Valproic acid suppresses growth and enhances cisplatin cytotoxicity to larynx cancer cells

A Grabarska1,*, M Dmoszyńska-Graniczka1, W Jeleniewicz1, M Kiebus1, E Nowosadzka1, A Rivero-Muller2, K Polberg3, A Stepulak1,3

Abstract

Introduction
Larynx cancer is the most common head and neck neoplasia in developed nations. The treatment of advanced larynx cancer include chemotherapy, often using cisplatin. The limitations of many antineoplastic drugs are their low delivery into respiratory tissues, thus the need of higher doses which results in severe side effects, and the development of drug resistance by cancer cells. Therefore the need of drugs and drug combinations that potentiates each other, increasing safety by reducing doses and act on several pathways at the same time, acting more specifically on cancer cells. Histone deacetylase inhibitors are a promising new class of anticancer agents. Among them valproic acid is a well-established drug in psychiatry with a potential use in cancer treatment. Here we study the mechanisms of action of valproic acid on larynx cancer cells.

Materials and methods
Viability, proliferation and migration of laryngeal cancer cell lines (RK33 and RK45) were studied by methylthiazolylidiphenyI-tetrazolium bromide (MTT) method, 5-bromo-2-deoxyuridine (BrdU) incorporation assay and the wound assay, respectively. Acetylation of histone H3 was detected by Western blotting.

Flow cytometry was used to determine cell cycle progression. Apoptosis was assessed by means of nucleosomes released to cytosol and the genes expression by quantitative polymerase chain reaction (qPCR) method.

Results
Valproic acid treatment resulted in reduced proliferation and migration, increase in apoptosis, and cell cycle arrest in larynx cancer cells tested in our study. Valproic acid treatment induced the hyperacetylation of histone 3 K18 (H3 K18ac), resulting in de novo expression of CDKN1A and simultaneously repressing of CCND1, thus affecting cell cycle progression. Moreover, valproic acid significantly potentiates the antineoplastic activity of cisplatin on larynx cancer cells, at low doses by increasing apoptosis, cell cycle arrest and reduced migration.

Conclusion
Overall, our results suggest that valproic acid can potentially be used as a therapeutic agent against larynx tumours, but it can also be used in combination with current treatments of choice to enhance their efficiency on cancer cells while reducing side effects.

Introduction
Larynx cancer is the most common head and neck squamous cell carcinoma in developed countries. Men are more likely to get that cancer than women1. The choice of laryngeal cancer treatment depends on the stage of tumour development, localization, histopathological type and may involve surgery, radiotherapy, chemoradiotherapy or chemotherapy2. The most commonly used chemotherapeutic agents for laryngeal cancer are cisplatin (cis-diammine-dichloroplatinum, CDDP) and 5-fluorouracil (5-FU)3. 5-FU inhibits pyrimidine nucleotides synthesis required for DNA replication4 while CDDP is an alkylating agent that targets DNA and forms intrastrand crosslink DNA adducts which in turn lead to inhibition of replication and activation of apoptosis5. Despite their clinical use as anticancer agents, therapeutic potential of CDDP and 5-FU in larynx cancer is limited, partially due to inefficient delivery into the tumour tissue, requiring high doses which also results in severe side effects and de novo and acquired resistance of cancer cells to CDDP6. Therefore, development of novel anticancer molecules that more specifically inhibit the growth of larynx cancer cells, enhance the efficacy of currently used cytosstatics and in consequence improve patients survival is critical.

Histone deacetylase inhibitors (HDIs) are a promising new class of potential anticancer agents of natural and synthetic origin. Depending on structure, HDIs are divided into four main chemical classes, including hydroxamic acids (trichostatin A, oxamflatin, suberoylanilide hydroxamic acid - SAHA), cyclic peptides (depsipeptide, apicidin), short chain aliphatic acids (sodium butyrate, sodium phenylbutyrate, valproic acid) and benzamides (entinostat)7. HDIs function as competitive inhibitors by binding reversibly to catalytic site of histone deacetylases (HDACs) and thereby inhibit their activity8. This results in an increase of histone acetylation and consequently in the induction/repression of aberrantly suppressed/activated genes9. In contrast to many cytosstatics, HDIs display cytotoxic effects against both proliferating and resting cancer cells and have relatively low toxicity towards normal cells10. The antineoplastic mechanisms of action of HDIs are complex and not fully

Licensee OAPL (UK) 2014. Creative Commons Attribution License (CC-BY)

elucidated. To date, it has been known that HDIs alter gene expression patterns, induce cell cycle arrest, apoptosis or differentiation of tumour cells. Moreover, it has been reported that HDIs inhibit angiogenesis and enhance the sensitivity of cancer cells to radiotherapy and chemotherapy. Combined treatment with anticancer agents, including gemcitabine, paclitaxel, CDDP, doxorubicin and etoposide, geldanamycin and bortezomide has been shown to have promising therapeutic effects.

