Synergistic Antitumor Effects of Novel HDAC Inhibitors and Paclitaxel In Vitro and In Vivo

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Abstract

Preclinical studies support the therapeutic potential of histone deacetylases inhibitors (HDACi) in combination with taxanes. The efficacy of combination has been mainly ascribed to a cooperative effect on microtubule stabilization following tubulin acetylation. In the present study we investigated the effect of paclitaxel in combination with two novel HDACi, ST2782 or ST3595, able to induce p53 and tubulin hyperacetylation. A synergistic effect of the paclitaxel/ST2782 (or ST3595) combination was found in wild-type p53 ovarian carcinoma cells, but not in a p53 mutant subline, in spite of a marked tubulin acetylation. Such a synergistic interaction was confirmed in additional human solid tumor cell lines harboring wild-type p53 but not in those expressing mutant or null p53. In addition, a synergistic cytotoxic effect was found when ST2782 was combined with the depolymerising agent vinorelbine. In contrast to SAHA, which was substantially less effective in sensitizing cells to paclitaxel-induced apoptosis, ST2782 prevented up-regulation of p21WAF1/Cip1 by paclitaxel, which has a protective role in response to taxanes, and caused p53 down-regulation, acetylation and mitochondrial localization of acetylated p53. The synergistic antitumor effects of the paclitaxel/ST3595 combination were confirmed in two tumor xenograft models. Our results support the relevance of p53 modulation as a major determinant of the synergistic interaction observed between paclitaxel and novel HDACi and emphasize the therapeutic interest of this combination.

Introduction

Epigenetic modifications and deregulation of gene expression have been linked to the development of malignant phenotype and tumor progression, likely as a consequence of aberrant silencing of multiple tumor suppressor genes [1]. The dynamic process of histone acetylation, regulated by the balance action of histone acetyltransferases (HAT) and deacetylases (HDAC), plays a critical role in modulation of gene expression [2,3]. HDAC inhibitors (HDACi) represent a promising class of antitumor agents which have been developed to reverse the silencing of critical regulatory pathways [4,5]. Indeed, the cellular response to treatment with HDACi shows pleiotropic effects involving cell cycle arrest, induction of apoptosis and differentiation, modulation of microtubule function, DNA repair, and angiogenesis [4,6,7]. Based on these effects and, in particular, the activation of proapoptotic pathways, HDACi may have interest in combination with conventional chemotherapeutic agents to enhance tumor cell chemosensitivity [8,9]. However, given the different isoenzyme specificity of the available HDACi, the rational use of their combination remains to be defined, because the specific role of the individual HDAC isoenzymes as therapeutic targets has not been clearly established [10,11].

In addition to the transcriptional effects, HDACi are also involved in acetylation status of non-histone proteins implicated in critical regulatory processes (e.g., tubulin and transcription factors) [12,13]. Recently, we have reported that HDACi of a novel series were very effective in inducing p53 and tubulin acetylation [14]. Since tubulin acetylation is expected to favour microtubule stabilization [15,16], which is recognized as a primary mechanism of action of taxanes [17], the present study was designed to explore the cellular/molecular basis of the interaction between paclitaxel and selected HDACi of the novel series [14]. Indeed, several studies have shown that the pan-HDACi SAHA enhances the growth inhibitory effect induced by paclitaxel against various human tumor cells [18–21]. In the present study we found that, in contrast to SAHA, novel HDACi (ST2785 and ST3595) and paclitaxel synergistically inhibit the proliferation of ovarian carcinoma cells with wild-type p53, and dramatically activated apoptosis. Similar results were observed by combining ST2782 with the microtubule depolymerising agent vinorelbine. In addition, experimental evidence we obtained in a panel of human solid tumor cell lines characterized by a different p53 gene status supports the implication of modulation of wild-type p53 in mediating the synergistic effect of the PTX/ST2782 (or ST3595)
combination. The efficacy of this combination was also confirmed in wild-type p53 tumor xenograft models.

