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Preclinical evidence for a beneficial impact of valproate on the response of small cell lung cancer to first-line chemotherapy

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ABSTRACT

Prognosis of small cell lung carcinoma (SCLC) is particularly poor, less than 5% of patients with extensive stage being alive after two years. We hypothesized that SCLC chemotherapy could be improved by using histone deacetylase (HDAC) inhibitors based on their ability to interfere with lysine acetylation and to alter gene expression. The goal of this study was to evaluate the anticancer efficacy of a HDAC inhibitor (valproate: VPA) on SCLC cells in combination with the standard chemotherapeutic first-line regimen (cisplatin + etoposide). We show that VPA induces apoptosis of small cell lung cancer cell lines and improves efficacy of cisplatin combined with etoposide. Both mitochondrial and death receptor pathways are involved in VPA-induced apoptosis. As expected for an HDAC inhibitor, VPA hyperacetylates histone H3. The mechanism of VPA pro-apoptotic activity involves induction of p21, inhibition of Bcl-xL, cleavage of Bid and phosphorylation of Erk and H2AX. In the presence of VPA, Bax is translocated from the cytoplasm to the mitochondria and cleaved in an 18 kDa isoform. Cytochrome c is released from the mitochondria into the cytosol. Transcriptomic analyses by microarray show that VPA modulates transcription of genes (Na^+/K^+ ATPase, Bcl-xL) involved in chemoresistance to cisplatin and etoposide. Finally, the efficacy of VPA combined with cisplatin and etoposide is supported by preclinical models of SCLC cells engrafted into SCID mice. Together, these data demonstrate that VPA augments anticancer activity of cisplatin and etoposide, two components of the standard first-line chemotherapy of small cell lung cancer.

1. Introduction

Lung cancer is the number one cause of cancer-related death worldwide, with more than 1 million deaths per year. Despite improvements in radiotherapy and chemotherapy, the five years survival rate remains below 15%. Incidence of small cell lung carcinoma (SCLC) has declined below 15% compared with 20–25% previously.[1–3] However, the outcome of extensive stage

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SCLC patients is the poorest of any histological subtype with an overall survival after 2 years of less than 5%. Because of the rapid dissemination of cancer cells, surgery for SCLC is rarely fruitful and extremely uncommon. For limited-stage, standard treatments include combined chemotherapy with concurrent radiotherapy, followed by prophylactic cranial irradiation (PCI).[4] Extensive-stage disease is treated with chemotherapy and prophylactic cranial irradiation when patients achieve complete or partial remission.[1] The gold standard chemotherapy for first-line presenting patients is a platinum-based regimen that includes cisplatin (a DNA crosslinking agent) and etoposide (an inhibitor of topoisomerase II).[1,4–8] Although rarely cured, SCLC is extremely sensitive to both chemotherapy and radiotherapy at least in the first-line setting, with response rates ranging between 60 and 70% in extensive-stage disease.[6] However, remissions are short-lived and second-line therapies yield response rates of about 20% and median survival times of 25 weeks.[9]

In this context, it is clear that improvements in chemotherapy for SCLC are needed, preferably at first presentation. Based on evidence from the literature[10–15] and from our laboratories[16–19], we hypothesized that chemoresistance may be due to epigenetic alterations leading to defects in apoptotic pathways. Therefore, modulating epigenetic changes may increase efficacy of SCLC chemotherapy. It is possible to interfere with epigenetic modifications, such as histone acetylation, using relatively specific and reversible inhibitors. Among a growing list of histone deacetylase (HDAC) inhibitors, the sodium salt of 2-propylpentanoic acid (VPA) has the major advantage of having been used for decades as an established treatment in humans for epilepsy and bipolar disorders. Moreover, VPA has appropriate pharmacokinetic properties and presents only moderate toxicity which is acceptable in the context of an anticancer treatment.[20–24] Analyses of VPA analogs have established a close correlation between antitumor properties and HDAC inhibition.[25,26] VPA binds to the catalytic pocket of lysine deacetylases, complexes with Zn^{2+} via its carboxyl group and inhibits their activity. By inhibiting HDACs, VPA favors hyperacetylation of histone *N*-terminal tails, restores a negative charge and decreases their affinity for DNA, leading to decondensation and transcriptional activation.[27–29] In cells, VPA modulates a broad range of activities including proliferation, apoptosis and differentiation.[30–35]

With the aim of improving first-line treatment of SCLC, we evaluated the capacity of VPA to increase the anticancer effect of cisplatin and etoposide in cell cultures and in xenograft mouse models.

2. Materials and methods

2.1. Cell culture conditions

BKT cells provided by Dr. Barbara Campling (18) were derived from biopsies isolated from patients with SCLC. Although these cells are tumorigenic in mice, their growth in culture is very slow. Human SCLC cell lines (H146, H526, H69) were purchased from the ATCC (Manassas VA). All cell lines were cultivated in RPMI 1640 medium supplemented with 10% (5% for BKT) fetal calf serum (FCS), 2 mM L-glutamin, 100 U/ml of penicillin and 100 μg/ml of streptomycin and maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cells were incubated with VPA (Sigma–Aldrich), cisplatin and etoposide (Bristol–Myers Squibb) alone or in combination.

2.2. Cell viability assay

As a surrogate of cell growth, viability was evaluated using the MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation assay, Promega). Lung cancer cell lines (1.10^4 cells/ml) were incubated in presence of different concentrations of VPA (0–10 mM), cisplatin (0–100 μM) or etoposide (0–100 μM). After 48 and 72 h of culture, 20 μl of tetrazolium containing reagent were added to each well in a 96-wells plate format. After 2 h incubation at 37 °C, the plate was analyzed using a colorimetric microplate reader at a wavelength of 490 nm.

