RNA was isolated from SCC-15, ORL-48, ORL-156, and ORL-166 cell lines using TRI-reagent (Molecular Research Centre, Cincinnati, OH) according to the manufacturer’s instructions. The quality and quantity of the extracted RNA was evaluated using the Nanodrop (Nanodrop Technologies, Wilmington, DE). Reverse transcription was performed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) and 1 μl of the synthesized cDNA was used for subsequent quantitative polymerase chain reactions (qPCR).

Quantitative Polymerase Chain Reaction. All qPCRs were performed on the ABI 7000 Sequence Detector (Applied Biosystems, Foster City, CA). Relative
expression of p53 was quantified by TaqMan qPCR assay (ID Hs01034252_g1/p53 exons 3&4; Applied Biosystems) using actin as an endogenous control (Hs99999903_m1; Applied Biosystems). To examine the expression of cell cycle regulators (p21, p16\(^{Nk4a}\), CDK2, CDK4, CCND1, and CCNE1) and markers of terminal differentiation (IVL and SPRR1b) and ORL-48 cells to determine the expression level of cell cycle regulators (p21, p16INK4a, CDK2, CDK4, CCND1, and CCNE1) and markers of terminal differentiation (IVL and SPRR1b), qPCR was performed in 25 μl volume reactions as described above. Primers used in these experiments were either designed using Primer Express 2.0 (Applied Biosystems) or obtained from previous studies (Table 1).\(^{53,54}\)

**Table 1. List of primers used for qPCR and methylation study.**

| Gene | Sequence |
|------|----------|
| CCND1 | (F) 5′ CCC TGA CGG CGG AGA AG 3′  |
|       | (R) 5′ CGG CGG CGG CGG AGA AG 3′  |
| CCND1 | (F) 5′ CCC TGA CGG CGG AGA AG 3′  |
|       | (R) 5′ CGG CGG CGG CGG AGA AG 3′  |
| CDK2  | (F) 5′ GGT GTG GCC AGG AGT TAC TTC T 3′  |
|       | (R) 5′ GCT TGG TCA CAT CCT GGA AGA 3′  |
| CCNE1 | (F) 5′ CTG GAT GTT GAC TGC CTT GAA TT 3′  |
|       | (R) 5′ CGG ACC CCC CTG AAG TG 3′  |
| CDK4  | (F) 5′ AAT GGT GTG CGG CCG AGT ATG GA 3′  |
|       | (R) 5′ ATG CAA TTG GCA TGA AGG AAA 3′  |
| p21   | (F) 5′ CTG GAG ACT TCT AGG GTC GAA 3′  |
|       | (R) 5′ CGG CGT GTG GAG TGG TAG AA 3′  |
| IVL   | (F) 5′ GCA AGA ATG TGG GCA ACA GC 3′  |
|       | (R) 5′ TGC TCT GGG TTT TCT GCT TT 3′  |
| SPRR1b | (F) 5′ GAA TGT GTG ATG AAG ATG TCT TCT C 3′  |
|       | (R) 5′ CAG ATG AAT TCT GAG CAG CTT GAA 3′  |
| p16\(^{Nk4a}\) | (F) 5′ GAG CAG ACAT GGA GCC TTC TCC 3′  |
|       | (R) 5′ GCG GAA AAA GCC CTT ACA TCC C 3′  |
| RAR-β2 | (F) 5′ TGG AGA AGC AGC GGG ATT CG 3′  |
|       | (R) 5′ GAC CAA TCC AAC CGA AAG AA 3′  |
| RAR-β2 (Me) | (F) 5′ TGG AGA AGC AGC GGG ATT CG 3′  |
|       | (R) 5′ GAC CAA TCC AAC CGA AAG AA 3′  |
| RAR-β2 (UnMe) | (F) 5′ TGG AGA AGC AGC GGG ATT CG 3′  |
|       | (R) 5′ GAC CAA TCC AAC CGA AAG AA 3′  |

**Abbreviation:** qPCR, quantitative polymerase chain reaction.

**Methylation-Specific Polymerase Chain Reaction and RAR-β2 Expression.** Genomic DNA was isolated from SCC-15 and ORL-48 cells using the QIamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) after treatment with 1 mM VPA or 0.5 μM 5-Aza-dC. Methylation of RAR-β2 was investigated using the CpGenome DNA Modification Kit (Chemicon, Temecula, CA). After DNA modification, PCR was performed using primers either targeting methylated DNA or unmethylated DNA as described elsewhere.\(^{54}\) The amplicons were electrophoresed on a 2% agarose gel for assessment of methylation status. After treatment with VPA, RNA was also extracted from SCC-15 and ORL-48 cells to determine the expression level of RAR-β2 using qPCR as described above. Primers used in these experiments are tabulated in Table 1.

