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# Suberoylanilide hydroxamic acid partly reverses resistance to paclitaxel in human ovarian cancer cell lines

Adriano Angelucci [a], Marianna Mari [b], Danilo Millimaggi [b], Ilaria Giusti [b], Gaspare Carta [c], Mauro Bologna [a], Vincenza Dolo [b,*]

[a] Department of Experimental Medicine, University of L'Aquila, Via Vetoio-Coppito 2, I-67100, L'Aquila, Italy
[b] Department of Health Sciences, University of L'Aquila, L'Aquila, Italy
[c] Department of Surgical Sciences, University of L'Aquila, L'Aquila, Italy

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ABSTRACT

*Objectives.* The purpose of this study was to determine whether the addition of the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) to paclitaxel (PTX) can sensitize PTX-resistant human ovarian cancer cell lines (CABA-PTX and IGROV-PTX) *in vitro*.

*Methods.* SAHA was studied in combination with paclitaxel in PTX-sensitive and PTX-resistant human ovarian cancer cell lines. Using cell proliferation analysis, immunofluorescence, and flow cytometric assays, we can determine whether the resistance was partly removed when the cells were treated with a combination of SAHA and PTX. Cells were also assayed for cytochrome *c* release. The levels of acetylated tubulin, β-tubulin, and HDAC6 were quantified by Western blots.

*Results.* SAHA in combination with PTX led to a more pronounced inhibition of cell growth compared with PTX alone. In addition, the combined exposure to PTX and SAHA resulted in a marked arrest in the G2/M phase of the cell cycle and in a significant increase in the percentage of apoptotic cells. The expression of acetylated tubulin was dramatically increased by exposure to the combination of PTX and SAHA. These data paralleled the findings of an increased expression of HDAC6 in the presence of PTX in PTX-resistant cell lines.

*Conclusions.* The results of this study suggest the existence of a novel resistance mechanism based upon the upregulation of HDAC6 and that the histone deacetylase inhibitor SAHA holds promise to overcome PTX resistance in ovarian cancer cell lines.



## Introduction

Dysregulation of the cell cycle machinery is an essential characteristic of various malignancies including ovarian cancer, a leading cause of gynecologic cancer mortality worldwide [1]. Management of ovarian cancer has improved significantly with the advent of more aggressive surgery and combination chemotherapy performed with paclitaxel (PTX) and carboplatin. Unfortunately, the use of PTX in ovarian cancer chemotherapy is often associated with the development of drug resistance [2,3]. Resistance to PTX involves multiple mechanisms including altered intracellular drug levels, variations in tubulin structure, altered signal transduction, and apoptotic pathways [4].

The development of cancer has been associated with epigenetic alterations such as histone deacetylation. Histone acetylation modifies specific lysine residues on histones and plays a key role in several cellular functions such as chromosome remodeling, gene transcription, and cell proliferation [5]. Chemotherapy resistance requires for its development significant changes in gene expression. Therefore, it has been suggested that epigenetic-mediated changes may be the responsible driving force for chemotherapy resistance. Epigenetic therapy with histone deacetylase inhibitors (HDACIs) holds promise to overcome resistance to chemotherapy [6]. The histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA; Zolinza/vorinostat) is capable of inducing early changes in gene expression profile [7] and is an important candidate drug in cancer therapy [8]. Of note, treatment of cancer cell lines with HDACIs has pleiotropic effects, inducing inhibition of HSP90, cell-cycle arrest, and mitochondrial-mediated apoptosis [9]. One of the best characterized non-histone HDAC-targeted protein is alpha-tubulin, and it suggests a key role of HDAC6 in the direct control of microtubule dynamic in normal and pathologic conditions [9]. HDACIs are therefore suitable candidates for combination therapy [10,11]. Recent data have shown that SAHA potentiates PTX-induced apoptosis in ovarian cancer cell lines [12].

*Abbreviations:* ELISA, enzyme-linked immunosorbent assay; BCI, Berenbaum's combination index; FCS, fetal calf serum; HDACI, histone deacetylase inhibitor; HDAC6, histone deacetylase 6; PBS, phosphate-buffered saline; PTX, paclitaxel; SAHA, suberoylanilide hydroxamic acid.

* Corresponding author. Fax: +39 0862 433523.
*E-mail address:* vincenza.dolo@univaq.it (V. Dolo).

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The purpose of this study was to determine whether the addition of SAHA to PTX can sensitize PTX-resistant human ovarian cancer cell lines (CABA-PTX and IGROV-PTX) *in vitro*. We also wanted to investigate the possible mechanisms involved in this phenomenon.

## Materials and methods

### *Cell cultures*

Human ovarian cancer cells A2780, SKOV3 and IGROV I were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown as monolayers in RPMI 1640 (Euroclone, Devon, UK) with 10% v/v fetal calf serum (FCS), 2 mM glutamine, penicillin and streptomycin. The CABA I cell line was established from the ascitic fluid of an ovarian carcinoma patient prior to any drug treatment [13]. CABA I cells were maintained as monolayers in RPMI 1640 (Euroclone, Devon, UK) containing 5% v/v FCS. Paclitaxel-resistant clones (CABA-PTX and IGROV-PTX) were selected through exposure to paclitaxel (Sigma, St. Louis, MO, USA) *in vitro* (500 ng/mL for CABA I cells, and 100 ng/mL for IGROV I cells). The selective pressure with PTX was maintained at least for 3 months and the experiments with PTX-resistant clones were performed within 1 month after the end of the selection.

