

Effects of the histone deacetylase inhibitor valproic acid on the sensitivity of anaplastic thyroid cancer cell lines to imatinib

MARIA GRAZIELLA CATALANO¹, MARIATERESA PUGLIESE¹, ROBERTA POLI¹, ORNELLA BOSCO¹,
RAFFAELLO BERTIERI², NICOLETTA FORTUNATI³ and GIUSEPPE BOCCUZZI^{1,3}

¹Department of Clinical Pathophysiology, University of Turin, Via Genova 3, 10126 Turin,

²Medical Department, Novartis Farma, Largo Umberto Boccioni, 1, 21040 Origgio (Va),

³Oncological Endocrinology, ASO San Giovanni Battista, Via Genova 3, 10126 Turin, Italy

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Abstract. New therapeutic approaches are mandatory for anaplastic thyroid cancer. We investigated the ability of a new combined treatment using valproic acid (VPA), the only clinically available histone deacetylase inhibitor, and the tyrosine-kinase inhibitor imatinib mesylate to control the cell growth of anaplastic thyroid cancer cell lines. We showed that treatment with imatinib alone is unable to affect the cell growth of anaplastic thyroid cancer cells, whereas in ARO cells, the combined treatment resulted in a cytostatic effect, with clinically achievable doses of imatinib and VPA. The effect is mediated by G₁ growth arrest, acting through p21 expression and the impairment of AKT phosphorylation.

Introduction

Anaplastic thyroid cancer (ATC) is one of the most aggressive malignancies in humans. Multi-disciplinary treatments including radio- and chemotherapy poorly control the progression of this disease and patients rarely survive >1 year after initial diagnosis (1). Novel strategies to control this lethal malignancy are therefore mandatory (2,3). Imatinib mesylate is a molecular target agent that suppresses signal transduction pathways mediated through c-Abl, c-kit, and platelet-derived growth factor receptors (PDGFRs) (4). Imatinib is already used to treat patients with chronic myeloid leukaemia (CML) over-expressing Bcr-Abl mutant proteins (5) and those with gastrointestinal stromal tumours (GIST) harboring active forms of c-kit somatic mutations (6). Moreover, preclinical studies suggested a potential antitumour activity of imatinib in other solid tumours, such as ovarian cancer (7), prostate cancer (8), neuroblastoma (9)

and osteosarcoma (10). Only a few reports on the effect of imatinib on ATC cells are currently available, with contradictory results (11-13). Podtchecko *et al* (11) demonstrated that imatinib is a potential anticancer drug against undifferentiated thyroid carcinoma. However, achieving the necessary doses of imatinib which would have cytotoxic effects on ATC cells in patients is difficult. Moreover, even if the doses were achieved by higher dosing, it would result in dose-limiting toxicity (12). Thus, imatinib mesylate monotherapy appears to be ineffective in suppressing human ATC cell growth *in vitro*.

Reinforcement of the killing activity of drugs targeting DNA (2,14,15) and tubulin (3) has been reported for histone deacetylase inhibitors (HDIs), a potent class of antineoplastic agents. These drugs induce differentiation, growth arrest and apoptosis of transformed cells (16-18). Several HDIs such as SAHA (19), LAQ824 (20), MS-275 (21) and valproic acid (22) enhance imatinib-induced growth arrest and apoptosis in imatinib-sensitive and -resistant leukaemic cell lines.

We investigated the ability and underlying mechanisms of a new combined treatment using valproic acid (VPA), the only clinically available HDI, and imatinib mesylate to regulate the cell growth of ATC cell lines.

Materials and methods

Drugs, cell lines and culture conditions. Valproic acid was purchased from Sigma-Aldrich and imatinib mesylate was kindly provided by Novartis Pharma AG (Basel, Switzerland).

