Valproic acid enhances tubulin acetylation and apoptotic activity of paclitaxel on anaplastic thyroid cancer cell lines

Maria G Catalano¹, Roberta Poli², Mariateresa Pugliese², Nicoletta Fortunati² and Giuseppe Boccuzzi¹,²

¹Department of Clinical Pathophysiology, University of Turin, Via Genova, 3, 10126 Turin, Italy
²Oncological Endocrinology, ASO San Giovanni Battista, C.so Bramante 88, 10126 Turin, Italy
(Correspondence should be addressed to G Boccuzzi; Email: giuseppe.boccuzzi@unito.it)

Abstract

The introduction of paclitaxel into multimodal therapy for anaplastic thyroid carcinoma has failed to improve overall survival. Toxicity rules out the high doses required, especially in older patients. The search for strategies to enhance paclitaxel antineoplastic activity and reduce its side effects is thus advisable. The study aimed to determine whether the histone deacetylase (HDAC) inhibitor valproic acid (VPA) improves the anticancer action of paclitaxel and elucidate the mechanisms underlying the effects of combined treatment. We examined the effect of VPA on the sensitivity to paclitaxel of two anaplastic thyroid carcinoma cell lines (CAL-62 and ARO), and the ability of the drug to determine tubulin acetylation and enhance paclitaxel-induced acetylation. The addition of as little as 0.7 mM VPA to paclitaxel enhances both cytostatic and cytotoxic effects of paclitaxel alone. Increased apoptosis explains the enhancement of the cytotoxic effect. The mechanism underlying this effect is through inhibition of HDAC6 activity, which leads to tubulin hyper-acetylation. The results suggest a mechanistic link between HDAC6 inhibition, tubulin acetylation, and the VPA-induced enhancement of paclitaxel effects, and provide the rationale for designing future combination therapies.

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Introduction

Anaplastic thyroid cancer (ATC) is one of the most lethal malignancies, with a rapidly fatal clinical course (Pasieka 2003). Management of ATC has not yet been standardized; multimodal treatments including surgical resection associated with radiotherapy and combination chemotherapy are currently used (Kebebew et al. 2005), but efforts to improve survival have been disappointing (Brignardello et al. 2007).

The introduction of paclitaxel, which had been reported to produce a response rate of about 50% (Ain et al. 2000), has not modified overall survival, because neurological and hematological side effects limit the use of this drug, especially in older patients; incidence and severity of toxicity appear to be related to both peak concentration and total duration of treatment (Markman 2003). Paclitaxel is a microtubule-targeting drug, and it is now recognized that binding the drug to microtubules, by powerfully stabilizing their dynamics without increasing the polymerization mass, is sufficient to lead to mitotic arrest and apoptosis (Jordan et al. 1996). Indeed, all stages of mitosis require highly dynamic microtubules in the spindle, ensuring timely and precise chromosome movement. Strategies or drugs that enhance paclitaxel effect on microtubule stability might improve the drug antineoplastic activity, reducing the incidence of the severe side effects associated with the use of high doses. Indeed, the identification of drugs able to increase antitumoral effects of paclitaxel is now under investigation (Yeung et al. 2000, Vivat-Hannah et al. 2001, Copland et al. 2006).

Histone deacetylase inhibitors (HDI) are a class of potent antineoplastic agents that induce differentiation, growth arrest, and apoptosis of transformed cells (Marks et al. 2001, Vigushin & Coombes 2002, Rosato & Grant 2003). Moreover, reinforcement of the killing activity of drugs targeting DNA has recently been reported (Marchion et al. 2004, 2005, Catalano et al. 2006) for HDI, but the mechanism underlying this effect is poorly understood. The study aimed to determine whether the histone deacetylase (HDAC) inhibitor valproic acid (VPA) improves the anticancer action of paclitaxel and elucidate the mechanisms underlying the effects of combined treatment. We examined the effect of VPA on the sensitivity to paclitaxel of two anaplastic thyroid carcinoma cell lines (CAL-62 and ARO), and the ability of the drug to determine tubulin acetylation and enhance paclitaxel-induced acetylation. The addition of as little as 0.7 mM VPA to paclitaxel enhances both cytostatic and cytotoxic effects of paclitaxel alone. Increased apoptosis explains the enhancement of the cytotoxic effect. The mechanism underlying this effect is through inhibition of HDAC6 activity, which leads to tubulin hyper-acetylation. The results suggest a mechanistic link between HDAC6 inhibition, tubulin acetylation, and the VPA-induced enhancement of paclitaxel effects, and provide the rationale for designing future combination therapies.
understood. Besides, histones and other non-histone proteins such as transcription factors p53, E2F1, and α-Jun, α-tubulin is also a substrate of histone deacetylases (HDACs; Zhang et al. 2003). Notably, it has been demonstrated that the state of tubulin acetylation is linked to microtubule dynamics and has a role in regulating microtubule stability (Matsuyama et al. 2002).

