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ORIGINAL ARTICLE

Valproic acid as a therapeutic agent for head and neck squamous cell carcinomas

Rafael B. Erlich · Danny Rickwood · William B. Coman · Nicholas A. Saunders · Alexander Guminski

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Abstract
Purposes Here we investigate if valproic acid (VA) can enhance the efficacy of commonly used therapies for head and neck squamous cell carcinomas (HNSCC) and the molecular mechanisms that may be related to its anticancer effects.
Methods Proliferation and viability of distinct cell types subjected to VA treatment alone or in combination regimens were measured through BrdU incorporation and LDH release, respectively. Molecular markers compatible with histone deacetylase inhibitory activity of VA were assessed through western blots assays in lysates obtained from cultured cells and tumour biopsies.
Results Treatment of all cell types with VA resulted in a dose-dependent increase in histone H3 acetylation and p21 expression, as well as dose-dependent cytostasis. In contrast, the cytotoxic response to VA was variable and did not correlate with cytostasis, histone acetylation or p21 induction. The variability in response to VA was also observed in tumour biopsy samples collected from patients prior to and following a 1 week oral course of VA. In addition, we found that a combination of a clinically achievable concentration of VA plus cisplatin caused a threefold to sevenfold increase in cisplatin cytotoxicity in vitro.
Conclusions VA acts as a histone deacetylase inhibitor (HDI) in SCC cells and normal human keratinocytes (HKs), potentiates the cytotoxic effect of cisplatin in SCC cell lines and decreases the viability of SCC cells as opposed to HKs. Taken together, the results provide initial evidence that VA might be a valuable drug in the development of better therapeutic regimens for HNSCC.

Keywords Valproic acid · HNSCC · Histone-deacetylase inhibitor · Combination therapy

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the developed world with an annual incidence of more than 500,000 cases worldwide, representing 3.2% of all newly diagnosed cancers in the United States alone [21, 30]. Tobacco use and alcohol intake are well established as the major risk factors for development of HNSCC but the molecular mechanisms by which these carcinogens induce transformation and malignant progression are not fully understood [10, 13]. Irrespective of the etiologic agent, HNSCC are associated with multiple genetic defects, which in turn lead to deregulation of basic biological processes such as proliferation, differentiation and apoptosis [15, 40]. Although important advances in the surgical and radiological treatment of HNSCC have occurred in the last decades, HNSCCs are still associated with severe disease- and treatment-related morbidity and have a 5-year survival rate of approximately

R. B. Erlich · D. Rickwood · N. A. Saunders (✉) · A. Guminski
Epithelial Pathobiology Group,
Cancer Biology Programme, Diamantina Institute for Cancer, Immunology and Metabolic Medicine, University of Queensland,
Princess Alexandra Hospital, 4th floor, Building 1, R wing,
Ipswich Rd, Woolloongabba, Brisbane, QLD 4102, Australia
e-mail: nsaunders@uq.edu.au

W. B. Coman
ENT Department, Princess Alexandra Hospital,
Brisbane, QLD, Australia

A. Guminski
Oncology Department, Princess Alexandra Hospital,
Brisbane, QLD, Australia

50% [10, 15, 21]. Further improvements in curative rates in HNSCC will require significant advances in the development of chemotherapeuthic drugs and strategies.

Histone deacetylase inhibitors (HDIs) are an emerging class of drugs that have shown promise as anticancer agents when used alone or in combination with conventional therapies [4]. HDIs regulate the acetylation status of both histone and non-histone proteins [16, 45]. Whilst the precise mechanism of action of HDIs is unknown, there is a growing body of evidence that the HDI effects on both histone and non-histone proteins are important for their anti-proliferative and pro-apoptotic activities [5, 18, 29, 31, 32, 47]. Several subsequent studies have related the anticancer effects of VA and other HDIs to their ability to increase acetylation in amino-terminal residues of histones, which leads to changes in chromatin structure resulting in the reduction of oncogene expression [4, 25, 37]. Earlier studies from our laboratory showed that HDIs are effective against squamous cell carcinomas (SCCs) in vitro and may have potential as anticancer agents for the treatment of HNSCC [5, 39]. Valproic acid (VA), a short-chain fatty acid first synthesized in 1882 and discovered serendipitously as an anticonvulsant in 1962, has been widely employed for the treatment of some forms of epilepsy, bipolar disorders and migraine for more than 30 years [2]. More recently, VA was shown to (1) induce differentiation, (2) inhibit the proliferation of cancer cells and (3) inhibit tumour angiogenesis at doses associated with histone-deacetylase activity. [12, 19, 26, 34, 46].