**Cell Cycle Analysis.** Cell cycle analysis was performed by flow cytometry after treating SCC-15 and ORL-48 cell lines with 0.5 mM, 1 mM, or 3 mM VPA. Cells were harvested at 24, 48, and 72 hours. Briefly, floating and adherent cells were collected, washed twice with cold PBS containing 0.01% BSA, and fixed overnight with 75% ethanol. The cells were resuspended in PBS containing 20 μg/mL of DNase-free RNaseA and 10 μg/mL propidium iodide for 30 minutes at room temperature in the dark. Cell cycle distribution was measured using the fluorescence activated cell sorter Calibur (Becton Dickinson, Franklin Lakes, NJ). DNA content was quantified using the Cell Quest software.

**Senescence-Associated β-Galactosidase Assay.** SCC-15 and ORL-48 cells were seeded at 1E4 cells/mL in 6-well plates. Upon treatment with 1 mM VPA for 48, 72, and 96 hours, both untreated and treated cells were fixed and stained for senescence-associated β-galactosidase (SA-β-gal) expression using the Senescence Detection Kit (BioVision, Mountain View, CA) according to the manufacturer’s instructions. The development of cytoplasmic blue color stain was detected under an inverted microscope at ×200 magnification and cells were counted in 6 random fields. The number of senescent cells was expressed as a percentage relative to the total number of cells counted for each field.

**Statistical Analysis.** All experiments were performed in triplicate. Data were obtained from at least 3 independent sets of experiments and expressed throughout as means ± SEM. Statistical evaluation was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Comparisons between groups were analyzed using 1-way analysis of variance with Bonferroni adjustment. Statistical significance was set at p < .05.

**RESULTS**

**Valproic Acid Inhibits Growth in Selective Head and Neck Squamous Cell Carcinoma Cell Lines at Physiologically Achievable Doses.** The acute growth-inhibitory effects of VPA measured by MTT assay varied between cell lines. Although SCC-15 and ORL-215 were sensitive to VPA (IC\(_{50}\) [96 hours]: 1.28 and 1.38 mM, respectively), ORL-48, ORL-166, and ORL-156 were relatively resistant to VPA and even at the highest concentration (3 mM), growth inhibition was observed in only 40%, 4%, and 25% of the cells, respectively (Figure 1A–E). Notably, we demonstrated that VPA had limited growth-inhibitory effects on normal oral keratinocytes, where 68.8% and 99.6% of cells were still alive for ORL-295N and ORL-310N, respectively (Figure 1F). The chronic growth-inhibitory effects of VPA also varied between the cell lines. However, VPA caused significant growth-inhibition in 3 of the cell lines (SCC-15, ORL-166, and ORL-215) after 96 hours of treatment with IC\(_{50}\) ranging between 0.46 to 1.79 mM. SCC-15 and ORL-166
exhibited the largest growth-inhibitory effects whereas ORL-48 and ORL-156 were not sensitive to VPA (Figure 1G). We attempted to perform growth-inhibition assays on ORL-295N and ORL-310N normal oral keratinocytes, but both the untreated and treated cells senesced before the assay could be completed.

**Valproic Acid Caused Histone Hyperacetylation and p21-Dependent Terminal Differentiation.** To determine the molecular mechanism underlying response to VPA, we compared the cell lines that were sensitive to VPA-induced cytostasis (SCC-15 and ORL-166) to those that were resistant (ORL-48 and ORL-156). We demonstrated that 4 hours upon VPA treatment, the acetylation of histone 3 (H3) was observed for both sensitive and resistant cells (Figure 2A) suggesting that VPA could act as an HDACi in all HNSCC cell lines. By contrast, p21 expression was increased upon VPA treatment in SCC-15 and ORL-166 cell lines but not in ORL-48 and ORL-156, and this effect seems to be independent of p53 (Figure 2A). In addition, we also found an increase in the expression of 2 terminal differentiation markers (involucrin [IVL] and SPRR1b) in both sensitive and resistant cell lines. The increase in IVL was significantly greater in SCC-15 and ORL-166 (6.9-fold and 14.8-fold, respectively) in contrast to ORL-48 and ORL-156 (2.3-fold and 4.4-fold, respectively; Figure 2B), suggesting that VPA induces terminal differentiation in sensitive cell lines. Although it seems that VPA could induce the expression of SPRR1b in both sensitive and resistant cell lines, the resistant cell lines had less than 5% of SPRR1b expression in comparison to confluent normal oral keratinocytes despite VPA treatment (Figure 2B).