2.3. Detection of apoptosis

Apoptosis was quantified by flow cytometry after ethanol fixation and propidium iodide incorporation. Cells were plated at 5×10^5 per ml and treated with 1 mM of VPA, 10 μM of cisplatin and 10 μM of etoposide alone or in combination. After 24 h of culture, cells were collected by centrifugation at 500 g for 10 min, washed twice with phosphate-buffered saline (PBS)-10% FCS and fixed with 70% cold ethanol. After incubation at −20 °C for at least 1 h, cells were washed twice with PBS-10% FCS and treated for 30 min at 37 °C with RNAse A (50 μg/ml, Sigma–Aldrich). Then, cells were incubated for 10 min in the dark in PBS containing 20 μg/ml of propidium iodide (PI, Sigma–Aldrich) and analyzed by flow cytometry (FACS Aria, Becton Dickinson). Cell doublets were excluded from the analysis using the (FSC-H/FSC-W) gating method. Ten thousand events were collected and analyzed with the FACS Diva Software. Cells in sub-G1 phase were considered as apoptotic cells.

To determine whether the effect on apoptosis induced by the combined treatment was additive, synergistic or antagonistic, a Synergy Index (SI) was calculated using the following formula: SI = specific apoptosis upon combined treatment/sum of specific apoptosis of single agent treatment. The percentage of specific apoptosis was determined using the following formula: specific apoptosis = (drug induced apoptosis – spontaneous apoptosis)/(100 – spontaneous apoptosis).

To assess the role of caspases in apoptotic pathways, 5×10^5 cells were incubated with or without 20 μM of total pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-CH_2F (Z-VAD-fmk, Becton Dickinson), 20 μM of negative control (Z-FA-fmk, Becton Dickinson), 40 μM of caspase 8 specific inhibitor Z-Ile-Glu(OMe)-Thr-Asp(OMe)-CH_2F (Z-IETD-fmk, Calbiochem) or 40 μM of caspase 9 specific inhibitor Z-Leu-Glu(OMe)-His-Asp(OMe)-CH_2F (Z-LEHD-fmk, Calbiochem), all compounds being diluted in dimethylsulfoxyde (DMSO).

2.4. Western blot analysis

After 24 h of culture, 2.10^6 cells were washed twice in cold PBS and lysed in cytosolic buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 pH 7.2, 100 μM PMSF, 200 μg/ml digitonine and protease inhibitors (Complete Mini, Roche) on ice for 5 min. After centrifugation at 1000 g for 5 min at 4 °C, the supernatants (i.e. the soluble

fraction on Fig. 3) were collected and stored at –80 °C. The pellets were solubilized in 50 mM Tris–HCl (pH 7.5), 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl and 0.1% SDS supplemented with a protease inhibitor cocktail (Complete, Roche). Tumor lysates were prepared using a tissue homogenizer and lysis buffer consisting of 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS supplemented with a protease inhibitor cocktail (Complete, Roche). After 30 min incubation on ice and centrifugation at 13,000*g* for 5 min at 4 °C, the supernatants containing soluble proteins were collected and stored at –80 °C. Protein concentrations were quantified using the Micro BCA Protein Assay kit (Pierce). Twenty micrograms of proteins were migrated on a 15% SDS–polyacrylamide gel and transferred onto a nitrocellulose membrane (GE Healthcare). After blocking with TBS containing 4% of non-fat dry milk, the membrane was incubated overnight at 4 °C with primary antibodies. Optimal antibody concentrations were provided by the manufacturer and tested experimentally: anti-acetylated histone H3 (dilution 1/10,000, Upstate), anti-actin (dilution 1/1000, Sigma–Aldrich), anti-Bax (dilution 1/250, Dako Cytomation), anti-p21 (dilution 1/500, Santa Cruz Biotechnology), anti-Bid (dilution 1/500, Becton Dickinson), anti-Bcl-2 (dilution 1/100, Dako Cytomation), anti-phospho-Bcl-2 (Ser70) (dilution 1/500, Cell Signaling), anti-Bcl-xL (dilution 1/500, Cell Signaling), anti-caspase 8 and anti-caspase 9 (dilution 1/500, Cell Signaling), anti-cytochrome *c* (dilution 1/1000, Becton Dickinson pharmingen), anti-Erk (dilution 1/10,000, Sigma–Aldrich), anti phospho-Erk (dilution 1/1000, Cell Signaling), anti-γH2AX (dilution 1/1000, Abcam) and anti-VDAC1 (dilution 1/100, Abcam). Membranes were then incubated for 1 h with 10,000 fold diluted polyclonal horseradish peroxidase-conjugated secondary antibodies (either goat anti-mouse or swine anti-rabbit immunoglobulin/HRP purchased from Dako Cytomation) and revealed using the ECL Plus Western Blotting Detection kit (GE Healthcare).

2.5. *Analysis of caspase activity*

Human SCLC cell lines (H69, H146, H526) were cultivated with VPA, cisplatin and etoposide alone or in combination for 24 h. Tumors were excised from the SCID mice in treatment and homogenized in lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS supplemented with a protease inhibitor cocktail; Complete, Roche). After 30 min incubation on ice and centrifugation at 13,000*g* for 5 min at 4 °C, the supernatants containing soluble proteins were collected, quantified using the Micro BCA Protein Assay kit (Pierce) and stored at –80 °C. Lysates were then incubated in white-walled 96-wells plate in the presence of reconstituted caspase-Glo 3-7, caspase-Glo 8 or caspase-Glo 9 reagents (sample–reagent dilution: 1:1). After 1 h of incubation at room temperature and in the dark, the luminescence of each sample was measured in a plate-reading luminometer.

2.6. *Microarray analysis*

H526 cells were cultivated for 4 h in the presence or the absence of 1 mM of VPA. After two washes in PBS, cells were lysed in Trizol RNA Isolation Solution (Ambion). RNA was extracted accordingly to the manufacturer's protocol, precipitated with isopropanol and washed with ethanol 75%. A second separation between aqueous and organic phase was performed on Phase Lock Gel (5 prime) to optimize RNA recovery and purity. All the extracted RNAs were assessed for quantity and purity on the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and stored at –80 °C until amplification step.

