Antitumour effect of valproic acid against salivary gland cancer *in vitro* and *in vivo*

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Abstract. Salivary gland cancer (SGC) has a comparatively poor prognosis and is prone to frequent recurrence and metastases. Therefore, the development of more effective chemotherapy against SGC is desirable. The aim of the present study was to investigate the antitumour effects of valproic acid (VPA) against SGC *in vitro* and *in vivo*. Two human SGC cell lines (HSY and HSG cells) were used in the present study. The effects of VPA on the proliferation of SGC cells *in vitro* were assessed by MTT assay. Cancer cells treated with VPA were subjected to cell cycle analysis by flow cytometry. In addition, the expression levels of p21 and p27 were examined by real-time RT-PCR to identify the mechanisms of the antitumour effect of VPA on SGC. The effects of VPA on cancer growth *in vivo* were evaluated in a xenograft model. VPA inhibited the proliferation of SGC cells in a dose-dependent manner *in vitro*. Degenerated cancer cells were observed at high concentrations of VPA. In the cell cycle analysis, VPA induced cell-growth inhibition and G1 arrest of cell cycle progression in both cancer cell lines in a time- and dose-dependent manner. VPA markedly upregulated the mRNA expression levels of both p21 and p27 in both SGC cell lines in a time-dependent manner. In the xenograft model experiment, VPA treatment markedly inhibited the growth of salivary gland tumours when compared with the growth of the untreated controls. VPA may be a valuable drug in the development of better therapeutic regimens for SGC.

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Abbreviations: VPA, valproic acid; SGC, salivary gland cancer; HDAC, histone deacetylase

Key words: valproic acid, salivary gland cancer, antitumour activity, epigenetics, histone deacetylase inhibitor

Introduction

Salivary gland cancer (SGC) is a rare cancer type of the head and neck region where cancer cells form in major or minor salivary glands (1-3). Generally, surgical resection is considered to be the most common and successful therapeutic approach for SGC performed today (1,4); however, SGC frequently recurs locally and metastasises to the regional lymph nodes and distant organs, which results in poor prognosis (4-6). Although other treatment approaches for SGC may include radiation therapy and chemotherapy, SGC exhibits low sensitivity to irradiation and chemotherapy. Therefore, the development of more effective chemotherapeutic drugs and strategies against SGC are desirable.

Cancer has long been known to be a primarily genetic disease initiated and progressed by alterations in genes, such as oncogenes and tumour suppressor genes. However, recent reports indicate that various types of cancers are caused by epigenetic changes, which are heritable changes in gene expression that occur without alteration in DNA sequences (7-9). These epigenetic changes, including DNA methylation and histone modifications, modify the structure of chromatin and affect the accessibility of regulatory proteins to DNA (10,11). Histone acetylation is one such epigenetic change and has been reported to play important roles in the initiation and progression of cancers (12,13). Histone deacetylation can cause the tightening of chromatin and can block transcriptional activation. Moreover, it is reversible and is regulated by a balance between the opposing activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC). HAT is an enzyme that catalyses the acetylation of N-terminal lysine residues in histone proteins, which reduces the affinity of histone for DNA and increases the accessibility of transcriptional regulatory protein to chromatin (10). On the other hand, HDAC is an enzyme that catalyses the removal of acetyl groups from histones, and increased HDAC activities are usually associated with transcriptional repression (10).

Recently, altered HDAC activities have been shown to be present in many types of cancers, possibly representing an attractive target for cancer therapy (7-9,12,13). HDAC inhibitors induce cell cycle arrest and apoptosis of cancer cells by blocking deacetylases leading to the accumulation of

hyperacetylated histones and alteration of gene transcription (7,9,11,14,15,17,18). Various classes of HDAC inhibitors have been identified and are currently being tested in phase I/II clinical trials (12,13). Valproic acid (VPA), which is widely used for epilepsy and manic-depressive psychosis, has also recently garnered attention as a specific inhibitor of HDAC activity (7,19). It is known that chromatin with hyperacetylated core histones adopts a relaxed conformation with the unfolding of the associated DNA and chromatin remodelling during the nucleosome packing of DNA, which are key steps in the regulation of many genes that play crucial roles in cell growth and differentiation (10). VPA has been reported to show antitumour effects against many types of cancers such as acute myeloid leukaemia, breast and prostate cancers and head and neck squamous cell carcinomas. The objective of the present study was to define the biological and therapeutic effects of VPA in treating SGC.