Valproic acid has a serum half-life of 9–18 h, can be administered orally and in contrast to most HDIs, is able to penetrate the blood-brain barrier as demonstrated by its long clinical history as an anti-epileptic [27, 33, 42]. While tumours of hematopoietic origin and selected solid tumours may undergo differentiation following VA exposure, for most solid tumours the primary effect of VA is a reversible cytostatic response [22]. Given that VA elicits minimal cytotoxic effects upon normal cells, its use in combination with more established anti-neoplastic agents may prove to be its most useful application. For example, preclinical studies have demonstrated that VA enhances tumour cell radiosensitivity in vitro and in vivo [8] and is able to suppress the cutaneous radiation syndrome, leading to an enhanced therapeutic ratio in cancer radiotherapy [11]. For this reason, several clinical trials and preclinical studies are currently investigating the effectiveness of VA as an anticancer agent as a monotherapy [1] or in combination therapeutic regimens (see http://www.cancer.gov).

In the present study we investigate the effects of VA on normal keratinocytes as well as a panel of keratinocyte-derived human HNSCC cell lines. In addition we also examined whether VA given orally to HNSCC patients is able to inhibit HDAC activity at clinically achievable doses. Finally, we undertake a series of in vitro studies to determine whether VA can potentiate the effects of cisplatin, radiation or taxol in vitro.

Materials and methods

Chemicals

Valproic acid was purchased from Sigma (US) and prepared as a stock solution using phosphate buffer saline. Cisplatin was obtained from DBL (Aus), HRP-conjugated anti-mouse and anti-rabbit antibodies were purchased from Chemicon (Aus). Anti-p21 monoclonal antibody (EA-10) was obtained from Calbiochem (US). Anti-acetyl histone H3 polyclonal antibody (06-599) was obtained from Upstate (US). Anti-actin polyclonal antibody (A-2066) was obtained from Sigma (US). Anti-tubulin polyclonal antibody (2,148) was obtained from Cell Signaling (US).

Maintenance of cells

Normal human keratinocytes (HKs) were isolated and cultured from neonatal foreskins following circumcision as previously described [23]. The SCC15, SCC25 and Cal27 tumour cell lines were grown and maintained in Dulbecco's modified Eagle medium-F12, supplemented with penicillin/streptomycin/glutamine solution (10 ml/l, Gibco), hydrocortisone (0.4 μg/ml), gentamycin (10μg/ml) and fetal bovine serum 10% (v/v) (Gibco). Culture flasks were maintained at 37°C in 5% CO_2 and 95% air. Exponentially growing cells were detached from the culture flasks with 0.25% trypsin/ethylene-diamminetetraacetic acid (EDTA) and seeded at different densities depending on the assay.

Proliferation and cytotoxicity assays

Human keratinocytes and tumour cells were seeded at 10^4 cells per well and maintained in their respective culture media in 96-well plates overnight. Cells were exposed to different VA concentrations for 24 h. For proliferation assays a BrDu (6 h) pulse was added (10 μM final concentration) after 18 h of VA treatment. BrDU incorporation measurements were performed with the Cell Proliferation assay kit (Roche, US, #11647229001) according to the manufacturer's protocol. Measurement of lactate dehydrogenase (LDH) release after VA treatments for 24 h was performed with the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, US, #G1780) according to the manufacturer's protocol. For reversibility assays HKs and Cal27 cells were seeded, respectively, at 4×10^4 and 2×10^4 cells per well and maintained in their respective culture media in six-well plates overnight. The next day (day 0)

cells were subjected to distinct treatments as described in the figure legend. At specified time points cells were trypsinised and counted in a hemocytometer. Culture media was replaced after 48 h. When needed, VA was replaced after 48 h.