**Materials and Methods**

**Drugs and antibodies**

ST2782 (N-hydroxy-3-(4’-hydroxybiphenyl-4-y]-acrylamide, also known as RC307) and ST3595 were prepared as previously described [14]. SAHA was provided by BIOMOL International LP (Plymouth Meeting, PA). Paclitaxel (PTX, Indena, Milan, Italy), nocodazole (Calbiochem EMD Chemicals, Inc., La Jolla, CA) and ST2782 were dissolved in dimethyl sulfoxide (DMSO); vinorelbine (Pierre Fabre, Castres, France) was dissolved in H2O. In all experiments the highest final concentration of DMSO in culture medium was 0.5%. For in vivo studies, ST3595, ST2782 and SAHA were dissolved in a mixture of DMSO and cremophor, suspended in sterile, distilled water (10+5+85%) and delivered orally. PTX was dissolved in a mixture of ethanol and cremophor (50+50%) employing a magnetic stirrer and stored at 4°C. Before treatment, the drug was diluted in saline (10% of ethanol-cremophor). After dilution, the drug was kept in ice and administered i.v.

We used primary antibodies against p53 (Dako, Glostrup, Denmark); p21WAF1/Cip1 (Neomarker, Union City, CA); caspase-3 (CPP32), cleaved caspase-3 (Asp175), acetylated p53 (lys382) (Cell Signalling Technology, Beverly, MA); PARP-1 (Oncogene Science, Uniondale, NY); cytochrome C (BD Pharmingen, Becton Dickinson, Franklin Lakes, NJ); actin, acetylated tubulin and β-tubulin (Sigma, St. Louis, MO); mitotic protein monoclonal 2 (MMP-2) (Upstate Biotechnology, Lake Placid, NY); Raf-1 (sc-7267), cyclin-B1 (sc-245), cdc-25C (sc-327) (Santa Cruz Biotechnology, Santa Cruz, CA); mitochondrial marker (Abcam, Cambridge, UK); topoisomerase II (Transduction Laboratories, Lexington).

**Cell culture and antiproliferative activity**

The following human cell lines: IGROV-1 [22] ovarian carcinoma and its cisplatin resistant subline IGROV-1/Pt1, selected by exposure to increasing drug concentrations [22], H460 human non-small cell lung cancer [23], HCT116 colorectal adenocarcinoma cell line [24], and SAOS osteosarcoma [25] were maintained in RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 10% FBS (Invitrogen, Carlsbad, CA). The U2OS osteosarcoma cell line [26] were cultured in McCoy’s (Lonza) supplemented with 10% FBS (Invitrogen). After 24 h, cells were exposed to ST2782 or to ST3595 to antimicrotubule agent or to their combination for 72 h. After treatment, adherent cells were trypsinized and counted by a cell counter (Coulter Electronics, Luton, UK). Drug interactions were analyzed using CalcuSyn software (Biosoft), which calculates the median effect equation. Analysis of drug interaction to determine the combination index (CI) was based on the multiple drug effect equation of Chou and Talalay [27]. CI = 1 indicates an additive effect; <1 synergy; >1 antagonism.

**Cell cycle analysis**

Cell cycle perturbations were analyzed at different times after the end of treatment, as described previously [28], by flow cytometry (FACSscan, Becton Dickinson) using CellQuest Software.

**Dual parameter analysis flow cytometry (cell cycle and apoptosis induction)**

Cells were fixed in 1% paraformaldehyde for 30 min in ice and, after the removal of the fixative, they were permeabilized in 70% ice cold ethanol for 30 min. After suspension in PBA (PBS containing 0.1% Tween-20 and 1% BSA), cells were incubated with a rabbit polyclonal anti-cleaved caspase 3 antibody (CPP32, diluted 1:500 in PBA, Cell Signaling) for 1 h, washed twice with PBA solution and then incubated with an anti-rabbit-Alexa Fluor 488 (1:500, Invitrogen). Cells were counterstained with propidium iodide for 1 hour and then a dual parameter analysis was performed by FACScan (Becton Dickinson). The green FITC-fluorescence, expressed on a logarithmic scale, indicated the CPP32 cleaved form. The PI-red fluorescence, was taken as index of DNA content.

**Assessment of apoptosis and mitotic cells**

Apoptosis was detected by TUNEL assay, using the in situ Cell Death Detection Kit fluorescein (Roche, Germany). Apoptosis was assessed by FACSscan, and the results were analyzed using the CellQuest software. To detect MMP-2 immunoreactivity, cells were processed as described previously [29] and examined by fluorescence microscopy. The number of mitotic cells was assessed on at least 300 cells in two different smears and referred to the whole cell population (floating and adherent cells).

**Western-blot analysis**

Cells were lysed as previously described [30]. The filters were incubated with primary antibodies and with peroxidase-conjugated secondary antibodies. Immunoreactive bands were revealed by using the enhanced chemiluminescence detection system from Amersham Biosciences (Piscataway, NJ).