Materials and methods

Valproic acid. Valproic acid sodium salt (VPA; Sigma Aldrich Co., St. Louis, MO, USA) was dissolved in Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} (DPBS; Sigma Aldrich) as a stock solution. For *in vitro* experiments, the VPA stock solution was diluted with the complete culture medium. For *in vivo* experiments, the VPA stock solution was supplemented in drinking water.

Cell lines and cell culture. Two human SGC cell lines, HSY and HSG cells, were used in the present study. They are adenocarcinoma cell lines derived from the parotid gland and submandibular gland, respectively (20,21). These two cell lines produce tumours when subcutaneously inoculated into nude mice. They were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich) supplemented with 10% fetal calf serum (FCS), 100 μg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere composed of 95% air and 5% CO_2 at 37˚C.

In vitro cell proliferation assay. The proliferation of SGC cells treated with VPA was assessed by MTT assay. Cells were seeded on 96-well plates (Falcon, Franklin Lakes, NJ, USA) at $2x10^3$ cells/well in DMEM containing 10% FCS. Twenty-four hours later, cells were treated with various concentrations (0, 1, 2, 5 and 10 mM) of VPA for 2 and 4 days. After VPA treatment, a 10 μl aliquot of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich) was added to each well, and the cells were incubated for 4 h. The blue dye taken up by cells was dissolved in dimethyl sulphoxide, and the absorbance was measured with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 540 nm. All assays were run in triplicate.

Mice and in vivo study. BALB/c nude mice were purchased from Japan CLEA Inc. (Osaka, Japan). The mice were maintained under pathogen-free conditions and were handled in accordance with the Guidelines for Animal Experimentation of Tokushima University. The experiments were initiated when the mice were 8 weeks of age and were performed as previously described (23). Five million HSY cells were inoculated subcutaneously into the backs of mice through 26G needles. Once palpable tumours were established at 1 week after the inoculation, VPA treatment was started by administering VPA (0, 0.2 and 0.4% w/v) in drinking water. The tumour volume and body weight of the mice were measured twice a week. Tumour volumes were calculated using the following formula: Tumour volume = 0.5 x L x W^2, where L and W represent the largest diameter and the smallest diameter, respectively. For each treatment, six mice were used.

Cell cycle analysis. The cell cycle distribution was analysed using a fluorescence-activated cell sorter (FACS) as previously described (23,24). Cells grown under subconfluent conditions were treated with VPA (0, 2 and 5 mM). After 12 or 24 h of VPA treatment, cells were collected and fixed with ice-cold 70% ethanol for 30 min and stored at 4˚C until further analysis. Then, cells were treated with propidium iodide (40 μg/ml) and RNase A (1 μg/ml) for 20 min at 37˚C. Samples were kept on ice, and the cell cycle analysis was completed by measuring the propidium iodide-stained DNA content with an Epics® XL-MCL cytometer (Beckman Coulter, Brea, CA, USA).

Real-time reverse transcription-polymerase chain reaction (RT-PCR). Cancer cells grown under subconfluent conditions were treated with VPA (0, 2 and 5 mM). After 24 or 48 h of VPA treatment, total RNA was prepared from the cells using Isogen® (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. The mRNA expression of p21 and p27 was examined by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as an internal control. Gene-specific products were measured continuously by an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) over 40 cycles. Experiments were performed at least three times.

Statistical analysis. All numerical data are expressed as the average of the values obtained ± SD. Significant differences between the means for the different groups were evaluated using one-way ANOVA, with the level of significance at $P<0.05$. All experiments were repeated two to three times, and similar results were obtained.