Reference RNAs were obtained by pooling equal amounts of total RNAs from 4 different SCLC cell lines. Five hundred ng of sample and reference RNAs were reverse-transcribed in parallel with the same Master Mix using polydT primer and the Moloney Murine Leukemia Virus–reverse transcriptase (MMLV–RT) and amplified by PCR. The cDNAs were labeled with Cy3 (reference) or Cy5 (sample) dyes using the Low Input RNA Linear Amplification Plus kit (Agilent technologies). RNA spike-in (Agilent Technologies) were included to provide positive controls for monitoring microarray steps from sample amplification to microarray processing. Quantity and dye incorporation of labeled cRNAs was assessed using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The labeled cRNAs were then hybridized on a Agilent oligonucleotide microarray (Two colours Whole Human Genome 4×44 K arrays, Agilent Technologies) for 17 h at 60 °C in a rotating oven (Agilent Technologies). The samples were randomized according to the treatment and the amplification run. After disassembling chambers, the slides were washed and analyzed by confocal laser scanning (Agilent Technologies). Grid positioning, spots localization, outliers flagging, fluorescence intensities quantification, background level evaluation and correction of the values according to the background followed by linear and Lowess data normalization were performed accordingly to the manufacturer's protocol (GE2_v5_95_Feb07 protocol, Feature Extraction software, version 9.5.3.1, Agilent Technologies). The statistical analyses were performed with the Genespring GX, version 7.3.1 (Agilent Technologies). Additional normalization steps were performed: (1) per spot (division by the control signal), (2) per array (normalization to percentile 50) and (3) per gene (normalization to median). Transcripts that were not present in at least one sample were excluded from additional analyses. Welch's *t* test was used to assess the statistical significance of the differences in mRNA expression observed between the different sample groups, excluding genes whose expression varied by a factor inferior to 2 across the sample set of interest. Moreover, we used a correction for multiple tests (CMT), according to Benjamini's test (FDR: false discovery rate corrections) and considered the threshold for statistical significance at 0.05. Gene pathways were analyzed with the Ingenuity program.

2.7. *Evaluation of regimen efficacy in SCID mice*

The Institutional Animal Care and Usage Committee (IACUC Protocol No. 672) at the University of Pennsylvania approved all animal protocols in compliance with the Guide for the Care and Use of Laboratory Animals. SCID mice (Jackson Laboratories) received a standard research diet throughout the experiment. BKT (6.5×10^6) and H69 (2.5×10^6) cells, embedded in

50% Matrigel Basement Membrane Matrix High Concentration (Becton Dickinson Biosciences), were implanted subcutaneously into the flanks of 7 weeks-old female SCID mice. When tumors reached a volume of 300–400 mm^3, mice were administered with daily intraperitoneal injections of VPA (400 mg/kg/day) or PBS as control. The first injection of VPA (at day 9 and 41 for H69 and BKT, respectively) was administered three days before starting the chemotherapeutic treatment to fit with pharmacokinetic properties. Intraperitoneal injections of cisplatin (3 mg/kg) and etoposide (40 mg/kg) were performed at days 12, 16, 22, and days 44, 49, 69 for H69 and BKT, respectively. The schedule of drugs injections was first determined in preliminary dose response experiments. Tumor volumes were calculated twice a week using the formula:

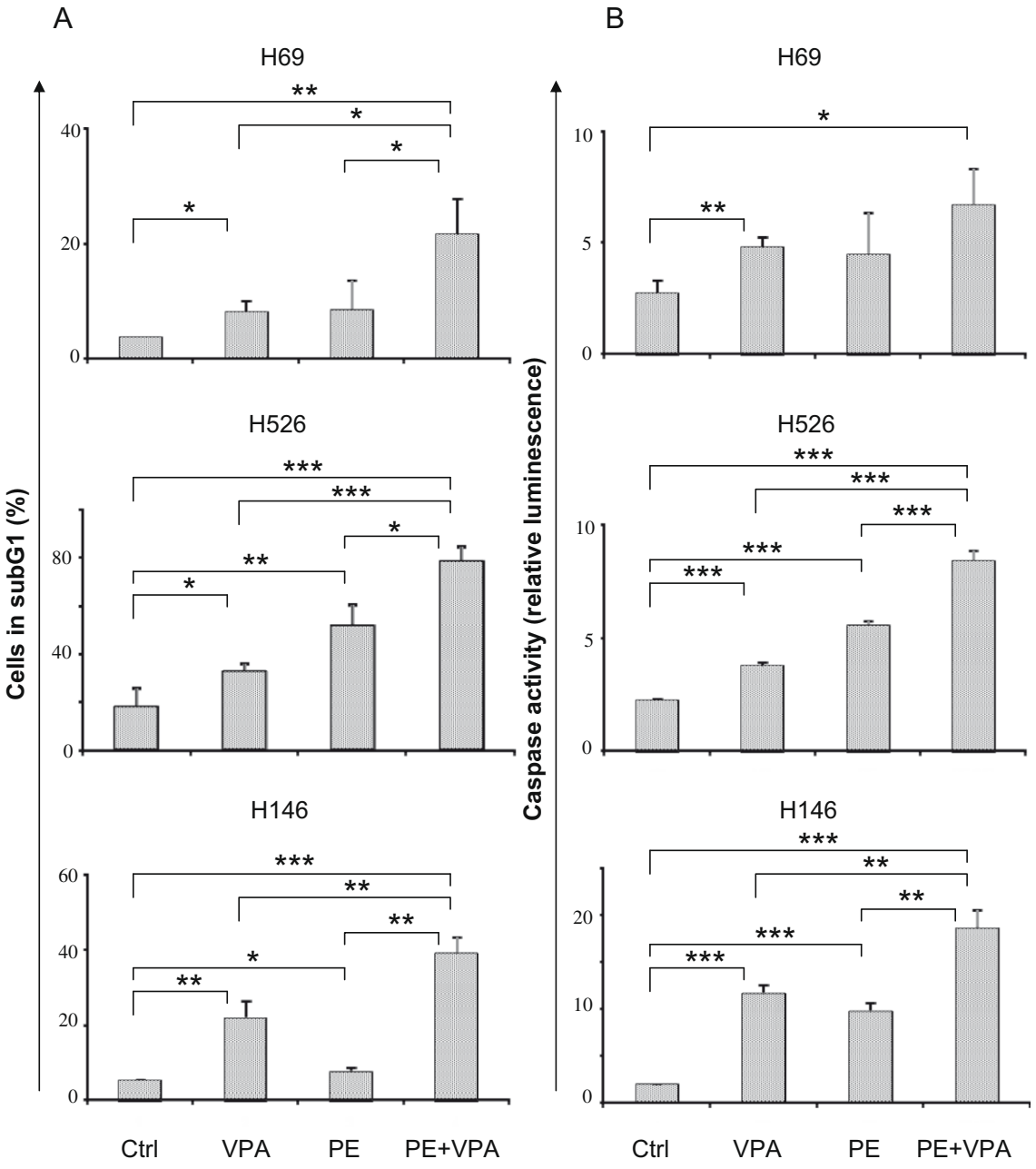

Fig. 1 – VPA synergizes with cisplatin and etoposide to induce apoptosis in SCLC cell lines. Three small cell lung cancer cell lines (H69, H526 and H146) were cultivated during 24 h with or without valproate (VPA, 1 mM) in combination with cisplatin (P, 10 μM) and etoposide (E, 10 μM). Apoptosis rates were determined by flow cytometry after ethanol fixation and propidium iodide incorporation (panel A). Cells in sub-G1 phase resulting from DNA fragmentation were considered to be apoptotic. The percentages of cells undergoing apoptosis are presented as means and standard deviations (±SD) of three independent experiments. Caspase activity was analyzed with caspase-Glo 3-7 reagent (panel B). Differences are considered to be significant (*$p < 0.05$), very significant ($p < 0.01$) and highly significant (***$p < 0.001$) according to the paired Student's t test.**

$1/6 \times \pi \times D \times d^2$ with D the largest diameter and d the smallest diameter. Groups of at least six mice were tested in each experimental condition.

2.8. Statistical analysis

All cell culture experiments were performed at least three times and data are shown as means ± standard deviations. Statistical significance was calculated using Student's *t* test and data were considered statistically significant (*), very statistically significant (**) and highly statistically significant (***) when $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

3. Results

3.1. VPA combined with cisplatin and etoposide and its effect on SCLC cell apoptosis

We first determined the response of SCLC cells to increasing doses of VPA (0–10 mM) using a viability assay (MTS). The IC50 determined at 72 h of culture was 0.4, 1.5 and 1 mM of VPA in H146, H526 and H69 cells, respectively (data not shown). These milimolar concentrations of VPA are clearly within the therapeutic dose achievable in patients.[36] Therefore, 1 mM VPA was combined with therapeutically relevant doses of cisplatin (P, 10 μM) and etoposide (E, 10 μM) and cells were analyzed by cytometer after propidium iodide staining in order to seek out the typical apoptotic DNA fragmentation. In H69, H526 and H146 cells, VPA significantly increased apoptosis induced by cisplatin and etoposide ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively, according to the paired Student's *t* test, Fig. 1A). In fact, the effect of VPA on the combined treatment was synergistic (calculated synergy index of 2.69, 1.25 and 1.84 in H69, H526 and H146 cells, respectively). In parallel, we measured caspase 3/7 activity and confirmed onset of caspase-dependent apoptosis (Fig. 1B).

We conclude that VPA synergizes with cisplatin and etoposide to induce apoptosis of SCLC cells.

3.2. Apoptosis induced by VPA combined to cisplatin and etoposide is dependent on extrinsic and intrinsic pathways

To determine if VPA/cisplatin/etoposide-induced apoptosis was dependent on either extrinsic (i.e. death receptor dependent) or intrinsic (i.e. mitochondria dependent) pathways involving caspases 8 and 9 enzymatic activities, respectively, pharmacologic inhibition with caspase inhibitors was performed. Compared to the Z-FA-fmk control (analog devoid of caspase inhibition activity), the pan-caspase inhibitor Z-VAD-fmk efficiently reduced apoptosis of H526 cells ($p < 0.001$ according to the paired Student's *t* test; Fig. 2). Similar results were obtained when the extrinsic or intrinsic pathways were selectively blocked using the caspase 8 inhibitor (Z-IETD-fmk, $p < 0.01$) or the caspase 9 inhibitor (Z-LEHD-fmk, $p < 0.01$), although the latter appeared to be slightly less potent ($^*p < 0.05$). Concomitant inhibition of both pathways resulted in almost complete inhibition of apoptosis. Similar conclusions were obtained with H146 and H69 cell lines (data not shown).