### *Cell proliferation assay*

Cells were grown to subconfluence in 96-well cell culture plates ($2\times10^3$ cells/well) and treated with SAHA (kindly provided by Merck & Co, Whitehouse Station, NJ, USA) and PTX. The cultures were maintained at 37 °C in 5% v/v $CO_2$ for 72 h. Cell proliferation was evaluated using the XTT assay (Sigma). The metabolic reduction of XTT by living cells produces a coloured, non-toxic formazan that is soluble in water; the amount of this substance (which can be measured by an ELISA reader at a wavelength of 450 nm) is directly proportional to the number of viable cells.

### *Drug interactions*

Drug interactions were assessed using Berenbaum's combination index (BCI) calculated from the equation $BCI = D1/Dx1 + D2/Dx2$, in which D1 and D2 are the concentrations of drug 1 and drug 2 that in combination give the same response as drug 1 alone (Dx1) or drug 2 alone (Dx2). A BCI of less than 1 indicates synergy, equal to 1, additivity, and greater than 1, antagonism [14].

### *Immunofluorescence assays*

Cells were grown to 90% confluence on glass coverslips and treated for 12 h. At the end of the reaction time, the cells were washed with phosphate-buffered saline (PBS), fixed in ethanol for 5 min at 4 °C, and then incubated for 1 h at room temperature with bovine serum albumin/PBS. Cells were subsequently incubated for 1 h at room temperature with anti-beta-tubulin (1:200; Sigma) or anti-acetylated tubulin (1:1000; Sigma). After washing with PBS, cells were incubated with the appropriate secondary antibodies (1:1000) which were conjugated to the fluorescent chromophore Alexa 488 (Molecular Probes, Eugene, OR, USA) at room temperature for 1 h. The images were acquired by a digital camera (DS-L1 image acquisition system, Nikon instruments, Melville, NY, USA) associated with a fluorescence microscope system (Leica microsystem, Wetzlar, Germany).

### *Flow cytometric analysis*

We evaluated cell cycle progression and apoptosis by flow cytometry according to standard procedures. Briefly, cells ($1\times10^6$) were washed with PBS and fixed for 30 min with 70% v/v ethanol at 4 °C. After washing with PBS, the cells were incubated in 1 mL DNA staining solution (PBS containing 200 μg/mL of RNAse A, 20 μg/mL of propidium iodide, and 0.1% v/v Triton X-100) at room temperature for 1 h. Suspended cells were analyzed on a FACScan instrument (Becton Dickinson, Franklin Lakes, NJ, USA) using the Cell Quest software (Becton Dickinson). Analysis was performed by evaluating at least 10,000 cells for each sample. All measurements were done using the same instrument settings. Apoptotic cells were detected by a quantifiable peak in sub-G1 phase corresponding to the red fluorescent light emitted by subdiploid nuclei of cells.

### *Cytochrome c release assay*

The cytochrome *c* release assay was performed as described previously [15]. Briefly, cells were harvested and permeabilized using a 50 mg/mL digitonin solution and incubated for 5 min on ice. Cells were subsequently fixed in 4% w/v paraformaldehyde, washed in PBS, and incubated for 1 h in blocking solution (3% w/v albumin, 0.05% v/v saponin in PBS). Cells were incubated overnight at 4 °C with 1 g/mL anti-cytochrome *c* antibody and subsequently with a secondary fluorescein isothiocyanate-labeled antibody for 1 h. Expression level of released cytochrome *c* was evaluated by flow cytometry as fluorescence intensity in the presence of the cytochrome *c* antibody, and reported as the ratio between this value and the background staining of cells incubated with fluorescent secondary antibody (IF). The loss of cytochrome *c* from mitochondria treated with 5 mmol/L solution compared with control cells was expressed in terms of ΔIF (IF control–IF treated cells).

### *Western blotting*

Cells were lysed in lysis buffer containing 50 mM Tris (pH 7.8), 150 mM NaCl, and 1% v/v NP-40. The protein concentrations of the lysates were determined as described previously. Cell lysates (40 μg) were resolved by 7.5–10% sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing or nonreducing conditions and then transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Nonspecific binding sites were blocked by incubation with 10% nonfat dry milk in TBST (Tris-Buffered Saline Tween) at room temperature for at least 1 h. The blots were probed with a monoclonal antibody raised against acetylated tubulin (mouse monoclonal anti-Acetylate Tubulin clone 6-11B-1 IgG2b isotype, 1:2000 diluition; Sigma), beta-tubulin (monoclonal anti-beta-tubulin mouse ascites fluid clone TUB 2.1, 1:200 diluition; Sigma) or anti-histone deacetylase 6 (H-300, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. A horseradish peroxidase-conjugated secondary antibody (Sigma) in blocking buffer was subsequently used. After washing, reactive bands were visualized using a chemiluminescence detection kit (ECL, Amersham-Pharmacia, Piscataway, NJ, USA) and analyzed using a public domain software (ImageJ v1.3 by Wayne Rasband; National Institutes of Health, Bethesda, Maryland, USA).

### *Statistical analysis*

Results are expressed as means ± standard deviation (SD) for at least three distinct experiments. Demonstration of significant differences among means was performed by Student's *t* test considering 0.05 (two-tailed) as the threshold value of *P*. All calculations were performed using Kaleidagraph 3.6 (Synergy Software, Reading, PA, USA).

## Results

### *Antiproliferative activity of PTX and SAHA in different human ovarian cancer cell lines*

We investigated the effects of PTX-induced inhibition of cell growth in different ovarian cancer cell lines, i.e. A2780, IGROV I, SKOV3, and