Currently more than 20 HDIs are under preclinical and clinical trials. Two of them - SAHA (Vorinostat, Zolinza®) and Depsipeptide (Romidepsin, Istodax®) have been approved by the U.S. FDA for the treatment of cutaneous T-cell lymphoma and peripheral T-cell lymphoma.

Histone deacetylase inhibitors have shown activity against multiple types of cancer cells, both haematological malignancies and solid tumours, however, their effect on larynx cancer cells is limited. The aim of this work was to determine the anti-cancer activity of valproic acid (VPA) in two different laryngeal cancer cell lines, RK33 and RK45 alone and in combination with CDDP.

Materials and methods
The protocol of this study has been approved by the relevant ethical committee related to our institution in which it was performed.

Reagents
Valproic acid and cisplatin (CDDP) were purchased from Sigma Chemicals. Stock solution of VPA (100 mM) and CDDP (1 mg/ml) were prepared in phosphate buffered saline (PBS).

Cell lines
Human larynx cancer cells (RK33 and RK45) were derived from patients with diagnosed larynx squamous cell carcinomas. Cancer tissue was removed from the larynx after total laryngectomy and established as stable cell lines, as previously described. Cell lines were maintained in an RPMI 1640 culture medium supplemented with 10% Foetal Bovine Serum (FBS) (Sigma), penicillin (100 µg/ml) (Sigma) and streptomycin (100 µg/ml) (Sigma).

![Figure 1](https://example.com/fig1.png)

**Figure 1:** Intracellular HDACs activity in RK33 and RK45 cells. The activity of HDACs was quantified by the Colorimetric HDAC Assay in cancer cells extracts after treatment with either culture medium alone (control), or VPA (1-10 mM) for 6 hours. Data are presented as mean ± SEM at each concentration. Student’s t-test revealed significant effect (**p<0.001**) of treatment with VPA compared to untreated culture. This effect was also concentration-dependent, as judged by analysis of variance ANOVA test (**p<0.001**), n=3 per concentration from three independent experiments.

![Figure 2](https://example.com/fig2.png)

**Figure 2:** Western blotting analysis of the histone H3 acetylation level at K9/14, K18 and K23 following 6 hours incubation of larynx cancer cells with either culture medium alone (control), or VPA (1-10 mM) (A). Total histone H3 was used as a gel loading control. Representative results for densitometric measurement of histone H3 acetylation level at K9/14, K18 and K23 (B).
Cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% CO2.

**Cell viability assessment**
Cancer cells were placed on 96-well plate (Nunc) at a density of 1 x 10^4 cells/ml (RK33) and 3 x 10^4 cells/ml (RK45). The next day, the culture medium was removed and cells were exposed to serial dilutions of VPA and/or CDDP in a fresh culture medium. Cell viability/proliferation was assessed after 72 hours by means of methylthiazolyldiphenyltetrazolium bromide (MTT) method in which the yellow tetrazolium salt (MTT) is metabolized by viable cells to purple formazan crystals. Tumour cells were incubated for 3 h with MTT solution (5 mg/ml, Sigma). Formazan crystals were solubilized overnight in sodium dodecyl sulphate (SDS) buffer (10% SDS in 0.01 N HCl) and the product was determined spectrophotometrically by measuring absorbance at 570 nm wavelength using an Infinite M200 Pro microplate reader (Tecan).

**Cell proliferation assay**
Cell Proliferation Elisa, BrdU Kit (Roche Diagnostics) was used following manufacturer’s instructions. Optimized amounts of RK33 (2 x 10^5/ml) or RK45 (5 x 10^5/ml) cells were placed on a 96-well plate (Nunc) (100 µl/well). On the next day, the cancer cells were stained with selected concentrations of VPA for 72 hours, followed by incubation for 2 hours with BrdU (100 µM) (10 µl/well). The culture medium was then removed and cells were fixed in FixDenat solution (200 µl/well) (30 minutes, room temperature - RT). Monoclonal anti-BrdU antibodies coupled with horseradish peroxidase were subsequently added (100 µl/well) (90 minutes, RT) and detected using tetramethylbenzidine substrate (TMB) (100 µl/well) (30 minutes, RT). 1 M sulphuric acid was added (25 µl/well) to stop enzymatic reaction, and quantitation was performed spectrophotometrically at 450 nm using Infinite M200 Pro microplate reader (Tecan).