Results

Effect of VPA on the proliferation of salivary gland cancer cells in vitro. We examined the effect of VPA on cell morphology and proliferation of SGC cells *in vitro*. The morphology of cancer cells treated with 0, 2 and 5 mM of VPA for 24 h was evaluated. As shown in Fig. 1, the morphology of the HSY cells was transformed from being cuboidal to being spindle-shaped in a dose-dependent manner. The results obtained for the HSG cells were similar to those obtained for the HSY cells (data not shown).

Next, the proliferation of cancer cells treated with 0, 1, 2, 5 and 10 mM of VPA was assessed by MTT assay. As shown in Fig. 2, VPA inhibited the proliferation of SGC cells in a dose-dependent manner. Treatment with 5 mM VPA induced a proliferation inhibition rate of ~40-70% in both cancer cell lines. Degenerated cancer cells were observed at a high

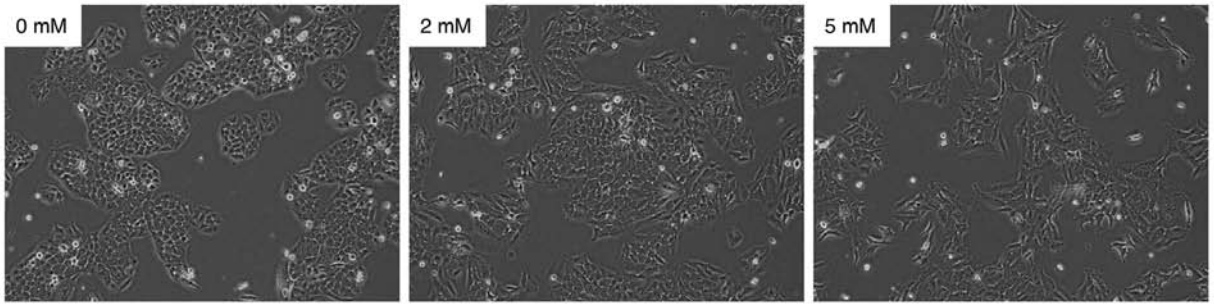

Figure 1. Phase-contrast micrographs of HSY cells treated with VPA. HSY cells were treated with 0, 2 and 5 mM of VPA for 24 h. The morphology of HSY cells was transformed from being cuboidal to being spindle-shaped in a dose-dependent manner. Original magnification, x40.

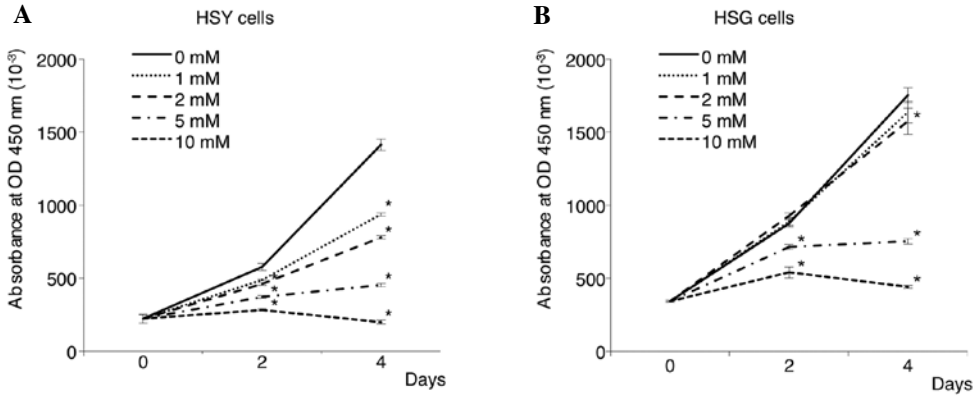

Figure 2. Effects of VPA on SGC cell proliferation. Salivary gland cancer (SGC) cells were seeded at a concentration of $2x10^3$ cells/well on 96-well plates. Twenty-four hours later, the cells were treated with various concentrations (0, 1, 2, 5 and 10 mM) of VPA for 2 and 4 days. *In vitro* cell proliferation was evaluated by the MTT assay. VPA inhibited the proliferation of SGC cells in a dose-dependent manner (A, HSY cells; B, HSG cells). Degenerated cancer cells were observed at a high concentration (10 mM) of VPA. *P<0.05, significant difference from the untreated control group.

concentration (10 mM) of VPA. In particular, the HSY cells were most sensitive to the inhibitory effects of VPA on cell proliferation.