Anaplastic thyroid carcinoma cell lines (CAL-62 and 8305C) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), while ARO cells were a kind gift from Professor Paola Cassoni (Pathology Service, Department of Oncology, University of Turin). CAL-62 cells were routinely maintained in DMEM-F12 (Invitrogen, Groningen, The Netherlands) supplemented with 10% heat-inactivated FCS (Euroclone, Wetherby, West York, UK). 8305C cells were maintained in Eagle's essential minimal medium (E-MEM) supplemented with 1% non-essential amino acids (Sigma) and 10% heat-inactivated FCS. The ARO cell line was maintained in RPMI-1640 (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated FCS.

Correspondence to: Professor Giuseppe Boccuzzi, Department of Clinical Pathophysiology, Via Genova 3, 10126 Torino, Italy
E-mail: giuseppe.boccuzzi@unito.it

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Table I. Primer sequences for real-time PCR.

	Forward	Reverse
ABL	5'-GCGTGAGAGTGAGAGCAGTCC-3'	5'-TGGATAATGGAGCGTGGTGATGAG-3'
c-kit	5'-TTCTCTGCGTTCTGCTCCTACTG-3'	5'-GGATGGATGGTGGAGACGGTTC-3'
PDGFR- β	5'-AATCCACAGCCCCGAGCAG-3'	5'-TAGTCCACCAGGTCTCCGTAGC-3'
AKT	5'-GCAAGGGCACTTTCGGCAAG-3'	5'-AGGCGGTTCGTGGGTCTGG-3'
p21	5'-ACCTGTCACTGTCTTGTACCCCTG-3'	5'-GGCGTTTGGAGTGTAGAAATCTG-3'
p27	5'-AGCAGACGCCCAAGAAGCC-3'	5'-GGATGTCCATTCCATGAAGTCAGC-3'
β -actin	5'-GCGAGAAGATGACCCAGATC-3'	5'-GGATAGCACAGCCTGGATAG-3'
β 2-microglobulin	5'-AGATGAGTATGCCTGCCGTGTG-3'	5'-TCAAACCTCCATGATGCTGCTTAC-3'
Ribosomal protein L13a	5'-GCAAGCGGATGAACACCAACC-3'	5'-TTGAGGGCAGCAGGAACCAC-3'

Cell viability and cytotoxicity. To assess the effect of VPA on imatinib antitumour activity, cells were seeded at 1×10^3 /well in 96-well plates (Corning, New York, NY, USA) in culture medium added with 10% heat-inactivated FCS. After 48 h, cells were exposed to 0.7 mM VPA for 24 h before the addition of imatinib (1-10 μ M). After a further 72-h incubation, cell viability was tested using the Cell Proliferation Reagent WST-1 (Roche Applied Science, Penzberg, Germany), following the manufacturer's instructions. Four replicate wells were used to determine each data point. Three response parameters were calculated for each cell line: the median growth inhibition (GI₅₀), total growth inhibition (TGI) and median lethal concentration (LC₅₀). GI₅₀ defines the concentration of the compound that inhibits 50% net cell growth, the TGI value is the concentration leading to total inhibition and LC₅₀ is the lethal dose of the drug for 50% net of cells. The dose of 0.7 mM VPA has been selected because it corresponds to plasma levels in patients treated for epilepsy.

Concerning imatinib doses, the higher concentration used in our study (10 μ M) is just beyond the mean plasma concentration (7.8 μ M) reached in CML patients treated with 600 mg of imatinib once daily in a phase II clinical study (23).

Apoptosis detection

Cell death detection ELISA. For apoptosis studies, 1×10^3 cells/well were seeded in 96-well plates and treated with VPA and imatinib (5 and 8 μ M), using the same schedule as for the antitumour activity. After different treatments, apoptosis was evaluated using cell death detection ELISA Plus (Roche Applied Science) following the manufacturer's instructions. Apoptosis was expressed as an enrichment factor, and calculated as a fraction of the absorbance of treated cells versus untreated controls.