**Tubulin polymerization assay**

Cells were exposed to drugs and, 24 h later, processed for the tubulin polymerization assay. Samples were prepared as described by Blagosklonny et al. [17] and Lanzi et al. [29] and separated by SDS-PAGE (10% resolving gel and 3% stacking gel), and tubulin distribution was assessed by immunoblot analysis using a mouse anti-β-tubulin antibody (Sigma).

**Analysis of cytochrome C release and subcellular fractionation**

Following treatment with single agents (IC50) or their combination, IGROV-1 cells were processed as described previously [28]. Cytosolic and mitochondrial fractions were analyzed by immunoblot analysis. In immunoblots, mouse monoclonal anti-mitochondrial marker (Abcam) and anti-topoisomerase II (Transduction Laboratories) antibodies were used as mitochondrial and nuclear markers, respectively.

**Small interfering RNA transfection**

Small interfering RNAs were synthesized by Invitrogen Corp. (Carlsbad, CA). The p33-siRNA consisted of a mixture of two siRNA duplexes targeting different regions of the p53 mRNA (Validated stealth RNAi DuoPak). As a negative control, Medium GC Duplex of Stealth® RNAi Negative Control Duplexes (Invitrogen) was used. Transfection of cells with siRNA duplexes was performed using Lipofectamine 2000 Reagent (Invitrogen). IGROV-1 cells were transfected with control siRNA or p33-siRNA at a final concentration of 100 nM for 24 h. After silencing, cells were replaced and treated with drugs on the second day as indicated. Gene silencing effects were monitored by Western blot also on the day of the treatment.

**In vivo antitumor activity**

Female athymic Swiss nude mice (7–10 weeks-old, Charles River, Calco, Italy). Were maintained in laminar flow rooms

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keeping temperature and humidity constant. Experiments were approved by the Ethical Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale Tumori (Internal reference INT_21/09 approved on 21 October 2009 Sent to Ministry of Health on 9 December 2009) according to the Italian law DL 116/92. The IGROV-1 human ovarian carcinoma was established as i.p. growing tumor xenograft [31]. Ascitic cells (2.5 x 10^6) suspended in 0.2 ml saline were inoculated s.c. in both flanks of each animal. Following s.c. injection of exponentially growing U2OS osteosarcoma cells (10^7 cells/0.2 ml saline). The tumor line was achieved by serial s.c. passages of fragments (about 3 x 3 x 3 mm). Groups of four mice bearing bilateral s.c. tumors were employed. PTX was delivered every fourth day for four times (q4dx4). HDACi were given qdx5/wx3w. Tumor growth was followed by biweekly measurements of tumor diameters with a Vernier caliper. Tumor volume (TV) was calculated according to the formula: TV (mm³) = d² x D/2, where d and D are the shortest and the longest diameter, respectively. Treatment started when TV was less than 100 mm³. The efficacy of the drug treatment was assessed as: i) Tumor volume inhibition percentage (TVI%) in treated versus control mice, calculated as: TVI% = 100-(mean TV treated/mean TV control x 100); ii) Complete regression (CR), i.e. disappearance of the tumor lasting at least 10 days. The toxicity of the drug treatment was determined as body weight loss and lethal toxicity. Deaths occurring in treated mice before the death of the first control mouse were ascribed to toxic effects. Student t test (two tailed) and Fisher’s exact test were used for statistical comparison of tumor volumes and CR, respectively.

Results

Cell growth inhibition studies

The combination of ST2782 with PTX [IC_{20} and IC_{50}, corresponding to drug concentrations producing 20% and 50% of cell growth inhibition assessed by dose-response curves after 72 h exposure] resulted in a marked growth inhibition of IGROV-1 cells when compared to single agents (Figure 1A). The sensitization was also evident at low concentrations of ST2782, which alone caused negligible effects. The analysis of drug interaction by Chou and Talalay method supported a synergistic interaction between PTX and ST2782 (Figure 1A). A similar interaction was found with a closely related analog, ST3595 (Figure 1B). Again, ST2782 enhanced the growth inhibition also of microtubule-destabilizing agents, including vinblastine and nocodazole (Figure 1C). The potentiation of PTX effect by ST2782 was less evident in p53 mutant IGROV-1/Pt1 subline, characterized by a hypersensitivity to PTX because of mutational inactivation of p53 (Figure 1A). Under the same conditions, the combination treatment of PTX with SAHA, a known pan-HDACi currently used in clinical therapy, resulted in additive effects in IGROV-1 cells (Figure 1D).