**Histone extraction and Western blotting analysis**
Tumour cells were placed on a 6-well plate (Nunc) at a density of 2 x 10^5/ml (RK33) and 3 x 10^5/ml (RK45). On the following day, the culture medium was removed and the cells were exposed to selected concentrations of VPA for 6 hours. After treatment, the cells were harvested, washed twice in ice-cold PBS and resuspended in lysis buffer – TEB (0.5% Triton X100, 2 mM PMSF and 0.02% NaN3 in PBS, pH 7.4). The cell lysates were centrifuged at 800 x g for 10 minutes at 4°C. The nuclear pellet was washed in TEB and then centrifuged as before. Histones were extracted from the pellet using 0.02 N HCl overnight at 4°C and centrifuged at 12000 x g for 10 minutes at 4°C.

The concentration of histones in supernatant was quantified spectrophotometrically using the Protein Assay Kit (BioRad) according to manufacturer instructions. For Western-blot analysis, extracted histones were solubilized in 3 x Laemml buffer (0.19 M Tris-HCl, pH 6.8, containing 30% glycerol, 3% SDS, 0.015% bromophenol blue, 3% β-mercaptoethanol), then boiled for 5 minutes at 100°C. Equal amounts of extracted histones were electrophoresed on 15% SDS-polyacrylamide gel and electroblotted to polyvinyl difluoride (PVDF) membrane. Next, the membrane was blocked with 5% non-fat dry milk and 0.05% Tween 20 in TBS, pH 7.5 for 2 hours at RT, then incubated with a primary antibody such as anti-acetylated histone H3 (Lys9/14, Upstate Biotechnology), anti-acetylated histone H3 (Lys18 and Lys23, Cell Signaling) overnight at 4°C. The next day, the membrane was washed 3 times with 0.05% Tween 20 in TBS, pH 7.5 for 2 hours at RT. The membrane was incubated with horseradish peroxidase-labeled secondary antibody followed by a colour development using tetramethylbenzidine and hydrogen peroxide in the presence of 0.02% H2O2. Densitometric analysis of the bands was made using the Gel Doc system (BioRad). The expression of acetylated histone H3 was normalized to α-tubulin.

**Cell migration assessment**
Cancer cell migration was studied by the wound assay. Optimized amounts of RK33 (3 x 10^5/ml) and RK45 (5 x 10^5/ml) cells were placed on 35 x 10 mm culture dishes (Nunc). Next day, cell monolayer was scratched by a pipet tip (P300), the medium and dislodged cells were aspirated and the plates were rinsed two times with PBS. Next, fresh culture medium was applied and the number of cells migrated into the wound area was determined in the control, and the cultures treated with selected concentrations of VPA (1-5 mM) after 24 hours. Then, plates were stained with the May-Grünwald-Giemsa method and observation performed by a Nikon Eclipse TS100 Microscope (Nikon Precision Inc). Cells that migrated to the wound area were counted on micrographs and results were expressed as a mean cell number migrated to the selected 40 fields taken from four micrographs.

![Figure 3](image)

**Figure 3:** Antiproliferative effect of valproic acid in RK33 and RK45 cells. The cancer cells were exposed to either culture medium alone (control), or VPA (0.5-20 mM) for 72 hours. Normalized cell viability and proliferation, measured by the MTT assay (A) and BrdU incorporation (B), respectively are presented as mean ± SEM at each concentration. Student’s t-test revealed significant effect (*p<0.05; **p<0.001) of treatment with VPA compared to control group. This effect was also concentration-dependent, as judged by analysis of variance ANOVA test (**p<0.001), n=40 per concentration from three independent experiments.

**Competition interests:** None declared.

**Authors:** Grabarska et al., 2014. Valproic acid suppresses growth and enhances cisplatin cytotoxicity to larynx cancer cells. Head Neck Oncol 2014 May 03;6(1):7.