Antitumour effect of VPA on salivary gland tumours in vivo. Based on the *in vitro* findings discussed above, to investigate the antitumour effect of VPA on salivary gland tumours *in vivo*, experiments with salivary gland tumour xenografts were performed. HSY cells were inoculated subcutaneously into the back of nude mice (n=5), and oral VPA (0.2 and 0.4% w/v) administration in drinking water was initiated at 1 week after the inoculation. Control mice drank water without VPA. As shown in Fig. 3, VPA treatment significantly suppressed tumour growth compared with the growth observed in the untreated controls. However, there was no significant difference between the 0.2 and 0.4% VPA groups. In addition, all of the VPA-treated mice without inoculation of cancer cells showed weight loss due to drug toxicity (data not shown).

Effect of VPA on the cell cycle distribution of salivary gland cancer cells. We next investigated the effect of VPA on the cell cycle distribution of SGC cells to explore the underlying mechanism of VPA-mediated cell growth inhibition. Cancer cells treated with 2 and 5 mM of VPA were subjected to cell cycle analysis by flow cytometry. As shown in Fig. 4, VPA treatment effectively altered the cell cycle distribution of the

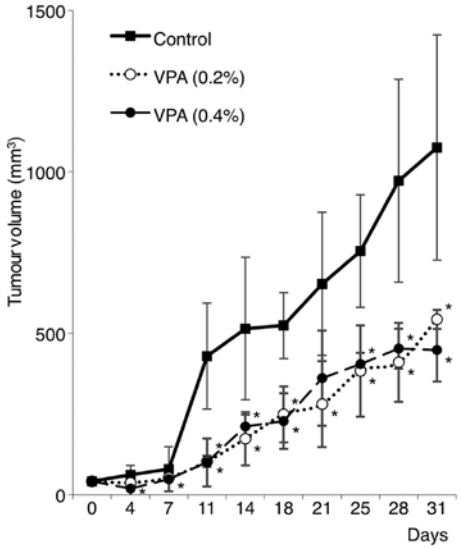

Figure 3. Antitumour effects of VPA on tumour xenografts. HSY cells ($5x10^6$ cells) were inoculated subcutaneously into the back of nude mice. Once palpable tumours were established at 1 week after the inoculation, VPA treatment was initiated by administering VPA (0, 0.2 and 0.4% w/v) in drinking water. Tumour volume and the body weight (data not shown) of mice were measured twice a week. The tumour volumes were calculated by the following formula: 0.5 x largest diameter x (smallest diameter)^2 in xenografted mice. For each treatment, 6 mice were used. VPA treatment significantly suppressed tumour growth relative to the growth observed for the untreated controls. However, there was no significant difference between the 0.2 and 0.4% VPA groups. *P<0.05, significant difference from the untreated control group.

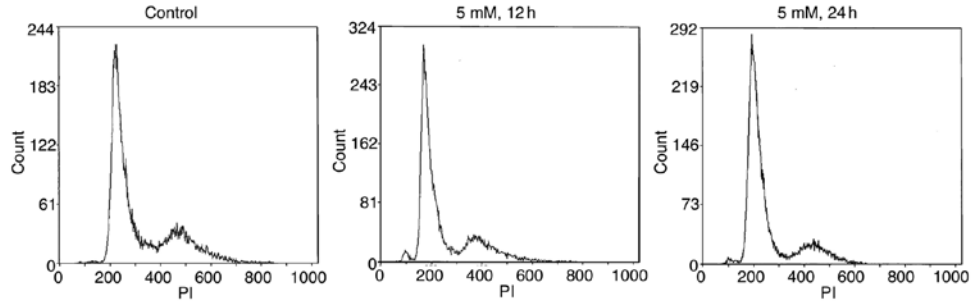

Figure 4. Flow cytometric analysis of treatment with VPA in salivary gland cancer cells (HSY cells). Cancer cells treated with 2 and 5 mM of VPA were subjected to cell cycle analysis by flow cytometry. VPA treatment effectively altered the cell cycle distribution of salivary gland cancer cells. The population of cells in the G1 phase was significantly increased in the VPA treatment group relative to the population observed in the untreated controls. Similar results were also observed for HSG cells (data not shown). *P<0.05, significant difference from the untreated control group.