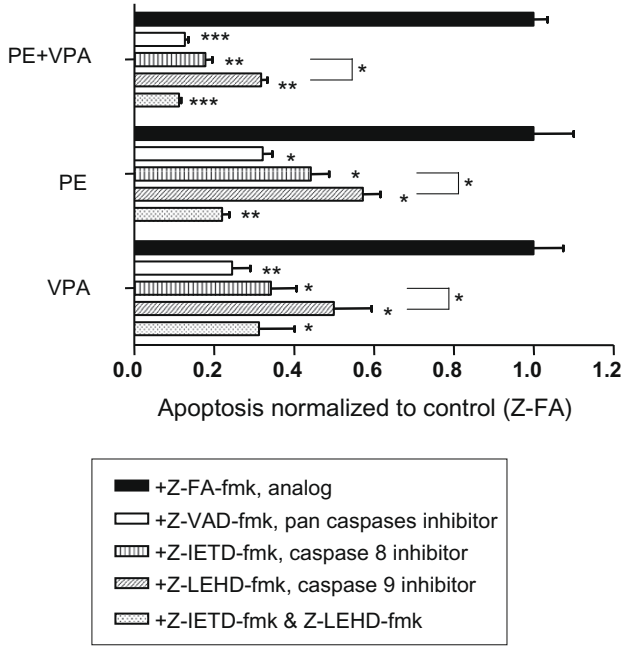

Fig. 2 – Apoptosis induced by VPA, cisplatin and etoposide is dependent on caspases 8 and 9. Different caspase inhibitors were added to H526 cell cultures for 2 h: a total pan-caspase inhibitor (Z-VAD, 20 μM), a negative control (Z-FA, 20 μM), a caspase 8 inhibitor (Z-IETD, 40 μM), a caspase 9 inhibitor (Z-LEHD, 40 μM) and a combination of caspase 8 and 9 inhibitors (Z-IETD + Z-LEHD, each 40 μM). Cells were then cultivated for 48 h with or without valproate (VPA, 1 mM) in combination with cisplatin (P, 10 μM) and etoposide (E, 10 μM). Cells in sub-G1 phase were considered to be apoptotic. The percentages of cells undergoing apoptosis from three independent experiments were normalized to the Z-FA control arbitrarily set to 1. Error bars are standard deviations (±SD). Similar results have been obtained with H69 and H526 cell lines.

These results demonstrate that apoptosis induced by VPA combined with cisplatin and etoposide in SCLC cell lines is caspase-dependent and involves both extrinsic and intrinsic pathways.

The mechanism of VPA-induced apoptosis involves hyperacetylation of histone H3, induction of p21, imbalance in pro- and anti-apoptotic modulators, release of cytochrome *c* from mitochondria and phosphorylation of H2AX and ERK1/2.

To further understand the mechanisms involved, the expression profile of selected proteins was obtained by western blot. Fig. 3 illustrates the analysis of H146 cells cultivated for 24 h with or without valproate (VPA, 1 mM) in combination with cisplatin (P, 10 μM) and etoposide (E, 10 μM). Protein levels in cell lysates were normalized using an anti-actin antibody and purity of mitochondrial and cytosolic fractions was assessed by expression of voltage-dependent anion channel 1 (VDAC1, Fig. 3). As expected for an HDAC inhibitor, VPA-induced hyperacetylation of histone H3 when added alone or in combination with cisplatin and etoposide. In the presence of VPA, the p21 cell cycle inhibitor was upregulated and Bid was cleaved generating the processed and active t-Bid

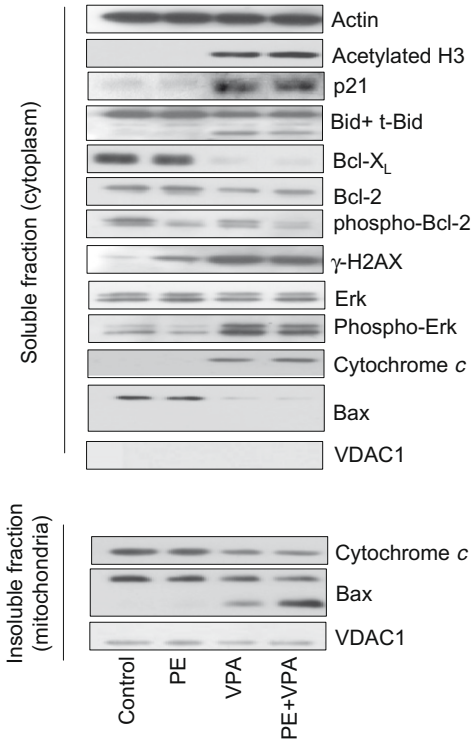

Fig. 3 – Protein expression profiling of small cell lung cancer cell lines. H146, H526 and H69 cells were cultivated during 24 h with or without valproate (VPA, 1 mM) in combination with cisplatin (P, 10 μM) and etoposide (E, 10 μM). After the culture, the cytosolic and mitochondrial fractions were analyzed by western blot with indicated antibodies. Protein concentrations were normalized according to actin levels. Purity of mitochondrial and cytosolic fractions was assessed by expression of VDAC1. Results are shown for the H146 cell lines. Similar results have been obtained with the other cell lines.

isoform (lower band). Under these conditions, expression of Bcl-xL was strongly reduced while the total levels of Bcl-2 remained unchanged. In contrast, phosphorylation of Bcl-2 slightly decreased in presence of cisplatin and etoposide. VPA-induced phosphorylation of H2AX (γ-H2AX) and Erk1/2 independently of cisplatin and etoposide treatment. Cytochrome *c* was released from the mitochondria into the cytoplasm (compare upper and lower panels on Fig. 3). Finally, the pro-apoptotic protein Bax was excluded from the cytoplasmic fraction and concentrated into mitochondria generating a cleaved 18 kDa isoform (lower band). Similar conclusions were obtained with H146 and H69 SCLC cell lines (data not shown).

Collectively, these protein profiling analyses thus demonstrate that VPA profoundly affects expression, processing, translocation or phosphorylation of important mediators of apoptosis.

3.3. *VPA modulates expression of several genes involved in apoptosis and drug sensitivity*

To assess the ability of VPA to alter cellular gene expression, we compared the transcriptome of H526 cells cultivated in the presence or the absence of VPA. The complete list of differentially expressed genes identified with Agilent microarrays is shown in data supplementary file 1. The reliability of these microarray data was confirmed by real time RT-PCR of five randomly selected genes in three different cell lines (H146, H526 and H69; data not shown). In the microarray list, expression of 273 genes were significantly up- or down-regulated by a factor >2. Genes such as TNFRSF12A, TNFRSF19, BCL2 binding component 3 and Bcl-xL are implicated in regulation of apoptosis. Interestingly, two genes Bcl-xL and Na^+/K^+ ATPase are also involved in chemoresistance to cisplatin + etoposide or platiniums, respectively (supplementary file 2 and reference).[37] Their transcriptomic network analyzed by Ingenuity is illustrated in Fig. 4 (see *).