**Valproic Acid Induced Growth Arrest and Senescence in Valproic Acid Responsive Cells.** To determine if the cytostasis observed in SCC-15 cells treated with VPA was due to cell cycle arrest, we analyzed both SCC-15 and ORL-48 cells treated with VPA using fluorescence activated cell sorter analyses. After 48 hours of VPA treatment, we showed that VPA significantly induces G1 arrest in SCC-15 but not in ORL-48 (Figure 2C; p < .01). As the cell cycle is governed by cyclin-dependent kinases (CDKs) and cyclins, we analyzed the expression of these genes after VPA treatment in both SCC-15 and ORL-48. The G1 arrest observed in SCC-15 was not associated with the downregulation of CDK2, CDK4, and CCNE1 (Figure 2D). Despite the downregulation of CCND1 observed at 8 hours after treatment, this effect was transient and subsequent upregulation of CCND1 was observed. Interestingly, the levels of CCNE1 increased in both SCC-15 and ORL-48 cells upon VPA treatment as early as 8 hours and remained high after 72 hours of VPA treatment (Figure 2D). Collectively, these data suggest that the G1 arrest observed in SCC-15 was induced by mechanisms other than the downregulation of cell cycle promoters.

We further determined if the G1 arrest was due to cellular senescence by staining treated cells for SA-β-gal activity. There was a significant increase in senescent cells in SCC-15, after 96 hours of VPA treatment but not in ORL-48 indicating that prolonged treatment with VPA-induced senescence in SCC-15 (Figure 2E and 2F). This was consistent with the expression of p16INK4a where in SCC-15 expression was persistently higher compared to ORL-48 (Figure 2G).

**Valproic Acid Treatment of Head and Neck Squamous Cell Carcinoma Cells Restored RAR-β2 Expression in ORL-48.** HDACIs have previously been shown to revert the epigenetic silencing of the RAR-β2 gene to sensitize cells to the effects of ATRA. In this study, we determined if VPA could restore RAR-β2 expression to increase the efficacy of ATRA in HNSCC cells particularly in ORL-48 where cells were not responsive to VPA treatment alone. We demonstrated that the RAR-β2 gene in both ORL-48 and SCC-15 were inherently methylated and the treatment with VPA did not reverse the methylation status of both cell lines completely (Figure 3A). However, a slight increase in RAR-β2 expression was observed in ORL-48 after 72 hours of VPA treatment (Figure 3B) indicating that VPA could in part revert the epigenetic silencing of RAR-β2 in these cells. Given that the expression of RAR-β2 can be induced by demethylation agent 5-Aza-dC, we also treated both cell lines with 0.5 mM 5-Aza-dC. Upon 5-Aza-dC treatment, the methylation status was partially reversed in ORL-48 but not in SCC-15 (Figure 3A).

Correspondingly, the expression of RAR-β2 in ORL-48 increased after 48 hours but remains unchanged in SCC-15 (Figure 3C). Collectively, these data indicate that both VPA and 5-Aza-dC could revert the epigenetic silencing of RAR-β2 in ORL-48 cells but not in SCC-15 suggesting that a combination of VPA and/or 5-Aza-dC and ATRA could be more effective in killing ORL-48 cells compared to VPA alone.

**Combination of Valproic Acid and All-Trans Retinoic Acid did not Increase Growth Inhibition in Head and Neck Squamous Cell Carcinoma Cancer Cell Lines.** To determine the effects of VPA and 5-Aza-dC in combination with ATRA on growth inhibition, we treated SCC-15 and ORL-48 cells with single agents or a combination of drugs. Consistent with the data described above, SCC-15 was very sensitive to VPA alone (23.6% survival). A combination of VPA + ATRA or 5-Aza-dC + ATRA did not improve growth inhibition significantly in SCC-15 compared to each single agent used (Figure 4A). The highest growth inhibition in SCC-15 cells was achieved by a combination of VPA and 5-Aza-dC, although this was not significantly higher than the effect caused by VPA alone (19.5% vs 23.6% survival; Figure 4A).