Western blotting

After the treatments described in the text, cells were washed twice with cold phosphate-buffered saline (PBS) and extracts were prepared by direct cell lysis in 1% NP40, 1% Tryton X-100, 1% Sodium Deoxycholate, 10 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.1% SDS and 5 mM EDTA in PBS, supplemented with Complete protease and phosphatase inhibitory cocktails (Roche, USA). For tumor lysates, freshly harvested tissue samples were homogenized in the lysis buffer described above. Samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Blots were exposed to primary antibodies and secondary antibodies following manufacturer instructions. The reaction was developed using enhanced chemiluminescence (Pierce, USA).

Radiation treatments

Cal27 and SCC25 cells were seeded into 96-well plates at 2.5×10^3 cells per well. 6 hours later cells were pre-treated with 1 mM VA or with vehicle for 24 h, after which they were irradiated using a MDS Nordion Gammacell 40 Exactor Irradiator (Caesium 137). Ninety-six hours irradiation, cell viability was measured using CellTiter-Blue™ (Promega) assay according to the manufacturers instructions.

Patient eligibility and biopsy procedure

Patients with advanced or recurrent HNSCC with tumour accessible for repeat biopsies were approached to participate in this study. All patients gave informed consent and the study was approved by the local ethics committee. Biopsies were taken from exposed oropharyngeal tumour by cutting forceps or from subcutaneous deposits by 3 mm punch biopsy. Tumour biopsies were performed before and after an oral course of VA. A staff pathologist used a tissue sample for diagnostic confirmation and the rest of the tissue was processed as described above.

Densitometries

Densitometric analyses of the immunoblots were performed using the ImageQuant software. Values are the ratio between acetyl-histone H3 bands and their respective loading controls.

Statistical analysis

The number of experimental replicates is given in figure legends. Data were analysed by ANOVA followed by post-hoc comparisons (Tukey's test) when multiple groups are compared. Dose-response data for combination treatments assays were analysed using Prism 4 software, which uses nonlinear regression to fit dose-response curves and then performs an *F* test on the log EC50 values.

Results

Valproic acid is cytotoxic in HNSCC cell lines but not in normal human keratinocytes

We examined the time and dose-dependent effects of VA upon proliferation of cancer cell lines and HKs. Proliferation was determined by quantitative measurement of BrDU (10 μM final concentration, 6 h pulse) incorporation and the results were normalized with respect to the rate of proliferation in untreated cells (Fig. 1a). After a 24 h treatment period, increasing concentrations of VA (1–10 mM) were able to inhibit proliferation of cancer cells and HKs in a dose-dependent manner. The HKs were more sensitive to VA-induced growth arrest than all tested cancer cell lines. Previous studies have shown that HDIs often exhibit tumour cell-specific cytotoxicity [4, 6]. Therefore, we tested for the cytotoxic effects of VA in cancer cell lines and HKs. Cytotoxicity was determined by quantitative measurement of LDH release to the culture media, and the results were normalized with respect to the rate of LDH release in untreated cells. In contrast to the effects upon proliferation, VA induced cell death in a dose- and cell type-dependent manner in most cancer cell lines but not in HKs or SCC15 cells (Fig. 1b). To investigate if the cytostatic effect of VA is reversible, we compared the proliferation of distinct cell types during a 4-day period in which cells were left untreated, treated with VA for the first 24 h only or treated for the whole duration of the experiment. Both HKs and cancer cells were able to recover from VA-induced cytostasis after removal of the drug (Fig. 1c, d), indicating that cells can recover from VA-induced proliferation arrest. These data indicate that VA-induced cytotoxicity is specific for a subset of HNSCC cell lines and is likely to require prolonged or continuous exposure to VA.