The aim of this work is to explore whether valproic acid (VPA), a widely used anticonvulsant drug which is a class I/II HDI (Gottlicher et al. 2001, Phiel et al. 2001), by inhibiting tubulin deacetylation, might enhance the effects of microtubule-targeting drugs. We report elsewhere that, at concentrations reached in the serum of patients treated for epilepsy, VPA promotes iodine uptake (Fortunati et al. 2004) and controls cell growth (Catalano et al. 2005) in poorly differentiated thyroid cancer cells. Here, we show that, in ATC cell lines, VPA clearly enhances paclitaxel effect on cell viability and apoptosis, by regulating the tubulin acetylation state.

Materials and methods

Cell lines and culture conditions

Anaplastic thyroid carcinoma (CAL-62) cell line was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Anaplastic thyroid carcinoma (ARO) cells were a kind gift from Prof. Paola Cassoni (Pathology Service, Department of Oncology, University of Turin, Turin, Italy). CAL-62 cells were routinely maintained in 25 cm² flasks at 37 °C, in 5% CO₂ and 95% humidity, with 100 IU/ml penicillin and 100 µg/ml streptomycin added, in DMEM-F12 (Invitrogen) supplemented with 10% heat-inactivated FCS (fetal calf serum; Euroclone, Wetherby, West York, UK). ARO cell line was maintained in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated FCS.

Antitumor activity

To evaluate the effect of VPA on paclitaxel antitumor activity, CAL-62 and ARO cells were seeded at 1 × 10⁵ cells/well in 96-well plates (Corning, New York, NY, USA) in culture medium plus 10% heat-inactivated FCS (Sigma). After 48 h, cells were exposed to 0.7 mM VPA for 24 h before the addition of paclitaxel (0.001–1 µM). After a further 72-h incubation, cell viability was assessed using the Cell Proliferation Reagent WST-1 (Roche Applied Science), following the manufacturer’s instructions. Four replicate wells were used to determine each data point. Three response parameters, median growth inhibition (GI₅₀), total GI (TGI), and median lethal concentration (LC₅₀), were calculated for each cell line. The GI₅₀ corresponds to the concentration of the compound that inhibits 50% net cell growth; the TGI value is concentration of the compound leading to total inhibition; and the LC₅₀ value is the concentration of the compound leading to 50% net cell death.

The dose of 0.7 mM VPA has been chosen because it corresponds to plasma levels in patients treated for epilepsy, and it is able to inhibit histone deacetylation (Catalano et al. 2006). Moreover, a dose–response curve showed that VPA alone has no effect on cell growth up to the dose of 1.5 mM (Catalano et al. 2006).

As far as paclitaxel doses are concerned, the higher concentration used in our study (1 µM) is within the range of the clinically attainable plasma paclitaxel concentration ranging approximately from 0.045 to 5 µM for various infusion durations and dose regimens in patients (Wiernik et al. 1987).

Apoptosis detection

Cell death detection ELISA

For apoptosis studies, 1 × 10⁵ cells were seeded in 96-well plates and treated with VPA and paclitaxel, using the same schedule as for the viability assay. After different treatments, apoptosis was evaluated using Cell Death Detection ELISA PLUS (Roche Applied Science) following the manufacturer’s instructions. Apoptosis was expressed as enrichment factor, calculated as a fraction of the absorbance of treated cells versus untreated controls.