Caspase activity assay. Cells (5×10^5) were seeded in 75 cm² flasks and exposed to VPA and imatinib as above. After drug treatments, caspase 3 was determined using a colorimetric assay kit (R&D Systems Inc., Minneapolis, MN, USA). Briefly, cells were lysed and incubated with the colorimetric substrate DEVD-pNA for 2 h at 37°C. After incubation, the chromophore was quantified spectrophotometrically at 405 nm.

Cell cycle analysis. Cells were treated with 0.7 mM VPA for 24 h, followed by treatment with 5 or 8 μ M imatinib for 48 h.

After treatment, cells were collected, fixed in 70% ethanol for 30 min on ice and incubated in propidium iodide solution (20 μ g/ml propidium iodide and 0.2 mg/ml RNaseA in PBS) for 1 h at room temperature. The cell population was analyzed by flow cytometry.

Gene expression evaluation with real-time PCR

RNA extraction. Cells (2×10^5 /well) were seeded in 6-well plates and treated as described above. Total RNA was extracted using TRIzol reagent (Invitrogen Ltd., Paisley, UK). DNase was added to remove genomic DNA residuals.

cDNA synthesis and PCR amplification for Abl, c-kit, PDGFR- β , AKT, p21 and p27. Total RNA (1 μ g) was reverse-transcribed with iScript cDNA synthesis kit (BioRad Laboratories, Inc.), following the manufacturer's protocol.

Primers were designed using Beacon Designer 5.0 software according to parameters outlined in the BioRad iCycler manual. Primer specificity was confirmed by BLAST analysis. The primer sequences are listed in Table I. Actin, β 2-microglobulin and ribosomal protein L13a were used as housekeeping genes.

Real-time PCR was performed using the BioRad iQ iCycler detection system (BioRad Laboratories, Inc.) with SYBR-Green as a fluorophore. The total reaction volume was 25 μ l and included 12.5 μ l iQ SYBR-Green Supermix (BioRad Laboratories, Inc.), 1 μ l of 10 μ M primer and 5 μ l of previously reverse-transcribed cDNA template. Conditions of RT-PCR included denaturation at 95°C for 5 min and 40 cycles of amplification (95°C for 15 sec and 60°C for 30 sec). A melt curve analysis was performed following every run to ensure a single product of amplification for every reaction. Reactions were carried out at least in triplicate for each sample. Relative expression levels were obtained using the GeneEx program (BioRad), after geometrical normalization against the three different housekeeping genes.

Western blot analysis. Cells (1×10^6) were seeded in 75 cm² flasks and treated as described for cell cycle analysis. After treatment, the cells were lysed in RIPA buffer (PBS, pH 7.4, 1% Nonidet P40, 0.1% SDS and 0.5% sodium deoxycholate) added with 100 μ g/ml PMSF, 30 μ l/ml aprotinin, 100 mM NaVO₄ and 0.7 VPA when required to maintain acetylation. The cells were extracted at 4°C for 30 min and centrifuged at