License OAPL (UK) 2014. Creative Commons Attribution License (CC-BY)

secondary antibody (Cell Signalling) for 1 hour at RT and visualized by the enhanced chemiluminescence (ECL) (Roche). Subsequently, the membrane was stripped with 100 mM β-mercaptoethanol and 2% SDS in 62.5 mM Tris-HCl, pH 6.8 for 20 minutes at 50°C, then blocked and probed with anti-histone H3 (Cell Signalling) and a secondary antibody, as described above. Densitometric measurement of protein level was performed using the GeneTools (Syngene). Obtained data were normalized to histone H3 expression.

Assessment of HDACs activity

Measurement of HDACs activity was performed using a HDAC Assay Kit (Upstate Biotechnology). The cells at a density of 2 x 10^5/ml (RK33) and 3 x 10^5/ml (RK45) were exposed to selected concentrations of VPA for 6 hours. After treatment, the cells were harvested and lysed in RIPA buffer (0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM Na3V04, 20 mM NaF, 0.5 mM DTT, 1 mM PMSF and protease inhibitor cocktail in PBS, pH 7.4) for 15 minutes at 4°C and then centrifuged at 8000 x g for 10 minutes at 4°C. Supernatant containing active HDACs was resuspended in 2 x HDAC assay buffer supplemented with 4 mM HDAC Assay Substrate and incubated for 1 hour at 37°C, followed by additional incubation with Diluted Activator Solution for 20 minutes at RT. During this step, the Activator Solution releases chromophore (p-nitroanilide) from the deacetylated substrate resulting in a yellow coloured product that was determined spectrophotometrically by measuring absorbance at 405 nm wavelength using an Infinite M200 Pro microplate reader (Tecan). Negative and positive controls were included in the assay, according to manufacturer instructions.

Assessment of cell death

Measurement of cell death was performed using a Cell Death Detection ELISAPLUS kit (Roche). RK33 and RK45 cells growing on 96-well plates were subjected to different concentrations of VPA alone or in combination with CDDP for 24 hours. Supernatants were removed and cells were lysed with lysis buffer for 30 min at RT. Subsequently, cell lysates were centrifuged at 200 x g for 10 minutes and 20 µl of the supernatants were transferred into the streptavidin-coated 96-well plate, followed by incubation with the immunoreagent (80 µl) containing anti-histone-biotin and anti-DNA-POD mouse monoclonal antibody for 2 hours at RT. The solution was removed and each well was rinsed three times with Incubation Buffer. Finally, 2,2’-azinobis(3-ethylbenzthiazoline-sulfonic acid) (ABTS) solution substrate was applied and incubated for 20 minutes at RT. Absorbance was measured at 405 nm wavelength using an Infinite M200 Pro microplate reader (Tecan).

Flow cytometry analysis

Experiments were performed using the FACScalibur™ flow cytometer (BD Biosciences), equipped with a 488-nm argon-ion laser. For cell cycle analysis, cells were treated with different concentrations of VPA for 24 hours and then fixed in ice-cold 80% ethanol at -20°C for 24 hours. After fixation, the cells were stained with propidium iodide utilizing the PI/RNase Staining Buffer (BD Biosciences) according to the manufacturer’s instructions. Acquisition rate was at least 60 events per second in low acquisition mode and at least 10,000 events were measured. Cell cycle analysis was performed by using a noncommercial flow cytometry analysing software - CytCher Version 1.0.2 for Windows (source: University of Wales) and WinMDI 2.9 for Windows (source:facs.scripps.edu/software.htm). The cells were acquired and gated by using dot plot FL-2 Width (X) versus FL-2 Area (Y)-gate to exclude aggregates and analysed in histograms displaying fluorescence 2-area (yellow/orange fluorescence: 585 nm).

RNA isolation and cDNA synthesis

Tumour cell lines, RK33 and RK45, were incubated on 6-well plates with the selected concentrations of VPA for 24 hours. Total RNA from the cells was isolated and digested with DNase using a High Pure RNA Isolation Kit (Roche) following the manufacturer’s instructions. Briefly, the cells were...
resuspended in PBS and lysed in Lysis/Binding Buffer. The cell lysates were transferred to a High Pure Filter Tube and centrifuged at 8000 x g for 15 seconds. Next, the RNA was incubated with DNase for 15 minutes at RT. Resulting RNA was rinsed once in Wash Buffer I, twice in Wash Buffer II, centrifuged at 8000 x g for 15 seconds and finally eluted using Elution Buffer.