Valproic acid modulates histone acetylation and p21 expression in HNSCC cell lines and HKs

Hyperacetylation of histones is a widely accepted marker for histone deacetylase (HDAC) inhibition, as is the expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$

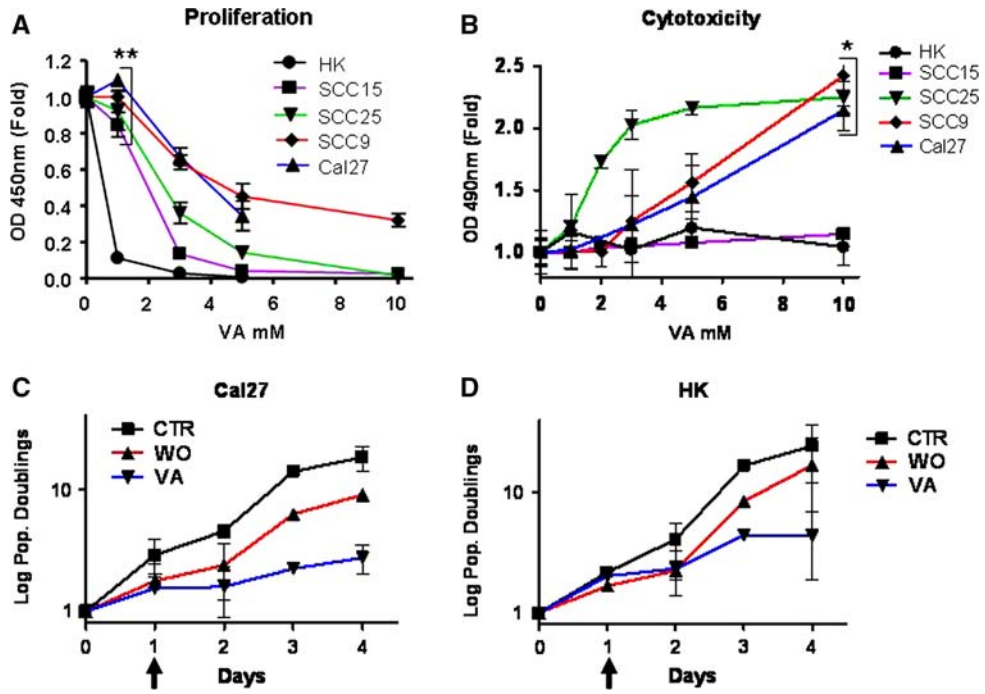

Fig. 1 Effects of valproic acid upon proliferation and viability of SCC cell lines and HKs. SCC cell lines (SCC9, SCC15, SCC25, Cal27) and HKs were subjected to VA treatment at distinct concentrations (1–10 mM) for 24 h. After this period, proliferation (**a**) and cytotoxicity (**b**) were determined as described in the text, *indicates $P < 0.05$ versus HK and **indicates $P < 0.001$ versus HK at the respective VA concentrations. Cal27 (**c**) cells and HKs (**d**) were cultured for four days in the absence of VA (*CTR*), in the presence of VA (6 mM for Cal27, 0.8 mM for HK) only for the first 24 h (*WO*) or in the presence of VA at the above mentioned concentrations during the whole experiment (*VA*). Cells were counted using a haemocytometer. *Arrows* indicate wash-out of VA from the WO group. Values are mean ± standard error of two independent experiments performed in triplicate

(p21). Western blots of lysates from HNSCCs and HKs showed that VA treatment induces histone H3 acetylation in a dose-dependent manner in all cell types, confirming its activity as an HDI. Interestingly, although p21 expression was also induced in all cell types, expression was higher in the cell lines sensitive to the cytotoxic effects of VA (SCC25 and Cal27) as opposed to the resistant ones (SCC15 and HKs) (Fig. 2). These data confirm that VA is an HDI in HNSCC cells and HKs. These data also show that histone H3 hyperacetylation may correlate with cytostasis but does not correlate with cytotoxicity.