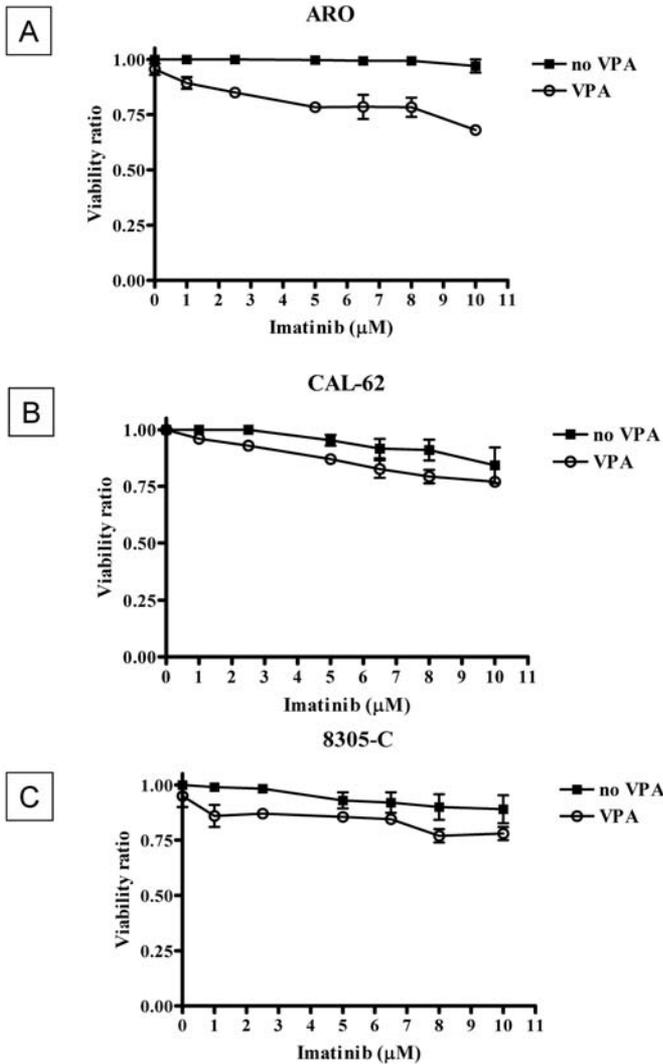


Figure 1. Sensitizing effect of VPA to imatinib in anaplastic thyroid cancer cells. (A) ARO, (B) CAL-62 and (C) 8305C cells were incubated for 24 h with 0.7 mM VPA before the addition of imatinib (1-10 μM). After a further 72-h incubation, cell viability was determined by the WST-1 method, and expressed as the ratio between treated cells and untreated controls (without imatinib or VPA).

4°C for 20 min at 15,000 x g. Aliquots of 50 μg of total protein were loaded to each lane of 12% (for p21 and actin) or 8% (for Akt and P-Akt) SDS-polyacrylamide gels and proteins were electrically transferred to PVDF membranes. Membranes were probed with the primary antibodies of polyclonal anti-p21 (sc-397, 1:200 dilution, Santa Cruz Biotechnology Inc.,

Santa Cruz, CA, USA); monoclonal anti-actin (clone AC-40, 1:1000 dilution, Sigma); polyclonal anti-phospho-Akt (Ser473; 1:1000 dilution, Cell Signaling Technology Inc., Danvers, MA, USA) and polyclonal anti-Akt (1:2000 dilution, Cell Signaling Technology Inc.). The secondary antibodies were diluted at 1:10000. Western blot analysis against actin was used to control protein loading.

Proteins were detected with the Pierce Super Signal chemiluminescent substrate following the manufacturer's instructions. Bands were photographed using the PC software 1D Kodak Digital Science.

Statistical analysis. Data are expressed throughout as means ± SEM, calculated from at least three different experiments. In viability experiments, the statistical comparison between cells treated with VPA plus imatinib, and VPA alone was performed with the Mann-Whitney U test. A comparison between the groups was performed with analysis of variance (one-way ANOVA) and the threshold of significance was calculated with the Bonferroni test. Statistical significance was set at P<0.05.

Results

Effect of VPA and imatinib on cell viability. As shown in Fig. 1 and Table II, treatment with imatinib alone up to the dose of 10 μM had no effect on the growth of ATC cell lines (CAL-62, ARO and 8305C). Pre-treatment with 0.7 mM VPA significantly increased the imatinib effect in ARO cells (P<0.05), whereas no potentiation was observed in CAL-62 and 8305C cells.