The RNA concentration was determined spectrophotometrically with a UV-VIS Genesy 10S spectrophotometer at 260/280 nm (Thermo Fisher Scientific). 3 μg of total RNA was reverse transcribed for 30 minutes at 50ºC using a oligo(dT) primer and Transcriptor High Fidelity cDNA Synthesis Kit (Roche) followed by 5 minutes of enzyme inactivation at 85ºC according to the manufacturer’s instructions.

qPCR
Quantitative Real-time expression analysis was performed using a LightCycler® 480 II instrument (Roche). Analysis was made utilizing UPL hydrolysis probes specific for CCND1 and CDKN1A genes labelled with FAM in duplex with a probe for reference gene GAPD labelled with Yellow 555 (Roche). The primers and probe sets were as following: CCND1 (For 5'-GAAGATCGTTGCCACCTG-3', Rev 5'-GAACCTCTGTTCGGACACTTCT-3', Probe 5'-TCTTGTGGAGTGGTAGAAA-3'), CDKN1A (For 5'-GGCGTTTGGAGTGGTAGAAA-3', Rev 5'-TCACTGTCTTGTACCCTTGTGC-3'), amplified in 45 cycles consisting of 10 seconds denaturation at 95ºC, 30 seconds annealing at 60ºC and 10 seconds extension at 72ºC. Amplification was performed in 10 μl of reaction mixture containing cDNA amount corresponding to 12.5 ng of total RNA, 1 x LightCycler® 480 Probes Master (Roche), and an appropriate set of 0.4 μM primers and 0.2 μM UPL hydrolysis probes for each target and reference duplex.

After 10 minutes of initial denaturation at 95ºC, cDNA was amplified in 45-55 cycles consisting of 10 seconds denaturation at 95ºC, 30 seconds annealing at 60ºC and 10 seconds elongation at 72ºC. Obtained fluorescence data was calculated using the relative quantification method with efficiency correction.

**Results**
VPA affected HDACs activity and histone H3 acetylation in larynx cancer cell lines

RK33 and RK45 cancer cells were treated with 1, 2, 5 and 10 mM VPA for 6 hours, then HDACs activity and H3 acetylation was assessed by colorimetric assay and Western blotting, respectively. As shown in figure 1, VPA reduced intracellular HDACs activity in a dose-dependent manner in both cell lines. Treatment with 10 mM VPA inhibited HDACs activity up to 70% in RK33 cells (**p<0.01) and up to 80% in RK45 cells (**p<0.001). The decreased activity of HDACs was accompanied by a dose-dependent increase of hyperacetylated histone H3 K9, K14, K18 and K23. When compared with the untreated control cells, a markedly enhanced acetylation of histone H3 K18 was observed in RK33 cells and H3 K23 in both RK33 and RK45 cells.

Furthermore, the differences in histone acetylation after VPA treatment were not the result of changes in total histone H3 expression, as demonstrated by reblotting PVDF membrane with total histone H3 antibody which recognize acetylated and non-acetylated histone H3 (Figure 2A and 2B).

VPA reduces the viability, proliferation and migration of larynx cancer cell lines

RK33 and RK45 cells were exposed to either culture medium (control) or VPA in the concentration range from 0.5 to 20 mM for 72 hours. Cell viability significantly decreased in both cancer cell lines treated with VPA in a dose-dependent manner as measured by means of the MTT assay. The lowest concentrations of VPA that elicited antiproliferative effect in tumour cell lines were 1 mM for RK33 (**p<0.01) and 0.5 mM for RK45 (p<0.05), whereas 20 mM concentration of VPA (**p<0.001) almost completely abolished larynx cancer cells viability (Figure 3A). Comparisons made by means of IC50 showed that RK33 cells were more sensitive to VPA (IC50 ~ 5.8
mM) in contrast to RK45 cells (IC50 – 8.5 mM). The solvent (PBS) did not influence the assays (data not shown).

The reduction in cell numbers, represented as viability, was attributed to decreased cell proliferation, as demonstrated by the BrdU incorporation assay. In this study, RK33 cells viability inhibition (MTT assay) was mirrored by the BrdU assay and even more pronounced effect on RK45 cells was displayed (Figure 3B).

The motility of RK33 and RK45 cells was assessed by wound assay method after 24 hours of VPA treatment. Our study showed that VPA reduced the migration of both larynx cancer cells in a dose-dependent manner (Figure 4C). We also observed that RK45 cells (control cells and VPA exposed cells) (Figure 4B) migrated more markedly to the wound area in comparison to RK33 cells (Figure 4A).