To explore the relevance of p53 gene status in the synergistic interactions between PTX and the two novel HDACi, we determined the antiproliferative effect of the drug combinations in a panel of human solid tumor cell lines harboring wild-type, mutant or null p53 (Table S1). A synergistic cytotoxic interaction between PTX and ST2782 was found in cell systems expressing wild-type p53 (H460 and HCT116) but not in those carrying a mutant (A431) or null (SAOS) p53. In addition, a moderate synergistic activity of the PTX/ST3595 combination was found in a wild-type p53 U2OS cell model of mesenchymal origin (osteosarcoma) (Table S1).

Cell-cycle perturbation and analysis of the cellular morphology

To investigate the cellular basis of the synergistic effects, we examined the cell cycle perturbation of IGROV-1 cells exposed to ST2782 or its combination with PTX or vinorelbine. At low concentrations (IC_{50}), PTX induced cell accumulation in G2/M phase (Figure 2A). On the contrary, ST2782 alone produced a marginal perturbation of cell-cycle progression. The concomitant exposure to both drugs determined an increase of G2/M phase arrest and appearance of cells in sub-G1 phase, thus suggesting the induction of cell death. This effect was still evident at a low non-toxic concentration of ST2782 (3 μM). After 24 h of treatment, the morphological analysis of MMP-2 immunoreactivity in cells treated with the combination evidenced a decrease in the percentage of mitotic cells in both cell lines as compared to PTX alone, supporting a G2 phase arrest with a concomitant increase of the apoptotic cells (Figure 2B). Similar effects were found also following treatment with vinorelbine (Figure 2C) and nocodazole (data not shown). Additional biochemical investigations in cells treated with the ST2782/PTX combination were performed to support cell cycle arrest in G2 phase. In IGROV-1 cells exposed for 24 h to the PTX/ST2782 combination, using IC_{50} concentrations for each agent, the G2 arrest was confirmed by the decrease of mitosis-specific phosphorylated epitopes recognized by MMP-2 antibody, lack of phosphorylation of Raf-1, Bcl-2 and cdc25C, clearly detected by loss of mobility shift, and reduced level of cyclin B1 as compared to PTX alone (Figure 2D). Moreover, under the same conditions, the combined treatment resulted in increased caspase 3 (CPP32) activation associated with cleavage of PARP-1, according to the synergistic effects observed in cell growth experiments (Figure 1).

Sensitization to apoptosis by PTX/ST2782 combination

We tested the hypothesis that the sensitization effect of ST2782 was related to enhanced cell susceptibility to antimicrotubule agent–induced apoptosis. Under the same treatment conditions used for the analysis of cell-cycle perturbation, exposure to each single agent resulted in a barely detectable CPP32 activation in IGROV-1 cells, thus indicating that, at the tested concentrations (IC_{50}), the effect of single-drug treatment was predominantly cytostatic (Figure 3). In contrast, IGROV-1 cells treated with the drug combination exhibited a dose-dependent activation of CPP32, associated with cleavage of PARP-1, which was already detectable after 24 h (Figure 3A). Moreover, consistent with an enhanced activation of caspase-dependent apoptotic pathways, the PTX/ST2782 combination caused the cytosolic release of cytochrome C, a caspase activator, which was associated to a decrease in the mitochondrial cytochrome C content. A weak CPP32 activation and cytosolic release of cytochrome C were found in the IGROV-1/Pt1 cell lines. The analysis of apoptosis, determined by TUNEL assay (Figure 3B), revealed a marginal (if any) induction of apoptosis in the presence of each single agent after 72 h exposure. On the contrary, the combined treatment produced a remarkable increase of apoptosis only in IGROV-1 cells (Figure 3B). SAHA combined with PTX induced a lower appearance of apoptotic cells as compared to the combination of PTX and ST2782 (Figure 3B), according to a marginal sensitization observed in cell growth inhibition experiments (Figure 1D) and to a slight induction of CPP32 and PARP-1 cleavage (Figure 3C). A cytosolic release of cytochrome C with a weak decrease in the mitochondrial cytochrome C content was found after 48 h exposure to SAHA/PTX combined treatment in IGROV-1 cells (Figure 3D). When combined with vinorelbine, ST2782 induced the cleavage of CPP32 in IGROV-1 cells.
Figure 1. Antiproliferative effects of ST2782 alone (■) or in combination with PTX (A) or vinorelbine (C, VIN) or nocodazole (C, NCD) at two indicated concentrations corresponding to IC_{20} (t) or IC_{50} (A), against ovarian carcinoma cells treated for 72 h. Similar experiments were performed with the combination of PTX/ST3595 (B) or PTX/SAHA (D). Each point of the dose-response curves is the mean±SD of three independent experiments. Each panel reports the combination index values (CI) calculated according to “Chou Talalay method”.