By contrast, in ORL-48, the effects of VPA or 5-Aza-dC alone did not seem to differ by much at 120 hours posttreatment as determined by MTT assays (Figure 4A). Interestingly, the growth-inhibitory effect of 5-Aza-dC in ORL-48 cells was obvious after the drug
FIGURE 1. Growth-inhibition profile for head and neck squamous cell carcinoma (HNSCC) cell lines and normal oral keratinocytes exposed to increasing concentrations of valproic acid (VPA). Cells were incubated with 0.25, 0.5, 1.0, 1.5, or 3 mM of VPA and MTT assays were performed at 48, 72, 96, and 120 hours (A–E) or at 96 hours (F) after treatment. The percentage of viable cells exposed to various concentrations of VPA was normalized against that of untreated cells. Data are the mean from 6 experiments. (G) Dose–response effects of VPA on clonal proliferation of HNSCC cell lines. Cells were incubated with 0.25, 0.50, 1.0, 1.5, 2.0, or 3.0 mM VPA for 96 hours. The percentage of surviving cells exposed to various concentration of VPA was normalized against that of untreated cells. Data are mean from 3 experiments.
Valproic acid (VPA) restored histone (H3) acetylation in both sensitive and resistant cell lines. Acetylation of H3 is associated with the increase in p21 expression in SCC-15 and ORL-166 but not ORL-48 and ORL-156. The induction of p21 expression is independent of p53 expression.

VPA treatment in head and neck squamous cell carcinoma (HNSCC) cell lines increased the expression of terminal differentiation markers IVL and SPRR1b. The levels of involucrin (IVL) and SPRR1b in treated and untreated cells were expressed as relative levels in comparison to confluent cultures of normal oral keratinocytes (ORL-295N). The induction of IVL expression was significantly higher in sensitive (SCC-15 and ORL-166) in comparison to resistant (ORL-48 and ORL-156) cells.

VPA induced G1 arrest in SCC-15 but not ORL-48 (**p < .01). Cells were treated with 1 mM VPA for 48 hours and stained with propidium iodide and analyzed by flow cytometry.

Expression of CCND1, CDK4, CCNE1, and CDK2 were investigated by quantitative polymerase chain reaction (qPCR). Cells were treated with 1 mM VPA for 48 hours. Results represent the mean ± SEM of 3 independent experiments.

VPA induces cellular senescence in SCC-15 but not in ORL-48. The number of senescent cells after VPA treatment was determined using senescence-associated β-galactosidase (SA-β-gal) assay and expressed as a percentage of cells relative to the total number of cells analyzed (**p < .01, ***p < .001).

VPA increases the expression of p16INK4a. The p16INK4a expression in SCC-15 and ORL-48 were expressed as relative levels to untreated cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
was left on for much longer periods as monitored using the RTCA microelectronic cell sensor system (Figure 4B). Similar cell killing effects were not observed in ORL-48 cells treated with VPA for the same period of time (Figure 4B). Although we showed that both VPA and 5-Aza-dC could revert the epigenetic silencing of RAR-β2 in ORL-48, we did not see a significant increase in growth inhibition when either of these drugs was used in combination with ATRA (75.2% vs 60.2% and 51.8% vs 55.6% survival, respectively; Figure 4A), indicating that an increase in RAR-β2 expression does not always guarantee a response to ATRA. There were no significant differences between ORL-48 cells treated with 5-Aza-dC alone compared to those treated with 5-Aza-dC-ATRA and 5-Aza-dC-VPA-ATRA. However, VPA seems to work with 5-Aza-dC and ATRA to further enhance growth inhibition ($p < .05$), leading to maximum growth inhibition in comparison to the other combinations of drug treatment. Further, we also demonstrated significant increase in growth inhibition was achieved when cells were treated with 5-Aza-dC-VPA-ATRA in comparison to VPA-ATRA ($p < .001$) again strongly indicating that both demethylation and acetylation are required to improve growth inhibition with ATRA in this cell line (Figure 4A).