Histone acetylation within tumours following VA treatment

The neurologic effects of VA exclude its use at maximal HDI concentrations in patients. This may limit the anticancer potential of VA in a clinical setting. For this reason, we tested the histone-deacetylase inhibitory activity of VA in HNSCC patients at clinically achievable concentrations of VA. Tumour lysates were obtained from biopsies performed on patients presenting with HNSCCs at distinct stages and with distinct clinical features (Table 1). Tissue samples were collected before and after patients undertook an oral course of VA for one week (Table 2) and histone H3 acetylation levels were measured in biopsies that yielded adequate samples for western blot assays (three out of six patients; Fig. 3). Although serum VA concentration of all patients clearly increased following the one week oral course, an increase in histone acetylation levels in the tumours was only observed in one out of three patients, suggesting that there is no direct relationship between serum VA levels and histone hypercetylation of tumours in vivo. These data suggest that the ability to achieve clinically relevant histone deacetylase inhibition in HNSCC tumours is variable and complex.

Valproic acid enhances the anticancer activity of cisplatin in HNSCC cell lines

The patient data indicated that achieving cytotoxic doses of VA in HNSCC patients may be difficult. However, recent in vitro and in vivo data suggests that combinations of HDIs plus traditional cytotoxic therapies may improve the cytotoxic profile above that of the agents delivered as monotherapies [4]. Specifically, we examined the cytotoxic effects of a low concentration of VA (1 mM), when combined with varying doses of cisplatin, taxol or radiation (Fig. 1b). Dose-response curves of cytotoxicity assays

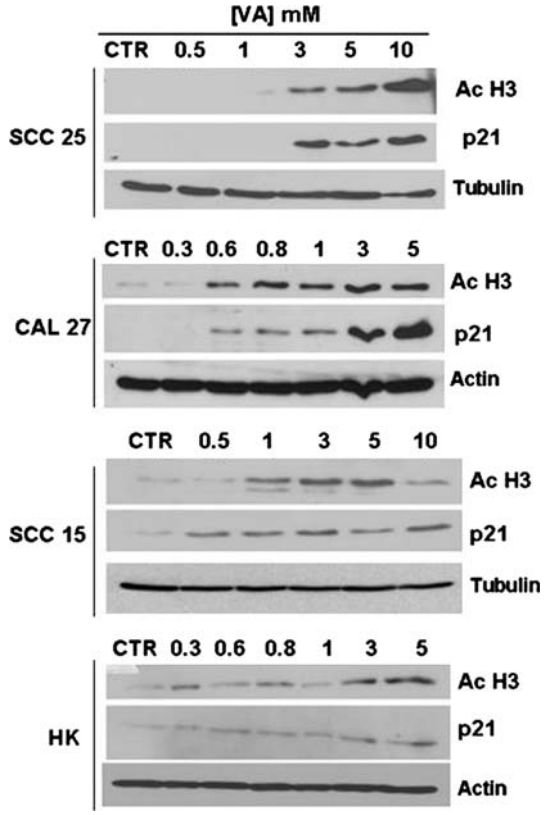

Fig. 2 Valproic acid induces histone acetylation and modulates p21 expression. SCC cell lines and HKs were subjected to VA treatment at distinct concentrations for 24 h and collected for immunoblot analysis of acetyl histone H3 and p21. Each blot is representative of two independent experiments with actin or tubulin used as loading controls. CTR, cells exposed to vehicle only (PBS)

showed that VA caused a marked decrease in the EC50 for cisplatin in both cell lines (SCC25; from 131.6 to 18.7 nM and Cal27; from 28.6 to 10 nM, $P < 0.0001$) (Fig. 4a). On the other hand, the EC50 for paclitaxel and radiation in cells co-treated with VA was not different from cells treated with paclitaxel or radiation alone (Fig. 4b, c). These data indicate that the clinical use of VA in combination regimens might help to minimize the undesirable side effects of cisplatin, a commonly used therapeutic agent, and may also improve clinical outcomes.

Discussion

The present study showed that (1) VA inhibits proliferation of HKs and HNSCC cells, (2) HNSCC cells and HKs vary in their response to VA-induced cytotoxicity, (3) VA-induced histone acetylation and p21 expression in HK and HNSCC cell lines do not correlate with cytotoxicity, (4) VA sensitizes HNSCC cells to cisplatin-induced cytotoxicity and (5) it may be feasible to induce histone acetylation