**VPA-induced apoptosis to larynx cancer cell lines**

Previous studies of HDIs revealed that apoptosis is one of the major mechanisms of cancer cell death. Thus, we determined whether increasing concentrations of VPA (0.5-

![Figure 6: The viability of RK33 and RK45 cells after treatment with CDDP alone (A) or in combination with 1 mM VPA (B and C) measured by the MTT assay. Results on Figure 6A are presented as mean ± SEM of three independent experiments, n=40 per concentration. (p<0.05; **p<0.01; ***p<0.001 versus control; Student’s t-test). Data on Figure 6B and 6C were analyzed by means of linear regression. Asterisk (***) indicates marked decrease (p<0.001) in larynx cancer cells viability compared with cells treated with cisplatin alone.](image)

![Figure 7: Induction of apoptotic cell death in RK33 and RK45 cells after treatment with VPA and CDDP alone or in combination of both agents for 24 hours. Results are presented as enrichment of oligonucleosome fragments (fold increase). N=9 per concentration from three independent experiments (p<0.05; **p<0.01; ***p<0.001 versus control, Student’s t-test).](image)

![Figure 8: Combinative treatment of VPA and CDDP increases the growth inhibition and enhances apoptosis of both larynx cancer cell lines](image)

5 mM) were able to induce apoptosis of RK33 and RK45 cells, by means of mono- and oligonucleosomes released to cytosol by apoptotic cells. Our study showed that VPA triggered apoptotic cell death in both larynx cancer cell lines in a dose-dependent manner. We observed 2.5-fold (**p<0.01) and 2-fold (***p<0.001) increase of apoptosis at 2 mM concentration of VPA in RK33 and RK45 cells, respectively. However, a higher dose of VPA (5 mM) resulted in 14-fold (**p<0.001) enhance of apoptosis in RK33 cells, compared to only 3-fold apoptosis in RK45 cells (**p<0.001) (Figure 5).

**Combative treatment of VPA and CDDP increases the growth inhibition and enhances apoptosis of both larynx cancer cell lines**

We examined RK33 and RK45 cells viability upon treatment with CDDP alone or in combination with VPA.
We observed that increasing concentrations of cisplatin (0.05 – 10 µg/ml) inhibited the viability of both larynx cancer cell lines in a dose-dependent manner (Figure 6A).

The solvent (PBS) did not influence the assays (data not shown). Next, we studied if VPA could enhance the sensitivity of larynx cancer cell lines to cisplatin. The concentration of VPA used in this experiment was 1 mM which only marginally affected the viability of RK33 and RK45 cells, 94% (**p<0.001) and 89% (**p<0.001) viability, respectively (Figure 6A).

Cotreatment of CDDP and VPA resulted in significant decrease in the survival of RK33 (Figure 6B) and RK45 cells (Figure 6C) compared to cisplatin alone (**p<0.01). After combined therapy the mean IC50 values of CDDP were reduced from 1.1 to 0.3 µg/ml for RK33 cells and from 3.4 to 1.0 µg/ml for RK45 cells.

We also observed that concurrent administration of VPA (1 mM) and CDDP (0.2 µg/ml) augmented apoptotic cell death in both larynx cancer cell lines. Compared to cisplatin alone treated cells, our study showed almost a 3-fold increase of apoptosis (**p<0.01) in RK33 cells and 2-fold (**p<0.001) in RK45 cells after combinative treatment with both agents (Figure 7).

These results indicate that valproic acid can potentiate cisplatin cytotoxicity against larynx cancer cell lines.

**VPA influence on cell cycle progression in larynx cancer cells**

Since inhibition of cell proliferation resulted from decreased cell division, we performed cell cycle analysis by means of flow cytometry. FACS analysis revealed that incubation of RK33 and RK45 cells with VPA (0.5-2 mM) slow down cell cycle progression. It was observed that VPA induced an arrest of RK33 (Figure 8A) and RK45 (Figure 8B) cells in the G1 phase of the cell cycle, followed by reduction of cells number in S/G2 phase. Detailed data are showed in table 1.