Effect of VPA and imatinib on cell cycle progression and apoptosis induction. To get insight into the mechanisms underlying the antitumour effect of the combined treatment with VPA and imatinib in ARO cells, we evaluated the effect of the two drugs on cell cycle progression and apoptosis induction. As shown in Table III, neither VPA nor imatinib (up to the dose of 8 μM) had any effect on cell cycle progression (Table III) and apoptosis induction (Fig. 2) of ATC cells, when used alone. Pre-treatment with VPA resulted in G₁ cell cycle arrest in ARO cells, a total of 58.8% of VPA + 5 μM imatinib-treated cells were in G₁ compared with 48.1% of cells cultured with 5 μM imatinib alone (p<0.01) and a total of 66.0% of VPA + 8 μM imatinib-treated cells were in G₁ compared with 58.8% of cells cultured with 8 μM imatinib alone (p<0.001).

After combined treatment, we observed no apoptosis induction at any concentration used, nor were nucleosome

Table II. GI₅₀, TGI and LC₅₀ for imatinib (μM) after treatment with imatinib alone or combined with VPA.

	CAL-62		ARO		8305C	
	Imatinib	Imatinib + VPA	Imatinib	Imatinib + VPA	Imatinib	Imatinib + VPA
GI ₅₀	≥10	≥10	≥10	5	≥10	≥10
TGI	≥10	≥10	≥10	≥10	≥10	≥10
LC ₅₀	≥10	≥10	≥10	≥10	≥10	≥10

Table III. Effect of VPA and imatinib on cell cycle progression of ATC cell lines.

	G ₁	S	G ₂ /M
ARO			
Basal	52.8±2.3	38.1±1.5	9.2±0.9
0.7 mM VPA	49.7±2.2	40.3±2	10.0±0.3
5 μM imatinib	48.1±1.6	43.6±1.3	8.3±0.4
5 μM imatinib + VPA	58.8±1.3 (**)	36.3±1.4	5.0±0.2
8 μM imatinib	50.5±1.5	43.4±1.7	6.2±0.5
8 μM imatinib + VPA	66.0±1.0 (***)	30.8±1.4	3.2±0.5
CAL-62			
Basal	45.8±0.9	24.3±4.2	29.9±5.1
0.7 mM VPA	45.5±0.5	24.3±5.1	30.2±5.6
5 μM imatinib	47.2±0.4	24.1±4.5	29.6±5.0
5 μM imatinib + VPA	46.4±2.2	23.1±4.6	29.4±6.8
8 μM imatinib	45.5±4.2	24.8±3.5	29.6±7.8
8 μM imatinib + VPA	48.6±0.4	23.4±3.1	24.8±9.9
8305C			
Basal	46.1±4	44.0±2.7	9.8±1.5
0.7 mM VPA	42.3±1.5	45.6±1.7	12.0±0.9
5 μM imatinib	44.9±6	46.2±3.5	8.9±2.5
5 μM imatinib + VPA	44.0±2.3	45.7±1.1	10.3±1.2
8 μM imatinib	45.9±1.7	44.6±1.0	9.6±0.7
8 μM imatinib + VPA	46.4±1.0	42.2±1.7	11.3±1.0