Table 1 Clinical features of patients

No	Sex	Age	Disease state	Prior treatment	Treatment after VA lead-in	Best response	Outcome
1	M	54	Stomal recurrence of laryngeal SCC within 6 months of completing initial treatment	Cisplatin chemo-radiation	Cisplatin with 5-fluorouracil	Partial response	Progressive disease at 6 months. Lost to follow-up
2	M	51	Recurrent laryngeal SCC with pulmonary metastases	Surgery, chemo-radiation	Methotrexate chemotherapy	Stable disease	Progressive disease after 8 weeks, died from disease
3	M	47	Extensive oro-pharyngeal SCC. Considered unsuitable for curative intent treatment	Nil	Cisplatin with 5-fluorouracil	Partial response, then proceeded to definitive chemo-radiation	Alive at 12 months, no evidence of disease
4	M	57	Inoperable oropharyngeal SCC, prior tongue SCC	Glossectomy and post-operative radiotherapy	Cisplatin with 5-fluorouracil	Complete radiological response	Alive at 6 months, no evidence of disease
5	M	67	Oropharyngeal SCC	Nil	Cisplatin chemo-radiation	Complete response	Alive at 6 months, no evidence of disease
6	M	44	Loco-regional recurrence and pulmonary metastases from tonsil SCC	Cisplatin chemo-radiation	Methotrexate chemotherapy	Stable disease	Progressive disease after 8 weeks, died from disease

Oral valproate was started 1–2 weeks prior to definitive treatment and continued during conventional treatment

Table 2 Record of serum VA levels (mM, therapeutic range 0.3–0.6 mM)

Patient	Day 7			Day 14		
	VA dose (mg)	Albumen (g/l)	VA (mM)	VA dose (mg)	Albumen (g/l)	VA (mM)
1[b]	600 bid	42	0.8[a]	–		
2	–			600 bid	42	0.54[a]
3[b]	600 bid	32	0.55[a]	–		
4[b]	600 bid	38	0.92	400 bid	32	0.51[a,c]
5	600 bid	41	0.45	600 bid	43	0.46[a]
6	600 bid	40	0.36	240 bid	44	0.2[a]

[a] Indicates the post-VA biopsy date

[b] Indicates patients with informative pre- and post- biopsies

[c] Level taken day after biopsy, VA dose was omitted by the patient 24 h prior to biopsy

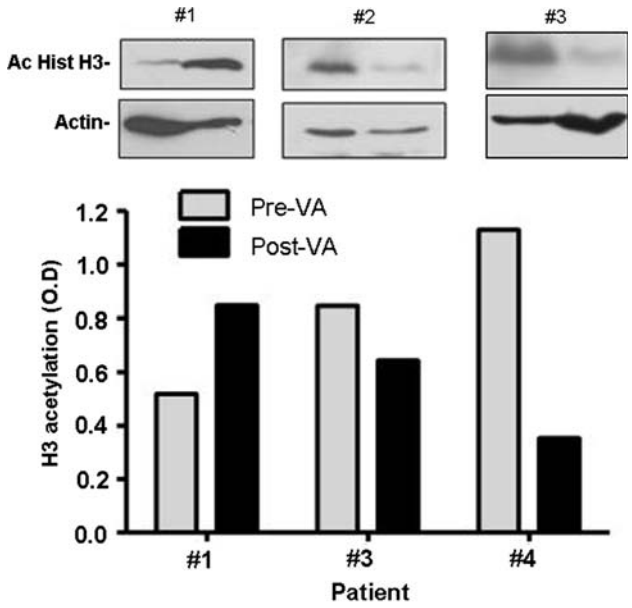

Fig. 3 Histone acetylation in tumours following VA treatment. **a** Western blots of samples from tumour biopsies obtained from patients before and after a 1-week oral course of VA with antibodies against acetylated histone H3. As loading controls, the membranes were re-probed with antibodies against actin. **b** Densitometry of acetyl-histone H3 western blots. Values are normalized to the respective loading controls

in a subset of HNSCC patients following an oral course of VA.