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Figure 2. Effect of ST2782 or antimicrotubule agents alone or their combination on cell cycle distribution (A) or apoptosis and mitosis (B and C) in IGROV-1 and IGROV-1/Pt1 cells. (A) FACS analysis of the cell cycle perturbation. Cells were treated for different times with subtoxic (antiproliferative) concentrations of ST2782 (10 and 3 μM) combined with PTX at IC50 (0.12 μM). B) and C) Apoptosis, determined by TUNEL assay and assessed by FACScan, and mitotic index, determined by MPM-2 staining and fluorescence microscopy analysis, in cells exposed for 24 h to IC50 of PTX (0.12 μM and 0.012 μM, for IGROV-1 and IGROV-1/Pt cells, respectively) or vinorelbine (0.047 μM) or ST2782 (10 μM) or their combination. The data, expressed as percent of total cell population, are the mean±SD of three independent experiments. Data were analyzed by t test (control vs treatment): *P<0.05, **P<0.005. D) Activation of mitosis-related factors in IGROV-1 cells exposed to cytotoxic concentrations of ST2782 (20 μM) and PTX (1.2 μM). Western blot analysis of cell lysates was performed at the end of treatment (24 h). Actin is shown as a control for protein loading. The size of caspase 3 (CPP32) and of its cleavage products is indicated.
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Expression of p53 and p21WAF1/Cip1

Since the transcriptional activity of p53 has a protective role in response to mitotic spindle damage [28], we have investigated the drug effects on p53 expression, following treatment with PTX, ST2782 alone or their combination for 24 and 48 h, at doses corresponding to IC50 or with ST2782 (IC50) alone or in combination (Figure 4). Tubulin acetylation was detectable only in polymerized form of PTX-treated cells, whereas ST2782 induced an acetylated tubulin in both soluble and polymerized form. The PTX/ST2782 combination markedly enhanced the acetylation of polymerized α-tubulin (Figure 4A). The analysis of the status of α-tubulin acetylation in whole cellular extract of IGROV-1 and IGROV-1/Pt1 evidenced a comparable increase of the acetylated form of α-tubulin (Figure 4B).

(a) The role of p53 acetylation in mediating the synergistic cytotoxic effect in IGROV-1 cells was also investigated ST2782/vinorelbine combination (Figure 5E). After 48 h of combined treatment with low concentrations (IC50) of both drugs, cells showed a slight enhancement in the level of p53 acetylation compared to that observed following exposure to vinorelbine alone. In addition, ST2782 was able to prevent the up-regulation of p53 and p21WAF1/Cip1 induced by the microtubule depolymerising agent (Figure 5E).

To better understand the role of p53 in cellular response, we investigated the effects of the single agents and their combination in IGROV-1 cells following p53 down-regulation by siRNA (Figure 5F). As expected, following p53 silencing, cells exhibited an increased sensitivity to PTX (IC50: 0.22±0.12 μM in negative control cells versus 0.06±0.025 μM in p53-siRNA transfected cells), as also indicated by caspase activation (Figure 5F). However, in spite of an increased sensitivity to ST2782 itself (IC50: 6.12±1.9 μM in negative control cells versus 3.5±1.1 μM in p53-siRNA transfected cells), no synergistic interaction was evident in the combination (Figure 5F).

Since SAHA was less effective in sensitizing cells to PTX-induced apoptosis, we explored p53 and p21WAF1/Cip1 modulation by the SAHA/PTX combination (Figure 5G). When compared to PTX alone, no appreciable modulation of p53 and p21WAF1/Cip1 was detected, thus providing a further indirect support to the implication of p53 in the synergistic interaction between ST2782 and PTX. Relevant to this point is the observation that p53 was acetylated following exposure to ST2782 and ST3595 (a methoxy derivative of ST2782), but barely (if any) following exposure to SAHA (Figure 5H).