**DISCUSSION**

HDACIs have emerged as a class of promising drugs for the treatment of cancer. In vitro and in vivo studies have demonstrated the potent growth inhibitory effects of HDACIs in many types of cancers. In HNSCC, HDACIs have been shown to inhibit the growth of carcinoma cells and sensitize them to other chemotherapeutic drugs. However, many established HDACIs are limited in their clinical utility because of their toxicity. In this study, we demonstrated that VPA, a well-established anticonvulsant, has both acute and chronic growth inhibitory effects in some HNSCC cell lines at physiologically achievable doses. VPA had very potent effects on the HNSCC cell line SCC-15 by inducing the expression of p21 and inducing G0/G1 arrest. This is consistent with previous reports that HDACIs including VPA can cause cytotoxicity and growth inhibition in HNSCC cell lines. Although growth arrest in head and neck cancer cells treated with HDACI was shown to be associated with the downregulation of mitotic genes in a recent study, our study demonstrated that growth arrest in SCC-15 was not associated with the downregulation of CCND1, CDK2, or CDK4, but an upregulation of CCNE1. The upregulation of CCNE1 was not unexpected as previous studies have shown that HDACIs could increase the expression of CCNE1 by causing hyperacetylation around the specific Sp1 sites within the promoter. VPA has been demonstrated to promote differentiation in several types of cancer cell lines including those of hematopoietic, neuroblastoma, and follicular thyroid origin. Using markers of
terminal differentiation, we demonstrated that VPA induced the expression of IVL suggesting that the growth inhibitory effects of VPA was, at least in part, due to the induction of terminal differentiation in HNSCC cells. Interestingly, we found that SPRR1b expression was significantly lower in resistant cell lines in comparison to those that were sensitive despite VPA treatment. Whether or not this could be an indication of the inability of these cells to undergo terminal differentiation remains to be determined. Finally, prolonged treatment of SCC-15 with VPA causes cellular senescence. Taken together, these data suggest that VPA, at physiologically achievable concentrations, may be considered as an additional or alternative treatment modality for some HNSCCs.

Importantly, HDACIs have been reported to improve the efficacy of a wide spectrum of chemotherapeutic drugs including those that modify epigenetic changes such as retinoic acid and 5-Aza-dC. Retinoic acid inhibits growth and induces differentiation in vitro by activating the RAR-β2 receptor which is frequently inactivated in cancers through epigenetic modification. For HNSCC in particular, retinoic acid has been shown to suppress premalignant lesions and secondary tumors. However, the use of retinoic acid is limited by its toxicity as high doses are necessary to overcome the inactivation of RAR-β2 caused by epigenetic silencing. Therefore, the restoration of RAR-β2 expression by chromatin remodeling using a demethylation agent or histone deacetylase inhibitor should increase the efficacy of retinoid acids in the treatment of cancer.

Consistent with this reasoning, a previous study on leukemic cell lines demonstrated that retinoic resistant cells could be re-sensitized to retinoic acid using HDACIs. Ferrara et al also demonstrated that a combination of tricostatin A and retinoic acid-induced growth arrest and terminal differentiation in leukemic cell lines and primary blasts from patients in comparison to either single agent used alone. Furthermore, histone acetylation and cellular differentiation was observed in samples from patients with acute myeloid leukemia treated with a combination of VPA and ATRA and stable disease was achieved in 5 of 8 patients. Interestingly, in contrast to these reports, our study demonstrated that a combination
of VPA and ATRA does not improve cell killing significantly in HNSCC cell lines. In SCC-15, this is most likely because VPA could already induce growth inhibition in the majority of cells, therefore, the effects of ATRA did not seem significant. In addition, different gene targets may be activated in response to VPA in the different cell types, and the inherent genetic differences that exist between the cell lines dependent or independent of HDAC inhibition may also contribute to these different observations. Notably, although ORL-48 was relatively resistant to VPA compared to SCC-15 cells, it was evident that VPA could enhance growth inhibition when combined with 5-Aza-dC-ATRA and used in the sequence of 5-Aza-dC-VPA-ATRA. Collectively, these results indicated that epigenetic changes such as modulation of methylation and acetylation are important drivers in HNSCC development.

In summary, as VPA has been shown to have acute and chronic growth inhibitory effects in HNSCC cell lines, this may be particularly relevant for patients with HNSCC in whom secondary or recurrent tumors are frequent. Considering this, the efficacy of VPA shown in HNSCC cell lines and its well-documented toxicity profile makes it an attractive agent for the treatment of head and neck cancer. However, it should be noted that long-term treatment with VPA is probably necessary to observe the benefits, as we have shown that the effects of VPA are seen only after prolonged treatment whereas others have demonstrated the reversible effects of VPA in the short term.12,45 This is, however, not a concern as VPA has been proven to be safe to use as a long-term treatment as it is currently used to treat patients with epilepsy.37–39 Notably, the treatment of patients with HNSCC with VPA has confirmed some of the HDACI properties of the drug reported in in vitro systems45 albeit with some variations; therefore, the use of VPA treatment (like many other current treatment options) could be enhanced with the identification of molecular markers that would identify individuals who would most benefit from this treatment.

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