VA together with sodium butyrate and derivatives are short-chain fatty acids and are generally considered to be weak HDAC inhibitors. Despite their weak activities several of these agents have shown clinical efficacy against a variety of tumours [17]. Indeed, VA is able to inhibit class I HDACs (HDAC 1–3) and class IIa HDACs (HDAC 4, 5, 7 and 9) [19] and studies with teratocarcinoma and neuroblastoma cells have shown that VA effects upon proliferation and differentiation are linked to its HDAC inhibitory activity on HDACs [12, 19, 20, 34]. Whilst it is accepted that the therapeutic potential of VA and other HDIs is based on their ability to selectively target cancer cells, the molecular pathways that render normal cells resistant to the cytotoxic effects of HDIs remain to be fully elucidated [4, 7, 35]. Currently, VA is being tested in several clinical trials either as monotherapy or in combination regimens and it has been shown to be well tolerated and to be effective as an adjuvant therapy in pediatric patients with malignant gliomas [3]. Also, a clinical phase I study of VA as a monotherapy in patients with a range of solid tumours resistant to chemotherapy showed that the drug was well tolerated and that HDAC activity was reduced in most patients [1].

Whilst the mechanisms involved in VA-induced cytostasis and cytotoxicity were not specifically addressed in the present study, we are able to draw several conclusions. For example, VA-induced cytostasis is reversible and is associated with increased H3 acetylation and p21 expression. In contrast, VA-induced cytotoxicity did not correlate with p21 expression or histone H3 hyperacetylation. Significantly, the cytotoxic effects of VA alone varied between different HNSCC cell lines. The reason for the difference in cytotoxic responses in the HNSCC cell lines remains unclear. Previously, it was reported that the induction of p21 could protect cells from early cytotoxic effects of HDIs [44]. This, however, is not the case with the HNSCC cell lines, since the induction of p21 expression was clearly more pronounced in HNSCC cells sensitive to the cytotoxic effects of VA as opposed to HK and SCC15 cells. This suggests that the induction of p21 is unlikely to protect HNSCC cells from HDI-induced cytotoxicity.

Cisplatin is an effective therapeutic agent against head and neck cancer both alone or in combination regimens [10, 14, 28, 43]. Considerable evidence shows that cisplatin induces cell cycle arrest and apoptosis in cancer cells but unfortunately, as with other chemotherapeutic drugs, its use is limited by severe side effects. Another limitation for the chronic use of cisplatin is the appearance of resistant cells [24, 41]. For this reason, the development of molecules with the ability to sensitize cancer cells and prevent the development of drug-resistance is critical. Recently, Sato et al. [38] and Rikiishi et al. [36] showed that oral SCC cells can be sensitized to cisplatin by several HDIs. Our data show that treatment of two distinct HNSCC cell lines with a combination of VA and cisplatin caused a threefold to sevenfold decrease in the EC50 of cisplatin for SCC25 and Cal27, respectively. More importantly, in these experiments cells were treated with a clinically achievable concentration of VA. In this context, by allowing the use of smaller doses of cisplatin, consequently minimizing its side