Caspase activity assay

5 × 10⁵ cells were seeded in 75 cm² flasks and exposed to VPA and paclitaxel 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.

Western blot analysis of tubulin acetylation

Western blot for acetylated α-tubulin was performed in CAL-62 cells treated with VPA, paclitaxel, or the combined treatment. In a first series of experiments, CAL-62 cells were treated with increasing doses of VPA (0.2–0.5–0.7–1–1.5 mM) for different times (ranging from 24 to 72 h). In a second series, cells were treated first with 0.7 mM VPA for 24 h and then with 0.01–0.1–1 µM paclitaxel. After treatment, cells were harvested and lysed in the presence of 200 μl radio immunoprecipitation assay (RIPA) buffer (1% NP40, 0.5% deoxycholate sodium,
0.1% SDS in PBS (pH 7.4) with 10 mg/ml phenylmethylsulphonyl fluoride, 30 μl/ml aprotinin, and 100 mM sodium orthovanadate) and incubated on ice for 30 min; cells were then centrifuged for 20 min at 15 000 g at 4 °C, and clear supernatants used. SDS-PAGE was performed on 10% gels, loading 20 μg protein/well. Separated proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membrane and probed with a mouse monoclonal anti-acetyl-α-tubulin antibody (clone 6-11B-1, 1:8000 dilution, Sigma). The membrane was then stripped and reprobed with a mouse monoclonal anti-α-tubulin antibody (clone DM1A, 1:2000 dilution, Sigma) to check protein loading. Proteins were detected with Pierce Super Signal chemiluminescent substrate following the manufacturer’s instructions. Bands were photographed and analyzed using Kodak 1D Image Analysis software.

**HDAC6 assay**

The inhibitory effect of VPA (0.5–2 mM) on HDAC6 activity was tested on a recombinant HDAC6 protein obtained by BPS Bioscience (San Diego, CA, USA) and measured with a Fluorimetric Assay kit (Biomol, Plymouth, PA, USA) following manufacturer’s instructions. Briefly, in a 96-well plate, 30 nM HDAC6 was incubated with 30 μM Fluor de Lys substrate in HDAC assay buffer in the presence of VPA at various concentrations. Reaction was performed at 30 °C for 1 h and stopped by adding 50 μl developer plus 1 mg/ml Tricostatin A (TSA). After further 15-min incubation, plate was read by a Microplate Spectrophotometer (Mithras LB940, Berthold Technologies, Germany) at $\lambda_{\text{excitation}}$ = 355 nm and $\lambda_{\text{emission}}$ = 460 nm.

**Statistical analysis**

Data are expressed throughout as means ± S.E.M., calculated from at least three different experiments. In viability experiments, statistical comparison between cells treated with VPA plus paclitaxel and VPA alone was performed with the Mann–Whitney test. Comparison between groups was performed with ANOVA (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 on paclitaxel antitumor activity**

Figure 1 shows the effect on CAL-62 and ARO cell viability exerted by paclitaxel alone or after a 24 h pretreatment with VPA. Pretreatment of the cells with 0.7 mM VPA significantly increased paclitaxel effect starting at the concentration of 0.01 μM ($P < 0.05$). In both cell lines, the effect on cell viability obtained when VPA was added to cultures was the same as that obtained with a one-tenth or smaller dose of paclitaxel alone.

As shown in Table 1, VPA improved both the cytostatic and cytotoxic activities of paclitaxel in CAL-62 and ARO cells.

**Effect of VPA on paclitaxel-mediated apoptosis**

Since our results indicated that pretreatment of CAL-62 and ARO cells with VPA sensitized cells to paclitaxel cytotoxicity, we investigated whether the same effect was detectable on apoptosis induction. As shown in Fig. 2 and as we report elsewhere (Catalano et al. 2006), 0.7 mM VPA alone had no effect on apoptosis induction in these ATC cell lines. Nevertheless, it significantly enhanced paclitaxel-