**VPA altered expression of CCND1 and CDKN1A genes in larynx cancer cells**

As demonstrated above, VPA induced a cell cycle arrest at G1/S phase. Therefore, we analysed gene expression of CCND1 and CDKN1A engaged in G1/S transition in larynx cancer cell lines after treatment with VPA (1-5 mM). Our study showed that VPA down-regulated expression of CCND1 and up-regulated expression of CDKN1A in a dose-dependent manner. The highest used concentration of VPA (5 mM) decreased (**p<0.001) the expression of CCND1 by 2-fold in both larynx cancer cell lines (Figure 9B) and increased 4.5-fold (**p<0.001) and 20-fold (**p<0.01) the expression of CDKN1A in RK33 and RK45, respectively (Figure 9A).

**Table 1: Cell cycle analysis of larynx cancer cell lines treatment with VPA.**

<table>
<thead>
<tr>
<th></th>
<th>ctr</th>
<th>0.5mM VPA</th>
<th>1mM VPA</th>
<th>2mM VPA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RK33</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>70.67 ± 0.4559</td>
<td>76.27 (**) ± 0.56</td>
<td>86.59 (**) ± 0.9321</td>
<td>92.47 (**) ± 0.502</td>
</tr>
<tr>
<td>S</td>
<td>10.22 ± 0.1153</td>
<td>8.140 ± 0.01</td>
<td>4.928 (**) ± 0.4853</td>
<td>1.968 (**) ± 0.2611</td>
</tr>
<tr>
<td>G2</td>
<td>18.96 ± 0.5668</td>
<td>15.99 (*) ± 0.565</td>
<td>8.322 (**) ± 0.701</td>
<td>5.288 (**) ± 0.245</td>
</tr>
<tr>
<td><strong>RK45</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>60.57 ± 0.6829</td>
<td>65.98 (**) ± 0.4943</td>
<td>74.78 (**) ± 1.3220</td>
<td>84.30 (**) ± 0.3402</td>
</tr>
<tr>
<td>S</td>
<td>16.30 ± 0.5830</td>
<td>12.23 (**) ± 0.2139</td>
<td>8.823 (**) ± 0.6798</td>
<td>4.016 (**) ± 0.2730</td>
</tr>
<tr>
<td>G2</td>
<td>23.54 ± 0.2862</td>
<td>22.00 (*) ± 0.3852</td>
<td>16.48 (**) ± 0.6524</td>
<td>11.61 (**) ± 0.3404</td>
</tr>
</tbody>
</table>

(*p<0.05; **p<0.01; ***p<0.001; Student’s t-test, n=9 per concentration from three independent experiments).

Figure 8: Flow cytometric analysis of propidium iodide-stained nuclei following exposure of RK33 (A) and RK45 (B) cells to VPA (0.5-2 mM) compared to control (*p<0.05; **p<0.01; ***p<0.001; Student’s t-test, n=9 per concentration from three independent experiments).
Discussion

More than ten years ago, VPA was discovered as an HDAC inhibitor 19,20,21, and regarded as a potential therapeutic agent with new mechanisms of action in different diseases, including cancer 22. VPA is a short chain fatty acid, which inhibits the class I HDACs (HDAC 1-3) and class IIa HDACs (HDAC 4, 5, 7 and 9) 23.

In our study, VPA treatment decreased larynx cancer cells proliferation and HDAC activity accompanied by an increase of histone acetylation. Interestingly, treatment with a low dose of VPA resulted in a robust hyperacetylation of H3 K18, while a diminishing acetylation of other analysed histone 3 lysine residues. Selective hypoacetylation of H3 K18 has been linked to oncogenic transformation 24, aggressive cancer phenotype and patients poor prognosis 25,26.

H3 K18 hypoacetylation is regulated by the Akt kinase pathway through phosphorylation and inactivation of CBP histone acetyltransferase 27. Thereby, VPA-mediated increase of H3 K18ac observed in our study suggests the disruption of the oncogenic Akt kinase signalling in cancer cells.

Moreover, deacetylation of H3 K18ac by SIRT7 has been linked to anchorage-independent growth of cancer cells and escape from contact inhibition 24. The reduced migration of larynx cancer cells observed in our study could be related to H3 K18 hyperacetylation after VPA treatment.

The antineoplastic mode of action of VPA has been linked to induction of apoptosis and blockade of the cell cycle progression in G0/G1 phase in several prostate 28, endometrial 29, gastric 30 and bladder 31 cancer cell lines, as well as in leukaemia 32, which was similar to our results, demonstrating that the inhibition of larynx cancer cells growth was associated with G1/S cell cycle arrest, and reduction of cells number in the S/G2 phase.