3.4. *VPA prevents tumor growth in SCID mice treated with cisplatin and etoposide*

Finally, we investigated the ability of VPA to inhibit tumor growth in two models of SCID mice engrafted with human SCLC cells. BKT and H69 cells embedded in Matrigel were implanted subcutaneously into the flanks of 7 weeks-old female SCID mice. When tumors reached a volume of 300–400 mm^3, mice were given daily intraperitoneal injections of VPA (400 mg/kg/day) or PBS as control. Three days after the first VPA administration, intraperitoneal injections of cisplatin (3 mg/kg) and etoposide (40 mg/kg) were performed as described in materials and methods. Treatment with VPA alone (▲) slightly and moderately reduced growth of H69 and BKT tumors, respectively (Fig. 5, panel A). As expected, a regimen of cisplatin and etoposide (●) delayed tumor growth, although mice eventually relapsed. However, when VPA was added to this treatment (♦), the efficacy of cisplatin and etoposide was significantly improved in both models ($^{**}p < 0.01$ using the Student's *t* test, Fig. 5A), as confirmed by excised tumor weights (Fig. 5B). It is noteworthy that tumors from the VPA-treated mice appear to be less vascularized, suggesting that VPA affects angiogenesis. Importantly, the combined treatment did not induce any significant increase in weight loss (i.e. <15%, data not shown), compared to controls, indicating reasonable tolerability of the cisplatin/etoposide/VPA regimen. In VPA-treated mice, histone H3 was hyperacetylated in tumors harvested post-treatment (Fig. 5C), indicating HDAC inhibition. Finally, there was a non-statistically significant trend for increased activity of caspases 8 and 9 in tumors extracted from mice treated with the combined treatment ($p > 0.05$, Fig. 5D), suggesting onset of apoptosis.

Taken together, these data demonstrate that VPA increases antitumor efficacy of cisplatin and etoposide in two separate SCID mouse xenograft models.

4. Discussion

Despite improvements in treatments,[4–6,38] SCLC still has a very bad prognosis. Our report supports a potential new approach to improve SCLC treatment by combining chemotherapy with the HDAC inhibitor VPA. Surprisingly, very few studies have investigated such approaches in SCLC. Previous reports have described the sensitivity of SCLC cells to single

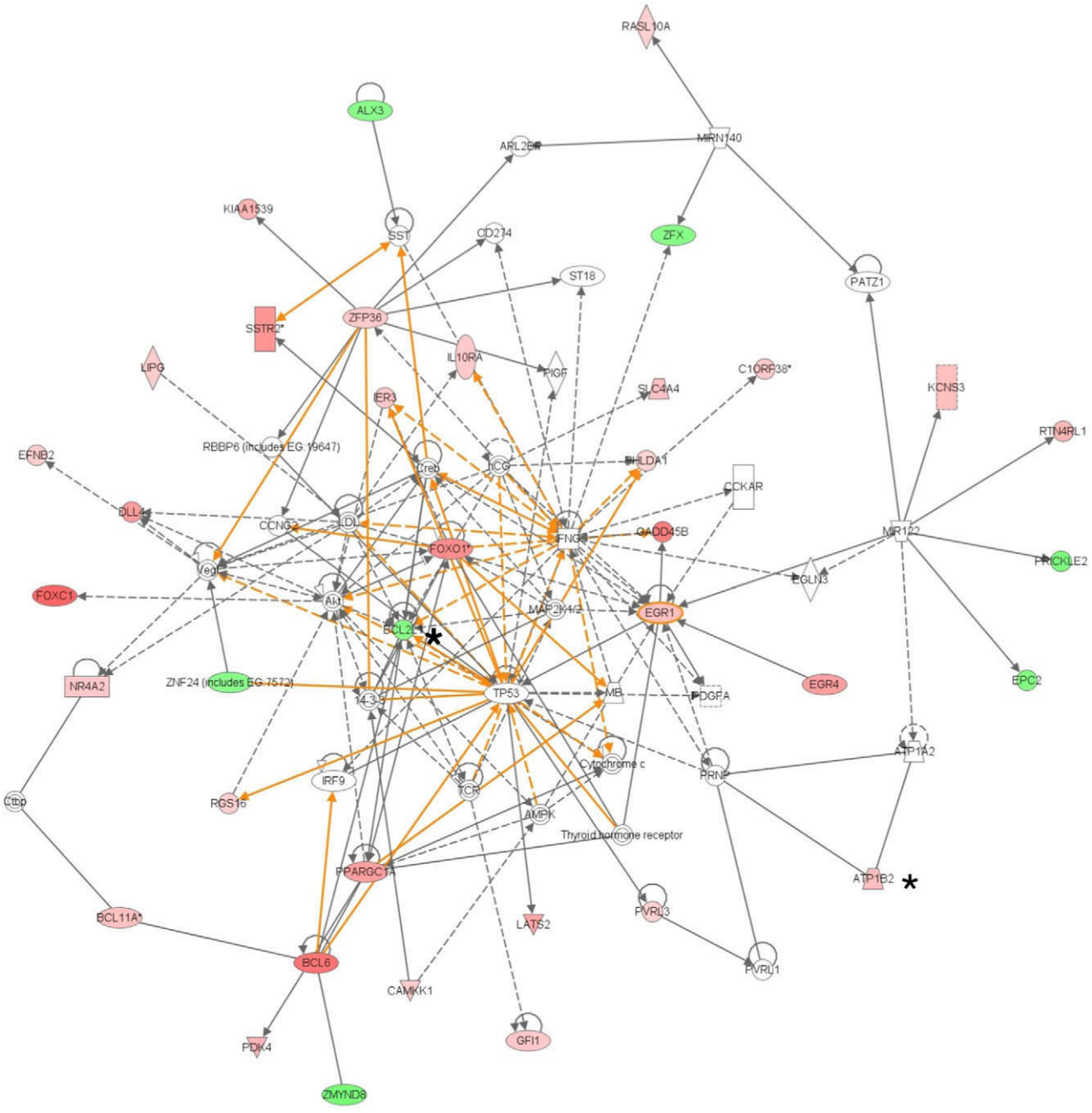

Fig. 4 – VPA modulates expression of several genes involved in apoptosis and drug sensitivity. Genes transcriptionally modified by VPA (supplementary file 1) and involved in resistance to cisplatin and/or etoposide (supplementary file 2) were analyzed with the Ingenuity software. Two genes (Na^+/K^+ ATPase and Bcl-xL) known to be involved in this type of chemoresistance are activated by VPA in H526 SCLC cells (indicated by *).