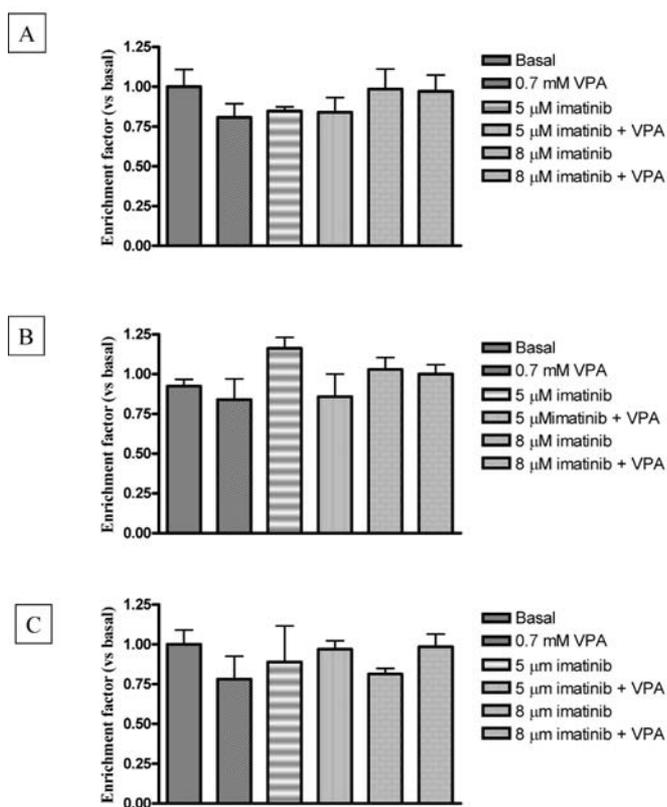


Figure 2. Effect of VPA and imatinib on apoptosis induction. ELISA detection of DNA-histone complexes. (A) ARO, (B) CAL-62 and (C) 8305C were treated with VPA (0.7 mM) for 24 h, followed by combined treatment with VPA and imatinib (5 and 8 μM) for a further 72 h. The enrichment factor was calculated as the ratio between the absorbance measurements of treated cells and the basal value (exposed to neither VPA nor imatinib).

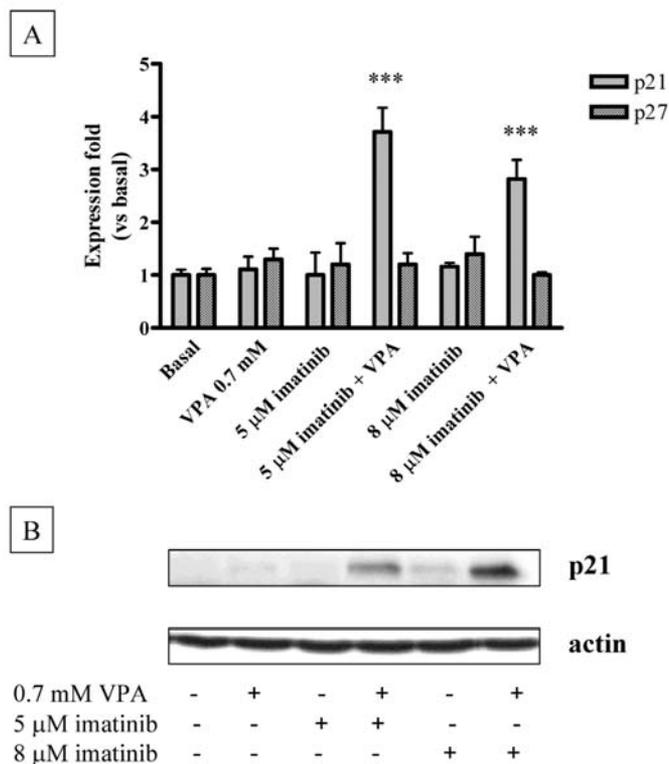


Figure 3. Effect of VPA and imatinib on the expression of p21 and p27. (A) mRNA expression changes of p21 and p27 in ARO cells after VPA and imatinib treatment. mRNA levels were determined with real-time PCR and normalized against actin, β2-microglobulin and protein L13a. Results are expressed as fold induction compared to untreated cells (basal). Significance vs basal, ***P<0.001. (B) p21 protein expression assessed by Western blot analysis with an anti-p21 antibody. Equal loading and transfer were verified by reprobing the membranes with an anti-actin antibody.