Since recent studies support a role of acetylated p53 in the transcription-independent regulation of apoptosis involving p53 translocation to mitochondria in stressed cells [32,33], we examined the subcellular localization of p53 following 24 h-exposure to the drugs (Figure 6). The combination of ST2782 with PTX, at the IC50 concentrations, induced a substantial translocation of acetylated p53 to mitochondria (Figure 6A). This subcellular distribution was associated with a concomitant release of cytochrome C (Figure 3). The combination of SAHA with PTX induced a lower extent of mitochondrial translocation of acetylated p53. ST2782 induced a marked down-regulation of the mutant p53 in IGROV-1/Pt1 (Figure 6B), barely detectable in the mitochondrial fraction.

In vivo antitumor activity

The antitumor efficacy of the combination of HDACi with PTX was investigated in the IGROV-1 ovarian carcinoma xenograft established with the same cell system used in cellular studies and in a different model (U2OS osteosarcoma) to explore whether the synergistic interaction may have a broad therapeutic interest, using well-tolerated doses of PTX (i.e., around ½ MTD), with the intermittent i.v. treatment schedule (usually 4×4×4), and of HDACi (100 mg/kg), with daily oral administration. Experiments performed with ST2782/PTX and ST3595/PTX combinations indicated an appreciable improvement of the antitumor efficacy over the single-agent therapy (Table 1). However, the therapeutic effects of ST3595 (a methoxy derivative of ST2782) were more consistent, likely as a consequence of a more favourable oral bioavailability and pharmacological profile (in particular, metabolic stability). Therefore, further in vivo experiments were performed with the methoxy analog. In contrast to ST3595, SAHA at the same dose was not effective in modulating the PTX activity, but it markedly worsened the toxicity of the combination (Table 1). The improved efficacy of ST3595/PTX combination over SAHA/PTX was dramatically evidenced in the human osteosarcoma U2OS, a model carrying wild-type p53 (Figure 7). ST3595 in combination with the taxane produced complete tumor...
Figure 3. Induction of apoptosis and analysis of apoptosis-related factors in IGROV-1 and IGROV-1/Pt1 and A431 cells exposed to the IC$_{50}$ PTX or vinorelbine (VIN) or ST2782 (ST) or SAHA or their combination. A) Cleavage of caspase 3 (CPP32) and PARP-1 and cytochrome C release. Western blot analysis was performed after 24 h exposure to drug concentrations used in experiments of Fig. 2A. β-Tubulin is shown as a control of protein loading. Release of cytochrome C was examined in cytosolic and mitochondrial extracts, prepared after 24 h exposure.
regression (with no evidence of disease at the end of the experiment) in all animals treated, without relevant manifestations of toxicity. In contrast, SAHA did not enhance the PTX efficacy but caused an appreciable increase of toxicity as evidenced by a marked body weight loss of animals treated with the combination.

**Discussion**

As observed for most target-specific agents, single-agent therapy with HDACi may not be sufficiently efficient to control tumor growth in the majority of solid tumors in spite of the claimed selectivity for tumor cells. It is now evident that, given the pleiotropic effects of HDACi, their therapeutic potential is expected to be best exploited through combination with other antitumor agents [8,9]. Indeed preclinical data with several tumor cell lines have shown synergistic effects when combining HDACi with various antitumor therapies [34–38]. The potentiation of the killing effects of DNA damaging agents could reflect modulation of DNA damage response [38]. In general, the ability of HDACi to enhance drug-induced cytotoxicity has been related to activation of proapoptotic pathways [7,8].

The antitumor effects of HDACi have been at least in part related to modulation of chromatin structure and gene expression resulting in reactivation of silenced genes. In addition to modulation of transcription, the biological effects of HDACi may be mediated by acetylation of nonhistone proteins, including transcription factors (e.g., p53), and by functional alterations of critical proteins (e.g., tubulin and Hsp90) [15,16,39]. The latter effects, which involve the inhibition of the cytoplasmically localized HDAC6 isoform [15,16,40,41], have been exploited to achieve a synergistic interaction between pan-HDACi and taxanes [21]. The antitumor efficacy of HDACi/PTX has been ascribed to cooperative effects on microtubule stabilization mediated by tubulin acetylation [21]. Based on this hypothesis, we have examined in ovarian carcinoma cells the interaction of paclitaxel with novel HDACi endowed with ability to induce hyperacetylation of p53 and α-tubulin. Our results show that the combination of the novel HDACi (ST2782 or ST3595) with PTX had a synergistic effect only in the IGROV-1 cells carrying wild-type p53, but not in the p53 mutant platinum-resistant subline IGROV-1/Pt1 in spite of a similar drug effect on α-tubulin acetylation. A synergistic activity of PTX combined with the two novel HDACi (ST2782 and ST3595) was also observed in additional tumor cell lines, H460, HCT116 and U2OS, expressing wild-type p53. Conversely, an antagonistic interaction was found in SAOS and A431 cell lines that harbor null and mutated p53, respectively. Moreover, in IGROV-1 cells a synergistic effect was found also with the combination of ST2782 and vinorelbine, a known microtubule destabilizing agent. These observations do not support a primary role of tubulin acetylation and polymerization in the synergistic effect of the combination.