| Table 1 GI50, total growth inhibition (TGI), and LC50 for paclitaxel (μM) after treatment with paclitaxel alone or combined with valproic acid (VPA) |
|---|---|---|---|
| CAL-62 | ARO |
| Paclitaxel | Paclitaxel + VPA | Paclitaxel | Paclitaxel + VPA |
| GI50 | 1.20 | 0.27 | 0.17 | 0.01 |
| TGI | 2.50 | 0.85 | 0.81 | 0.41 |
| LC50 | 4.20 | 1.40 | 1.45 | 0.88 |
mediated apoptosis in both cell lines at all concentrations tested. The induction of apoptosis was demonstrated by quantification of nucleosome formation (Fig. 2A and B) and was confirmed by caspase 3 activation (Fig. 2C and D).

**Effect of VPA and paclitaxel on tubulin acetylation**

It has been reported that HDI may promote acetylation of non-histone proteins, and that tubulin may also undergo acetylation as a consequence of HDI action (Blagosklonny et al. 2002). As reported in Fig. 3, VPA induced tubulin acetylation, the effect already being evident starting at 0.5 mM concentration and after 24 h treatment. Any significant further increase in tubulin acetylation was observed after exposure for longer times (data not shown). Tubulin acetylation is mainly controlled by HDAC6 (Zhang et al. 2003). Here, we demonstrate that VPA, a class I/II HDI, is able to inhibit HDAC6 activity (as reported in Fig. 3C).

Moreover, 0.7 mM VPA enhanced the ability of both 0.01 and 0.1 μM paclitaxel to induce tubulin acetylation (as shown in Fig. 4). Interestingly, 0.7 mM VPA had only a slight effect on the level of tubulin acetylation induced by 1 μM paclitaxel. It may thus be suggested that tubulin acetylation has almost reached its plateau with 1 μM paclitaxel treatment, and therefore, its acetylation level cannot be significantly enhanced by further VPA.

**Discussion**

We show in this study that the combination of VPA with paclitaxel enhances paclitaxel cytostatic and cytotoxic activity in two different ATC cell lines (CAL-62 and ARO). Notably, in the presence of VPA, we obtained the same effect on cell viability with a one-tenth dose of paclitaxel when compared with the dose needed when the drug is used alone. The effect is reached with VPA at a dose of 0.7 mM, which corresponds to plasma levels in patients treated for epilepsy and is without any serious side effects.

Apoptosis induction mediates paclitaxel cytotoxicity, and here we show that VPA treatment markedly increases paclitaxel-induced apoptosis, as demonstrated by both cytoplasmatic nucleosome formation and caspase 3 activation. Suppression of microtubule dynamics is recognized as the mechanism by which paclitaxel blocks mitosis and kills tumor cells (Jordan & Wilson 2004): rapid dynamics ensures timely and accurate chromosome movement. The suppression of spindle microtubule dynamics might slow or block mitosis at the metaphase–anaphase
transition, and facilitate apoptotic cell death. The effect on microtubule dynamics is at least partially due to paclitaxel effect on tubulin acetylation (Piperno et al. 1987), an established marker of microtubule stability (Schiff & Horwitz 1980, Jordan et al. 1993, Mollinedo & Gajate 2003). Here, we show that VPA is able not only to induce tubulin acetylation, when used alone, but also to markedly enhance the acetylating activity of paclitaxel. This latter effect may be related to a more efficient binding of paclitaxel to microtubules, depending on the VPA-induced suppression of microtubule dynamics (Zhou et al. 2004). Zhang et al. (2003) reported that HDAC6 is a microtubule-associated protein capable of deacetylating α-tubulin in vitro and in vivo, and that HDI treatment leads to hyperacetylation of tubulin and microtubules. We here demonstrate that VPA is able to affect HDAC6 activity; our results being in line with those obtained by Gottlicher, which reported an IC_{50} = 2.4 mM (Gottlicher et al. 2001), whereas Gurvich et al. (2004) measured an IC_{50} > 20 mM. Our data clearly show that VPA induces tubulin acetylation and maximally potentiates the acetylating activity of paclitaxel. The capability to induce acetylation of tubulin (Blagosklonny et al. 2002) has also been documented for TSA, a HDI, whose clinical use is hampered by its high toxicity (Sandor et al. 2002).