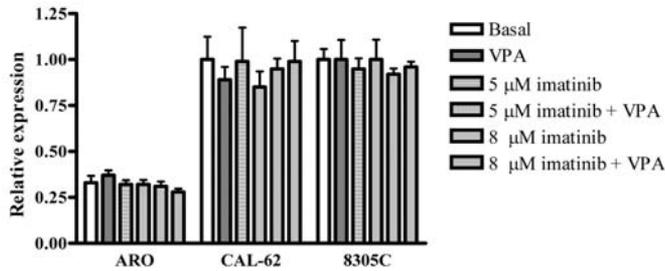


Figure 4. Effect of VPA and imatinib on the expression of Abl mRNA levels for c-Abl were determined with real-time PCR and normalized against actin, β 2-microglobulin and protein L13a. Results are expressed as fold induction compared to the higher mRNA expression level (CAL-62 cells exposed to neither VPA nor imatinib).

formation (Fig. 2) and caspase activation (data not shown) detected.

Effect of VPA and imatinib on p21 and p27 gene expression. To clarify the mechanism of VPA plus imatinib-induced G₁ cell cycle arrest in ARO cells, we examined the effect of the drugs on p21 and p27 gene expression involved in cell cycle control. p21 was up-regulated at the mRNA (Fig. 3A) and protein levels (Fig. 3B). Conversely, the mRNA expression of p27 was not changed upon treatment (Fig. 3A).

Effect of VPA and imatinib on the expression of c-Abl, c-kit, PDGFR and AKT. Neither c-kit nor PDGFR were expressed in any of the cells (data not shown). c-Abl mRNA expression was observed in the ATC cell lines used for the study. Notably, in ARO cells the expression was only one third versus CAL-62 and 8305C cells. VPA plus imatinib treatment had no effect on the modulation of the gene expression (Fig. 4). Regarding AKT expression in ARO cells, drug treatments determined the inhibition of AKT phosphorylation, without any effect on the amount of total AKT (Fig. 5B) and on the mRNA expression level (Fig. 5A). No modulation of AKT expression and phosphorylation was observed in either CAL-62 or 8305C cells (data not shown).

Discussion

In the present study, we showed that treatment with imatinib alone is unable to affect the cell growth of anaplastic thyroid cancer cells, which is in line with previous data by Dziba *et al* (12). Other authors (11) have previously reported that only cell lines harbouring mutated p53 are sensitive to the drug. However, although the cell lines used in this study are p53-mutated, they are resistant to imatinib when used alone up to the dose of 10 μ M.

We demonstrated that a combined treatment of valproic acid and imatinib at concentrations clinically achievable for the two drugs, affects cell viability of the ARO cells, where a cytostatic effect is observed. The underlying mechanism is cell cycle arrest in G₁, whereas no apoptosis induction is observable. Cell cycle arrest is the result of the activation of