The finding that the synergistic effects was produced by the combination only in wild-type p53 cells suggested the implication of functional p53 as a critical determinant of drug interaction. In

**Figure 4. Analysis of the tubulin polymerization status in IGROV-1 cells on extracts derived by cell fractionation (A) or by lysis of whole cells (B).** Cells were exposed to ST2782 (ST, 10 μM), PTX (0.1 μM) alone or their combination for 24 h. Cell fractionation was performed as indicated in the Materials and Methods section. Soluble cytosolic (S) or polymerized (P) α-tubulin was detected in cell fractions by Western blot analysis.

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Figure 5. Western blot analysis of modulation of p53 and p21\textsuperscript{WAF1/Cip1}. A) IGROV-1 cells were treated for 24 and 48 h with PTX (0.12 μM) or ST2782 (ST, 10 μM). B) IGROV-1 cells were exposed to a cytotoxic doses (IC\textsubscript{80}) of ST2782 (20 μM) and/or paclitaxel (1 μM) for 24 hours. C) IGROV-1/Pt1 cells were treated with PTX (0.012 μM) or ST2782 (ST, 10 μM) for 24 and 48 hours. D) A431 cells were treated with PTX (0.0035 μM) or ST2782 (ST, 3 μM) for 24 h and 48 h. E) IGROV-1 cells were exposed to ST2782 (10 μM) and vinorelbine alone (0.047 μM) or in combination for 48 h. F) Effects of PTX (0.12 μM) and its combination with ST2782 (10 μM) in IGROV-1-negative control cells and in p53-siRNA-transfected cells. The negative control cells are referred to cells transfected with RNAi-negative control duplex containing 48% GC. Actin is shown as a control of protein loading. Panel
IGROV-1 cells, the ST2782/PTX combination was associated with a partial p53 down-regulation and acetylation of the residual p53 protein. The acetylated form of p53 was found to be localized predominantly in mitochondria, where it may play a transcription-independent proapoptotic activity [32,33].

Our previous studies support a protective role of the transcriptional activity of p53 in response to mitotic spindle damage [28]. Down-regulation of p53 could result in a sensitization to PTX as a consequence of prevention of p21WAF1/Cip1 induction in response to PTX. Indeed, we have found that ovarian carcinoma cells selected for resistance to cisplatin and characterized by mutational inactivation of p53 are hypersensitive to PTX [42]. The results presented in this study indicated that ST2782 prevented the up-regulation of p21WAF1/Cip1 induced by both PTX, a microtubule polymerising agent and vinorelbine, a microtubule depolymerising agent. The modulation of p21WAF1/Cip1 expression in PTX-treated cells by ST2782 is reminiscent of the effect of pifithrin-α, a transcriptional inhibitor of p53 [24]. Relevant to this point is the observation that, in contrast to SAHA, ST2782 and ST3595 induced a dose-dependent down-regulation of p53. The mechanism of this effect is not clearly understood, but likely it is related to modulation of acetylation status of Hsp90, which, as is a protein substrate for the cytoplasmic HDAC6 isoenzyme, may be involved in p53 stabilization [39,43,44].