HDAC inhibitors: FR901228,[15,39] trichostatin A,[13] VPA,[14] and panobinostat (LBH589).[18] In the current report, we provide experimental evidence in cell culture (Fig. 1) and in animal models (Fig. 5) demonstrating that the standard first line treatment of SCLC is improved by VPA.

The present study shows that VPA combined with cisplatin and etoposide induces a caspase-dependent mechanism of apoptosis involving both intrinsic and extrinsic pathways (Fig. 2). Indeed, inhibition of caspase 8 or caspase 9 (or both) abrogates onset of apoptosis suggesting that both pathways are interconnected by the truncated t-Bid isoform. Consistently, protein profiling experiments indicate that VPA increases the levels of t-Bid (Fig. 3). The cleaved carboxy-terminal portion of Bid causes loss of membrane potential[40–42] and stimulates release of cytochrome c from mitochondria, as observed in Fig. 3. Bid can be cleaved not only by caspase-8, but also by other caspases (caspases 2 and 3) and other proteases (granzyme B, calpains and cathepsins).[43] Our data shows that the pan-caspase inhibitor Z-VAD-fmk totally inhibits cell death. If other proteases are required for Bid

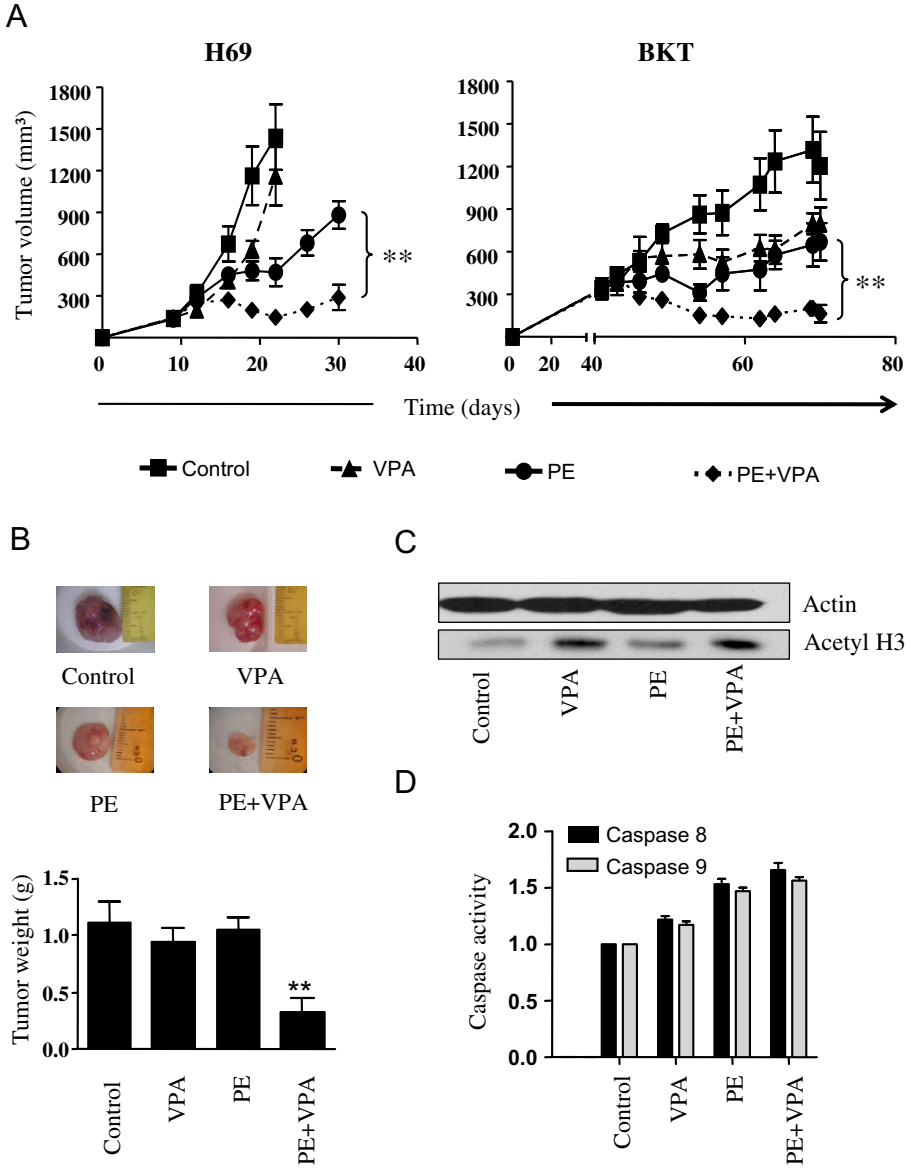

Fig. 5 – VPA prevents tumor growth in combination with cisplatin and etoposide in SCLC. Two human small cell lung cancer cell lines (H69 and BKT) were injected subcutaneously in SCID mice in the presence of 50% of matrigel. Groups of at least 6 mice were tested in each experimental condition. When tumors reached a volume of 300–400 mm^3, mice were administered with daily intraperitoneal injections of VPA (400 mg/kg/day) or PBS as control. Three days after the first VPA administration (at day 9 and day 41 for H69 and BKT, respectively), intraperitoneal injections of cisplatin (3 mg/kg) and etoposide (40 mg/kg) were performed (at days 12, 16, 22, and days 44, 49, 69 for H69 and BKT, respectively). The tumor volume (in mm^3) was calculated at regular intervals of time (in days, panel A). At the end of the experiment, H69 tumors were harvested and weighted (panel B). For ethical reasons, VPA-treated and control tumors had to be harvested earlier than PE and PE + VPA treated tumors, after reaching the maximal volume threshold. Tumor cell lysates were analyzed by western blot with indicated antibodies (panel C) and for caspase -8 and -9 activities (panel D). Results are presented as means and standard deviations (±SD) of 3 experiments on 3 different tumors. ** denotes the statistical significant difference according to the Student's *t* test $p < 0.01$.

cleavage as well as Bax cleavage, caspases therefore appear to be required to initiate the upstream process. Expression of the anti-apoptotic protein Bcl-X_L is drastically reduced while Bcl2 levels remain relatively constant. It is noteworthy that other HDAC inhibitors like FR901228 and panobinostat inhibit Bcl2 in SCLC cell lines indicating that slightly different mechanisms may be involved.[18,39] Since resistance to cisplatin of SCLC cells has been correlated to downregulation of Bcl2,[44] this observation may be particularly important for the design of efficient therapeutic regimens which would therefore include VPA rather than FR901228 or panobinostat.