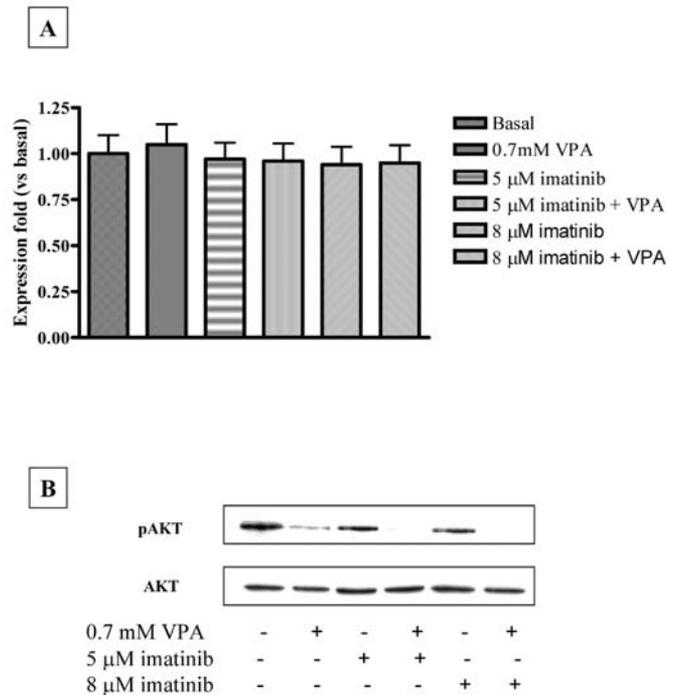


Figure 5. Effect of VPA and imatinib on AKT expression. (A) mRNA expression changes of AKT in ARO cells after VPA and imatinib treatment. mRNA levels were determined with real-time PCR and normalized against actin, β 2-microglobulin and protein L13a. Results are expressed as fold induction compared to the untreated control group. Phosphorylated AKT protein was analyzed by Western blot analysis. pAKT levels were determined using an antibody against phosphorylated AKT. Equal loading and transfer were verified by reprobing the membranes with an anti-AKT antibody.

p21 expression. HDIs induce cell-cycle arrest at G₁/S (24) and this is most often associated with the p53-independent induction of CDKN1A, encoding p21. We demonstrated elsewhere (25) that VPA is able to induce p21 expression in poorly differentiated thyroid cancer cell lines, but no induction was observed when the drug was administered alone in anaplastic thyroid cancer cells.

The tyrosine kinase inhibitor imatinib selectively suppresses the activity of Abl, PDGFR and c-kit. In our cell lines, PDGFR and c-kit are not expressed and neither the drugs used alone nor the combined treatment are able to induce their expression. In contrast, RT-PCR revealed the presence of c-Abl mRNA in the cell lines we used. Abl affects cell proliferation phosphorylating many targets, including AKT (26). AKT regulates the process of cell survival by multiple substrates including cyclin D, Myc, p27 and p21 (27). In detail, AKT phosphorylates the cell cycle inhibitor p21 at two different residues, resulting in the cytoplasmic localization of p21 and consequent promotion of the cell cycle (28). We showed that treatment with imatinib and valproic acid inhibits AKT phosphorylation. The question remains as to why valproic acid potentiates the action of imatinib only in ARO cells that express a lower level of Abl. Abl expression in ARO cells is one third lower than in CAL-62 and 8305C cells. VPA (22) as well as other HDIs, such as SAHA (19) and, more recently pivanex (29), have

been reported to decrease the mRNA and protein levels of Bcr-Abl in myeloid leukaemia cells, enhancing imatinib-induced growth arrest. However, to our knowledge, the different treatments are not able to modify the expression of Abl in any of the cell lines. We suggest that valproic acid acts downstream of Abl expression, directly working against the phosphorylation of AKT induced by Abl. The lack of effects on the other cell lines may be dependent, at least partially, on the overexpression of Abl. In this case, the phosphorylating activity of Abl on AKT may overwhelm the inhibitory effect of the combined treatment.

Therefore, cell cycle arrest observed in ARO cells after treatment with imatinib and valproic acid is the result of the induction of p21 expression and inhibition of AKT phosphorylation. p21 is a well-known cell cycle inhibitor and AKT phosphorylation in turn regulates the G₁/S transition by controlling the cell cycle inhibitor p21 at the post-translational level. AKT phosphorylates p21 at the residues Thr145 and Ser146. Thr145 residue phosphorylation results in the cytoplasmic localization of p21 promoting the cell cycle, whereas Ser146 site phosphorylation enhances the stability of the protein and increases the assembly of the cyclin D-CDK4 transition complex.

VPA was shown to be able to interfere with the AKT pathway, together with its ability to potentiate the activity of other anti-neoplastic agents with different mechanisms (2,3) which defines the multifaceted activities of this sole clinically available HDI.

In conclusion, although a phase II clinical trial (ClinicalTrials.gov Identifier: NCT00115739) using imatinib for the treatment of anaplastic thyroid cancer continues, the data from our investigations appear to discourage the monotherapeutic use of imatinib in this type of tumour. A combined treatment with imatinib and an HDI inhibitor such as valproic acid may be more effective. However, such treatment should be assessed in each case, as we observed the anti-tumour activity in only a single cell line.

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