**Table 1.** Antitumor activity of PTX alone or in combination with histone deacetylase inhibitors (ST2782, ST3595 or SAHA) against the ovarian carcinoma model IGROV-1.

| Drug treatment | Dose (mg/kg) | Schedule | TVI (%) | CR (%) | BWL (%) |
|---------------|--------------|----------|---------|--------|---------|
| PTX           | 36           | q7dx3    | 90      | 0/8    | 9       |
| ST2782        | 20           | qdx5d/wx3 | 30      | 0/8    | 7       |
| PTX+ST2782    | 98           |          | 0/8     | 9      |
| PTX           | 24           | q4dx4    | 90      | 2/8    | 5       |
| ST3595        | 100          | qdx5d/wx3 | 49      | 0/8    | 9       |
| PTX+ST3595    | 98           |          | 3/10    | 12     |
| SAHA          | 100          | qdx5d/wx3 | 64      | 0/8    | 5       |
| PTX+SAHA      | 87           |          | 1/8     | 22     |

*a* Tumor volume inhibition (%) in treated vs control mice, determined one week after the end of drug treatment.

*b* CR, complete response, i.e. disappearance of tumor lasting at least 10 days.

*b* BWL, body weight loss (%).

Figure 6. Analysis of mitochondrial p53 translocation induced by ST2782 or SAHA in absence or presence of PTX in IGROV-1 and IGROV-1/Pt1 cells. The cytosolic and mitochondrial extracts were prepared after 24 h of treatment with PTX alone or combined with HDACi. An antibody against a mitochondrial marker and an antibody against topoisomerase II were used as protein control to ensure a correct subcellular fractionation process. A nuclear extract (n.e.) was used as a positive control for topoisomerase II. A) Comparison of the p53 localization in cytosol or mitochondrial fraction after the combination of PTX (0.12 μM) with ST2782 (10 μM) or with SAHA (3 μM) for 24 h in IGROV-1 cells. One representative experiment of at least three is shown. B) IGROV-1/Pt1 cells were treated for 24 h with PTX (0.012 μM) or ST2782 (10 μM) alone or in combination. One representative experiment of at least three is shown.
The lack of synergistic effect between ST2782 and PTX in the p53 mutant IGROV-1/Pt1 and A431 cell lines, could reflect loss of p53 transcriptional function and marginal (if any) activation of p21WAF1/Cip1 (Figure 5C). Thus, the ST2782-induced down-regulation of mutant p53 may have a marginal impact owing to lack of its function. Again, p53 silencing in IGROV-1 cells caused a lack of p21WAF1/Cip1 up-regulation, an increase sensitivity to single-agent treatment and an absence of synergistic interaction in the combined treatment. The interpretation implicating p21WAF1/Cip1 upregulation as a protective event in response to taxane was also consistent with the moderate synergism between SAHA and PTX, because SAHA had not effect in the modulation of p21WAF1/Cip1 in paclitaxel-treated cells. Thus, the different profile of protein acetylation induced by SAHA and novel HDACi used in this study could account for the different cellular response to their combination with PTX (Figure 5F). However, the pleiotropic effects of HDACi do not allow a definitive explanation of the observed synergistic interaction with antimicrotubule agents.

The sensitization of wild-type p53 cells in vitro to PTX by ST3595 was confirmed in tumor xenograft models. The enhancement of the PTX antitumor efficacy by ST3595 was impressive in the osteosarcoma model resulting in complete tumor regression in all treated animals, without evidence of disease at the end of the experiment (after three months). These preclinical findings may have therapeutic implications also considering the use of nontoxic doses of PTX and the good tolerability of ST3595 following protracted oral administration.

In conclusion, given the current interest for combination therapy with HDACi, the present study provides a basis for a rational strategy to improve the taxane-based antitumor therapy.

Supporting Information

Figure S1 Dual parameter analysis of apoptosis induction and DNA content by flow-cytometry. Cells were treated for different times with subtoxic (antiproliferative) concentrations of ST2782 (10 μM) combined with PTX at IC₅₀ (0.12 μM). At each time, cells were fixed and processed as described in ‘Materials and method’ section. Green fluorescence (x-axis): cleaved caspase 3 (CPP32); red fluorescence (y-axis): propidium iodide. The upper quadrant represents cleaved CPP32-positive cells. In each panel equivalent phases of the cell cycle are indicated on the basis of DNA content by PI staining. (TIF)

Table S1 Combination index values (CI) of the PTX/ST2782 (or ST3595) combined treatment as a function of cell lines p53 gene status. (TIF)

Author Contributions

Conceived and designed the experiments: VZ FZ. Performed the experiments: VZ MDC. Analyzed the data: VZ MDC. Contributed reagents/materials/analysis tools: RC RN. Wrote the paper: VZ FZ NZ. Participated in discussions and critical reading of the manuscript: CP NZ. Carried out the synthesis of the drugs (ST2782 and ST3595): RC RN.

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