Figure 3 Effect of VPA on tubulin acetylation. A representative experiment of the accumulation of acetylated tubulin in CAL-62, treated with 0.2–1.5 mM VPA for 24 h (A), assessed by western blotting with an anti-acetyl-tubulin antibody. Equal loading and transfer were verified by reprobing the membranes with an anti-tubulin antibody. Histogram (B): semiquantitative analysis of western blotting results. Net intensity was determined as the ratio between treated cells and untreated control. Results are expressed as means ± S.E.M.; n = 3. Effect of VPA on HDAC6 activity (C). The activity was determined in the presence of 0.5–2 mM VPA by deacetylation of fluorescence-labeled acetylated lysyl substrate. Results are expressed as means ± S.E.M.; n = 3.

Figure 4 Combined effect of VPA plus paclitaxel on tubulin acetylation. A representative experiment of the accumulation of acetylated tubulin in CAL-62 cells, treated with VPA and paclitaxel (0.01–0.1–1 μM; A), assessed by western blotting with an anti-acetyl-tubulin antibody. Equal loading and transfer were verified by reprobing the membranes with an anti-tubulin antibody. Histogram (B): semiquantitative analysis of western blotting results. Net intensity was determined as the ratio between treated cells and untreated control (basal). Results are expressed as means ± S.E.M.; n = 3. Significance versus cells not exposed to VPA: *P < 0.05; **P < 0.001.
checkpoint, contributing to enhancing paclitaxel cytotoxicity and increasing drug selectivity on tumor cells. As we report elsewhere (Catalano et al. 2006), VPA also enhances the sensitivity of the anaplastic tumor thyroid cell lines to doxorubicin, a DNA-targeted drug widely used in treating ATC; we suggest that impairment of cell-cycle checkpoints might contribute to the enhanced killing activity of the combined treatment. In line with this observation, Dowling et al. (2005) reported that TSA, by inhibiting histone deacetylation, affects the expression of genes that encode for mitotic checkpoint proteins, and hampers mitotic checkpoint surveillance and regulation of post-mitotic progression.

Although we demonstrated that the effects of valproate are through tubulin acetylation, potential effects on the membrane transporters responsible for multidrug resistance cannot be excluded. However, recent studies have shown that HDIs increase the expression of MDR1 (Tabe et al. 2006) and its product P-glycoprotein (Robey et al. 2006).

The clinical use of paclitaxel is severely hampered, especially in older patients with thyroid anaplastic carcinoma, by its several dose-dependent side effects, i.e. myelotoxicity, neuropathy, anaphylaxis, and severe hypersensitivity reactions. Mechanisms underlying paclitaxel toxic effects still remain largely unknown; however, it has to be remarked that they chiefly depend upon high-dose regimes, and that a potential cofactor for these damages is the polyoxyethylated castor oil vehicle, Cremophor EL, possibly inducing per-oxidation products affecting neurons (Mielke et al. 2006). As far as toxic effects mediated by microtubule alterations are concerned, it has been reported that they mainly depend on microtubule polymerization (Zhou et al. 2004), whereas apoptotic cell death in tumor cells already appears after the suppression of spindle microtubule dynamics (Jordan et al. 1996, Jordan & Wilson 2004), without any increase of microtubule polymer mass formation. At the peak and steady-state plasma concentrations reached by the doses of paclitaxel in current clinical use (Wierink et al. 1987), the effect on microtubule polymer mass formation (Derry et al. 1995), and thus toxicity (Jordan et al. 1996), might be prevalent. Therefore, the use of VPA, a drug that may consent to reduce the paclitaxel dose maintaining the same killing effect on tumor cells, might allow more prolonged and effective treatment. Nevertheless, on the basis of the present results, VPA-induced tubulin hyperacetylation also on non-target tissues cannot be excluded, and translational and clinical studies will ultimately determine the clinical safety of this combination therapy.

Collectively, our data suggest a mechanistic link between HDAC inhibition, tubulin acetylation, and the VPA-induced enhancement of paclitaxel effects, and might provide insight into the design of future combination therapies, which include microtubule-targeting drugs.

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