Analysing genes expression of key proteins that regulate cell cycle progression, we demonstrate that increasing concentrations of VPA induced CDKN1A expression and simultaneously repressed CCND1, which is consistent with previous reports 29,30,33,34. CDKN1A expression is frequently silenced in human tumours by recruitment of HDAC-containing repressor complexes to its promoter site 35, while CCND1 is overexpressed 36,37,38,39,40. Thereby, reintroduction of p21 expression, together with inhibition of Cyclin D1 could be regarded as a more universal mechanism of VPA action on cancer cells.

VPA’s antiproliferative activity has been shown against a wide range of cancer cell lines in vitro, such as melanoma 41, acute lymphatic leukaemia 42,43, chronic myeloid leukaemia 44, gastric carcinoma 36, oral squamous cell carcinoma 33,44, prostate cancer 36,45, multiple myeloma 33,46, ovarian carcinoma 47,48, thyroid cancer 49, breast cancer 50, lung cancer cells 51, as well as Hep2 52, UM-SCC-2453, KB 54 laryngeal carcinoma cells and other head and neck squamous cell carcinoma (HNSCC) cell lines, including tongue (SCC15, SCC-9, SCC-25, Cal27) 24,44 and hypopharynx (FaDu) cancers 55,55 at concentrations similar to used in our studies.

Here we show that VPA differentially suppressed the growth of RK33 and RK45 larynx cancer cells at milimolar concentrations in a dose-dependent manner, which could be linked to different biological aggressiveness of these cell lines-derived cancers at the molecular level, since the original tumours had similar histopathological characteristics 18.

HDIs have been reported to improve the efficacy of chemotherapeutic drugs, including CDDP 44. It has been observed that VPA exhibited synergistic cytotoxicity with CDDP on HNSCC cells 29, and melanoma cells in vitro 41,56, as well as on ovarian cancer cells in vitro 48,57 and in vivo 48. A combination therapy using VPA and CDDP has not been tested in larynx cancer cells.

We found that combined treatment with both agents resulted in reduction of CDDP concentration necessary to obtain 50% inhibition of larynx cancer cells proliferation. Moreover, CDDP alone did not induce apoptosis in larynx cancer cells at the given concentrations. However, when administrated together with VPA CDDP enhanced cell death, particularly in RK33 cells, suggesting that VPA sensitized larynx cancer cells for apoptosis induction, what has been demonstrated for human ovarian cancer cells 48.

Molecular mechanisms that could be responsible for the enhanced sensitivity of cancer cells to cisplatin are not clear and has been linked to upregulation of reactive oxygen species and PTEN (tumour suppressor phosphatase and tensin homolog) protein level 57.
Conclusion
Taken together, VPA could be a potential chemotherapeutic agent against larynx cancer cells. VPA is commonly used in the treatment of some forms of epilepsy and seizures, bipolar disorders, migraine, clinical depression and schizophrenia, with a mild toxicity profile and safe, even when administrated over a long period of time. VPA levels in epilepsy patients are usually not above 0.7 mM. Limited toxicity was observed when the concentration was below 3.1 mM, and severe side effects developed when the concentration was above 5.9 mM. It should be noted that lower doses (1-2 mM) of VPA used in our study significantly suppressed cancer cells proliferation, induced cell cycle arrest, and apoptosis, as well as increase sensitivity of larynx cancer cells to cisplatin treatment.

Most chemotherapeutics, such as CDDP, display severe side effects. Thereby, reducing the CDDP dose in combined treatment with VPA to obtain the same therapeutic effect, could have significant benefit for the cancer patients. Additionally, VPA and CDDP in in vitro studies are applied once in 48-72 hours cell treatment time.

In patients, drug treatment can be administrated every day, thus, increasing drugs delivery and concentration level. Therefore, our findings suggest that VPA could be a novel attractive agent for treatment of laryngeal cancer that could be tested clinically both as monotherapy and in combination with cisplatin.

Acknowledgment
This study was supported by Medical University of Lublin DS440/2012-2013 grant and The Polish Ministry of Science and Higher Education Grant No NN405 617538.

Abbreviations list
CDDP - cisplatin (cis-diammine-dichloroplatinum); 5-FU - 5-fluorouracil; HDACs - histone deacetylases; HDIs - histone deacetylase inhibitors; RK45 - human larynx cancer cell lines; SAHA - suberoylanilide hydroxamic acid; VPA - valproic acid;

References

Licensee OAPL (UK) 2014. Creative Commons Attribution License (CC-BY)