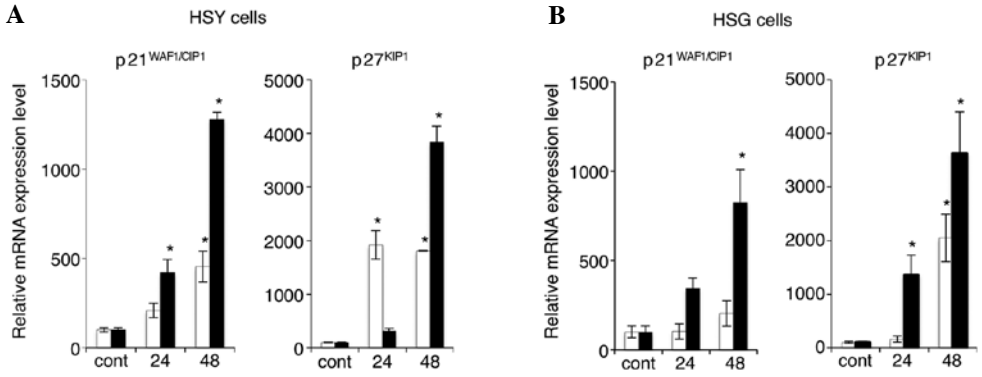

Figure 5. Effects of VPA on the mRNA expression of p21 and p27 in salivary gland cancer cells. Cancer cells grown under subconfluent conditions were treated with VPA (0, 2 and 5 mM) for 24 or 48 h. The mRNA expression levels of p21 and p27 were examined by real-time RT-PCR. GAPDH was used as an internal control. The data are expressed as the ratio of the GAPDH-normalised fold-change in the PCR product in control cells. Experiments were performed at least three times. VPA markedly upregulated the levels of p21 and p27 mRNA expression in both salivary gland cancer cell lines (A, HSY cells; B, HSG cells). The results are representative of the means of three independent data. *P<0.05, significant difference from the untreated control group.

Table I. Effect of VPA on cell cycle distribution of salivary gland cancer cells (HSY cells).

		VPA			
		2 mM		5 mM	
	Control (%)	12 h (%)	24 h (%)	12 h (%)	24 h (%)
G1	56.2±0.8	57.7±6.0	60.4±0.6[a]	67.1±1.6[a]	73.8±2.0[a]
G2	24.6±0.3	21.9±0.6	18.3±2.2[a]	21.2±0.1[a]	15.1±0.6[a]

[a]P<0.05, significant difference from the untreated control group.

HSY cells. Compared to the controls showing 56.2% cells in the G1 phase at 24 h, VPA treatment at doses of 2 and 5 mM resulted in 60.4 (P<0.05) and 73.8% (P<0.05) cells in the G1 phase of the cell cycle, respectively (Table I). The population of cells in the G1 phase was significantly increased in the VPA treatment group relative to the population in the untreated controls. Similar results were also observed for HSG cells (data not shown).

Mechanisms of the antitumour effect of VPA on salivary gland cancers. To further identify the mechanisms of the antitumour effect on SGC by VPA treatment, the expression levels of tumour-suppressor genes p21 and p27 were examined by real-time RT-PCR. Fig. 5 shows the mRNA expression levels of p21 and p27 in HSY and HSG cells after 24 or 48 h of treatment with 2 or 5 mM VPA. VPA markedly upregulated the levels of both p21 and p27 mRNA expression in both SGC cell lines. The expression of p21 and p27 mRNA was increased in a time-dependent manner. In particular, in HSY cells treated with 5 mM VPA, the mRNA expression levels of both p21 and p27 were increased appreciably.