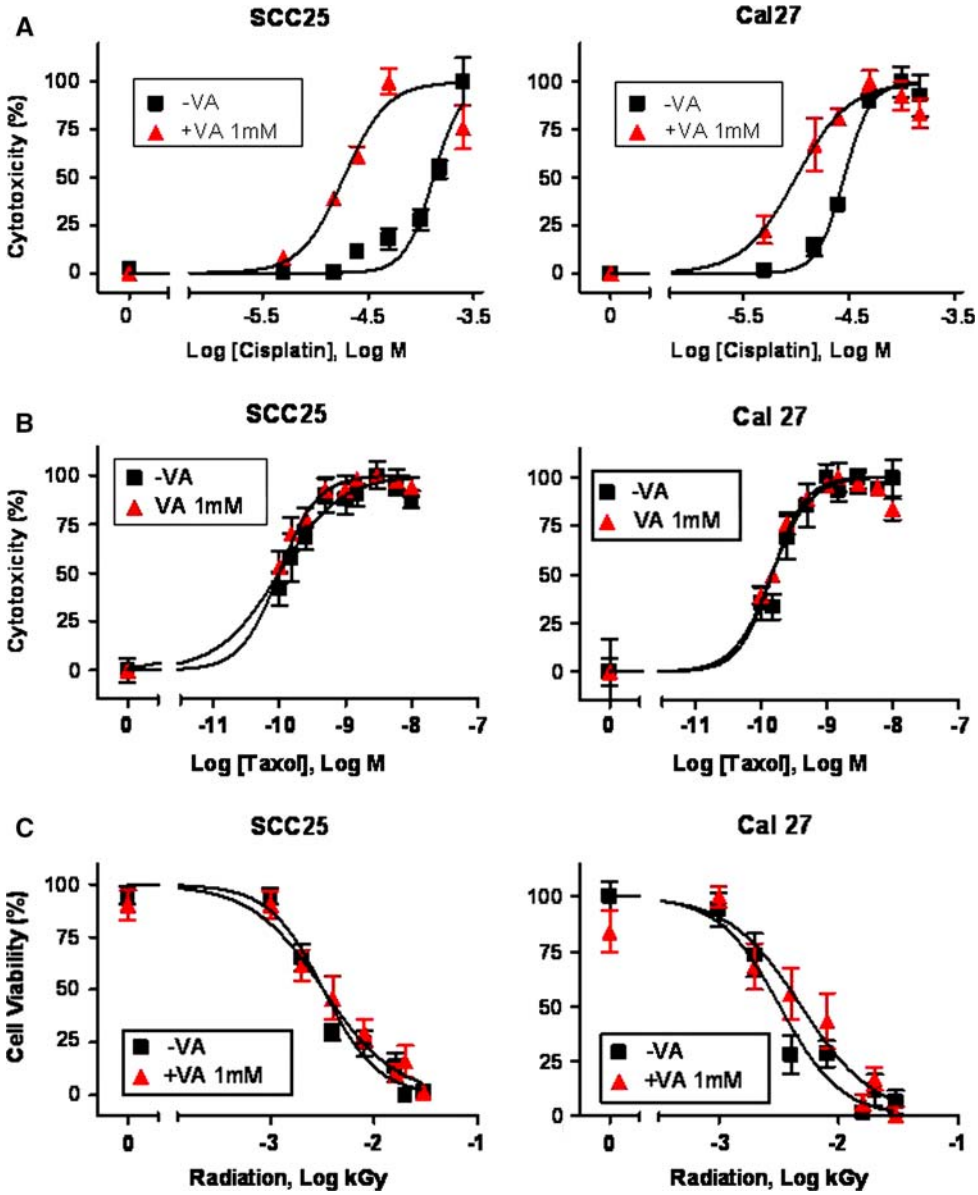

Fig. 4 Valproic acid augments cisplatin-induced cell death. **a**, **b** SCC25 and Cal27 cell lines were treated with increasing concentrations of cisplatin or paclitaxel alone (−VA) or in combination with VA 1 mM (+VA) for 24 h. Cytotoxic effects were determined as described in the text. Values are mean ± standard error of two independent experiments performed in triplicate. $P < 0.0001$ when comparing the EC50 values for cisplatin treatment alone to cisplatin in combination with VA 1 mM for both cell lines. **c** SCC25 and Cal27 cell lines were left untreated (−VA) or treated for 24 h with VA1 mM (VA), then cells were subjected to distinct radiation doses in the presence of VA. Cell viability was determined 48 h after beginning of VA treatment. Values are mean ± standard error of two independent experiments performed in triplicate

effects, VA may prove to be a valuable drug in the development of better therapeutic regimens for HNSCC.

The outcome for patients with advanced HNSCC remains sub-optimal and the integration of rational biological therapies such as HDIs require further investigation and validation. Although the number of patients was too small to determine the true relationship between exposure to VA and biological effect in HNSCC, we observed an increase in tumour tissue histone acetylation in one of the patients. Larger clinical studies will be required to determine the true probability and consistency of a molecular response to VA in the clinical setting. The observed in vitro synergy between cisplatin and VA suggest that this combination should be tested clinically with the goal of either increasing treatment outcomes or reducing toxicity by being able to reduce the dose for cisplatin for similar anti-cancer effect. Although VA did not show synergy with radiation in our study other HDIs have been reported to do so [9] suggesting that the molecular basis for VA-induced action may differ to those reported for other HDIs.

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