In addition to inhibition of Bcl-xL and cleavage of Bid, VPA also stimulates translocation of Bax from the cytoplasm to the mitochondria (Fig. 3). Bax is known to interact with the

anti-apoptotic Bcl-xL protein and dissociation of the complex activates apoptotic functions of Bax.[45,46] Interestingly, we also detected a particular 18 kDa cleaved isoform of Bax, which has been described to potently accelerate the apoptotic process.[47–50] The truncated Bax fragment does not interact with and is not inhibited by the anti-apoptotic Bcl-2 protein.[49] The amount of this cleavage product was clearly increased in presence of the combined treatment (versus VPA alone) and may thus be particularly important in explaining the augmented effects seen with the combination. Since the levels of t-Bid and cytochrome c are similar upon treatment with VPA and PE + VPA and because Bax cleavage is upstream of cytochrome c release, it is possible that cell death is also induced by the release of other mitochondrial apoptogenic proteins such as AIF or Endonuclease G.

Probably the most straightforward effect of VPA is the induction of hyperacetylation of histone proteins (Fig. 3), a process leading to chromatin decondensation and transcriptional gene activation.[51,52] An open conformation of chromatin may also favor access of cisplatin or etoposide to the DNA and increase their damaging activity.[53,54] Consistent with this model, the p21 cyclin-dependent kinase (CDK) inhibitor known to be activated in response to DNA damage is strongly induced by VPA (Fig. 3). Extensive phosphorylation of H2AX reveals that DNA double strand breaks occur in the presence of cisplatin + etopiside but also in presence of VPA alone (Fig. 3). Although our results support the link between HDACs inhibition, histone acetylation and modulation of apoptotic gene transcription, VPA as HDAC class I inhibitor, may induce acetylation of non-histone pro and anti-apoptotic substrates. For example, Ku70 inhibit apoptosis by sequestering Bax from the mitochondria.[55] When acetylated, it releases Bax, allowing it to translocate to the mitochondria and trigger cytochrome c release. Ku70 is targeted for deacetylation by class I/II HDAC and class III/sirtuin deacetylases.

We have observed that VPA induces reactive oxygen species which may trigger DNA damage in SCLC cells. Although the free radical scavenger *N*-acetylcysteine was not sufficient to inhibit apoptosis (data not shown), this mechanism provides a rationale to the synergism between VPA and DNA intercalating agents such as epirubicin or doxorubicin upon induction of apoptosis in cancer cell lines.[56,57] Transcriptome profiling (Fig. 4) shows that VPA modulates expression of a broad list of genes. As expected, some of them code for proteins involved in apoptosis: Bcl-xL (BCL2 like 1), Puma (BCL2 binding component 3) and upstream signaling receptors (TNFRSF12A and TNFRSF19). Other genes such as frizzled receptor proteins and HEY1 (hairy/enhancer-of-split related with YRPW motif 1) regulate cell cycle progression and differentiation. In particular, the Notch signaling pathway maintains stem cells through transcriptional activation of HES/HEY family members to repress tissue-specific transcription factors. Consistent with our data, another member of this family, HES1, has previously been shown to be activated by VPA.[14] It is also noteworthy that the gene encoding Na^+/K^+ ATPase (ATPase, Class I, type 8B, member 2) known to be involved in resistance to platiniums (supplementary file 2 and reference[37]) is activated by VPA (supplementary file 1). Activity of Na^+/K^+ ATPase is associated with increased intracellular accumulation and/or efficacy of cisplatin. Whether Na^+/K^+ ATPase affects cisplatin cellular uptake directly or through alteration of intracellular pH is still unclear. The relevance of this gene in the synergism between VPA and cisplatin definitely merits further investigation.

Together, our data are thus consistent with the hypothesis that VPA acts through HDAC inhibition, chromatin decompaction and DNA damage. Systemic treatment with etoposide plus a platinum agent has been recommended as the standard first-line therapy for SCLC since the 1980s.[58] Unfortunately, recent clinical trials failed to show superiority of newer regimens over etoposide and cisplatin.[59] Empiric combinations of these two compounds and other cytotoxics have resulted in heightened toxicity, but no increase in efficacy compared to etoposide and cisplatin alone. Improved understanding of SCLC biology has underscored the importance of the epigenome in cancer progression and opens prospects in novel therapeutics. In contrast to *in vitro* results (Fig. 1), we observed that VPA alone at 1 mM did not have the same efficacy *in vivo* as a chemotherapeutic agent (Fig. 5). However we show here that a combination of VPA with a standard first-line regimen may improve the response to treatment and perhaps ultimately improve the outcome of SCLC patients. The doses needed to induce apoptosis *in vitro* were approximately 1 mM, a dose that can be achieved clinically in patients receiving VPA plus chemotherapy.[60] The combined treatment appears to be well tolerated in SCID mice, with no significant weight loss or incidence of death from other causes. VPA has been combined with a series of compounds including cisplatin and other topoisomerase inhibitors (epirubicin) generating acceptable side-effects.[60,61] Our ongoing clinical trial of second-line treatment of SCLC with doxorubicin, cyclophosphamide and vindesine will further assess the combination of VPA with chemotherapy in SCLC patients (protocol 01081 at http://www.elcwp.org/). Based on the very promising and original data of the present study, the potential efficacy of a combination of VPA with cisplatin and etoposide as first-line treatment of SCLC merits to be tested in a well designed prospective clinical trial.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.03.021.

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