Discussion

SGC frequently spreads to regional lymph nodes and distant organs and its prognosis is quite poor (1-6). Among the multiple options available for SGC treatment, surgical resection is currently the primary choice and standard option (5,6). In addition, SGC shows low sensitivity to chemotherapy and radiotherapy, and the effectiveness of systemic chemotherapy against SGC has not been established. Therefore, it is apparent that the development of more effective chemotherapeutic drugs and strategies against SGC is necessary. Recent advancements in the field of cancer epigenetics have shown that the development of cancer involves epigenetic abnormalities along with genetic alterations (9,10). Histone acetylation is one of

these epigenetic changes and a regulator of gene expression; moreover, it plays an important role in carcinogenesis. Histone acetylation is associated with transcriptional activation and silencing and is regulated by a balance between the opposing activities of HAT and HDAC. The inactivation of HAT and activation of HDAC have been observed in various types of cancers (10). HDAC inhibitors have been reported to exhibit antitumour effects against various human tumour cells and, therefore, have become promising candidates for cancer treatment. Several HDAC inhibitors, such as vorinostat and depsipeptide, have undergone or are undergoing both phase I and phase II clinical trials as anticancer agents against haematological malignancies and solid tumours (12,13). However, there is no reported study on the implementation of such inhibitors against salivary gland cancer.

VPA has recently been shown to exhibit activity as an HDAC inhibitor. VPA has also been reported to prevent the growth of many human cancers, including neuroblastoma, erythroleukaemia, acute myelogenous leukemia (11,12), osteosarcoma (25) and carcinomas of the breast (8), skin (16), prostate (26), lung (27) and colon (28), suggesting that it can be a useful agent for the treatment of a wide variety of malignancies. In the head and neck region, the effect of VPA against squamous cell carcinomas has been reported (29); however, its effect against SGC has never been reported. Thus, in the present study, we investigated the antitumour effects of VPA against SGCs. It was clearly demonstrated that VPA inhibited the proliferation of SGC cells *in vitro* and that VPA treatment by oral administration effectively inhibited the growth of salivary gland tumours in xenograft models *in vivo*. Furthermore, we observed that VPA induced cell cycle arrest in the G1 phase with the upregulation of p21 and p27 expression in SGC cells.

Many researchers have investigated the underlying mechanism of cancer cell growth inhibition induced by VPA acting as an HDAC inhibitor (7,8,19,25). Gottlicher *et al* (30) demonstrated that VPA relieves HDAC-dependent transcriptional repression and induces the hyperacetylation of histones in several types of cancer cells *in vitro*, as well as reduced tumour growth and metastasis formation in animal experiments. The authors indicated that VPA is a powerful HDAC inhibitor and might serve as an effective drug for cancer therapy. On the other hand, Chen *et al* (31) reported that VPA inhibited the proliferation and invasion of bladder cancer cells by increasing histone H3 acetylation. In contrast to findings reported for bladder cancer, VPA had no effect on invasion or migration for prostate cancer cells. The authors concluded that VPA exerts its effect in a cancer type-specific manner. In the present study, VPA inhibited the proliferation of SGC cells *in vitro* and the growth of tumours formed by SGC cell inoculation. Although this antitumour effect of VPA against SGC is not fully understood, VPA might be able to serve as an effective drug for the treatment of SGC, acting as an inhibitor of HDAC.

To explore the underlying mechanism of VPA-mediated cancer cell growth inhibition, we examined the effect of VPA on the cell cycle phases and distribution of SGC cells treated with various concentrations of VPA. Our flow cytometric analysis data indicated that VPA induced the G1 arrest of the cell cycle progression in SGC cells in a time- and dose-dependent manner. Numerous reports have indicated that HDAC inhibitors induce cell cycle arrest, differentiation and apoptosis in many cancer cells by decreasing the activity of HDAC and leading to the accumulation of histone hyperacetylation (7,11,14,15). VPA has also been reported to exert its antitumour effects by inducing cell cycle arrest, differentiation and apoptosis of cancer cells. Greenblat *et al* (32) demonstrated that VPA activates Notch-1 signalling in human carcinoid tumour cells *in vitro* and *in vivo*, which results in the inhibition of cell growth via the induction of cell cycle arrest. VPA is also known to affect several different signalling pathways, including those of extracellular-regulated kinase (ERK)-AP-1, protein kinase C (PKC), glycogen synthase kinase-3 β (GSK-3β) and Wnt. In addition, VPA has recently garnered attention as a specific inhibitor of HDAC activity (7,19). In the present study, we did not examine the molecular pathway of VPA against SGC; however, VPA may act as an effective drug for the treatment of SGC through these molecular pathways.

Furthermore, to explore the underlying mechanism of the VPA-mediated G1 arrest of cell cycle progression, we examined the expression levels of tumour-suppressor genes p21 and p27 in SGC cells treated with various concentrations of VPA by real-time RT-PCR. Our data indicated that VPA upregulated the expression of p21 and p27 in SGC cells in a time-and dose-dependent manner. The cell cycle progression in mammalian cells is controlled by protein complexes composed of cyclins and cyclin-dependent kinases (CDKs), although cell cycle regulation is complex (11,28). In prostate cancer cells, Sidana *et al* (34) demonstrated that VPA treatment caused cell cycle arrest, as determined by the increase in p21 and p27 and decrease in cyclin D1 expression. p21, a cyclin-dependent kinase inhibitor, plays an important role in cell cycle progression and has been shown to be consistently induced by numerous HDAC inhibitors (11,28). It is well known that p21 is a transcriptional target of the tumour-suppressor gene p53 and a downstream effector of p53-dependent cell growth arrest. Induction of p21 expression may lead to the marked G1 arrest of cancer cells. In addition, p21 can also bind the proliferation cell nuclear antigen (PCNA), leading to an inhibition of DNA replication (33). We previously reported that a differentiation-inducing drug, vesnarinone, induced histone hyperacetylation and p21 gene expression, resulting in cell growth arrest in a human SGC cell line (24). In the present study, VPA induced G1 cell cycle arrest and upregulated p21 expression in SGC cells, suggesting that VPA may induce histone hyperacetylation. On the other hand, p27 is a p21-related cyclin-dependent kinase inhibitor that regulates the progression of cells from the G1 to S phase in the cell cycle (28). Downregulation of p27 has been associated with cancer progression and unfavourable results with respect to several malignancies. It is well known that reduced expression of p27 is frequently observed in various cancers, and a lack of p27 is suggested to be due to an enhancement in its degradation (13). We previously reported that the upregulation of p27 protein may exert growth-inhibitory effects by inducing G1 arrest and apoptosis in oral cancer cell lines. p27 also modulates the cell cycle progression and apoptosis of cancer cells, but its contribution to HDAC inhibitor-induced processes remains to be fully elucidated.

In the animal experiments performed in the present study, we administered VPA at a concentration of 0.2 or 0.4% w/v in drinking water. VPA treatment markedly suppressed the

growth of salivary gland tumours when compared with the growth observed in the untreated controls, and there was no significant difference between the 0.2 and 0.4% VPA groups. Shabbeer *et al* (8) demonstrated that the administration of 0.4% w/v VPA in drinking water was effective in inducing growth arrest, cell death, and senescence *in vivo* against prostate cancer. Furthermore, the authors indicated that the mean plasma level of VPA in mice was 0.4 mM, which is approximately the level obtained in human patients on VPA. Although we did not measure the plasma level of VPA in mice, this VPA concentration (0.4% w/v) poses no significant risk for inducing toxic effects, including thrombocytopenia and somnolence.

In summary, the present study demonstrated the possibility of using a new and safe anticancer therapy against SGC, supporting the use of HDAC as a molecular target for the expression of tumour-suppressor genes, such as p21 and p27. We provide evidence that suggests that VPA could contribute to the development of more effective chemotherapy against SGC. Further studies are needed to characterise the antitumour activity of VP*A in vivo* in other tumour types of SGC for clinical trials.

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