PRECLINICAL STUDIES

Histone deacetylase inhibitors enhance the anticancer activity of nutlin-3 and induce p53 hyperacetylation and downregulation of MDM2 and MDM4 gene expression

Chithra D. Palani · James F. Beck · Jürgen Sonnemann

Summary Nutlin-3, a small-molecule MDM2 inhibitor, restores p53 function and is, thus, an appealing candidate for the treatment of cancers retaining wild-type p53. However, nutlin-3 applied as single agent may be insufficient for cancer therapy. Therefore, we explored whether the anticancer activity of nutlin-3 could be enhanced by combination with histone deacetylase inhibitors (HDACi), i.e. vorinostat, sodium butyrate, MS-275 and apicidin. We found that nutlin-3 and HDACi cooperated to induce cell death in the p53 wild-type cell lines A549 and A2780, but not in the p53 null cell line PC-3, as assessed by Alamar Blue assay and flow cytometric analyses of propidium iodide uptake and mitochondrial depolarization. Combination index analysis showed that the effect was synergistic. For comparison, we tested nutlin-3 in combination with paclitaxel, revealing that nutlin-3 antagonized the cytotoxic activity of paclitaxel. To shed light on the underlying mechanism of the synergistic action of nutlin-3 and HDACi, we determined the acetylation status of p53 by immunoblotting and the mRNA levels of MDM2 and MDM4 by real-time RT-PCR. We observed vorinostat to induce p53 hyperacetylation, to reduce the constitutive gene expression of MDM2 and MDM4, and to counteract the nutlin-3-induced upregulation of MDM2 gene expression. In conclusion, our study shows that HDACi amplify the antitumor activity of nutlin-3—possibly by inducing p53 hyperacetylation and/or MDM2 and/or MDM4 downregulation—suggesting that treatment with a combination of nutlin-3 and HDACi may be an effective strategy for treating tumors with wild-type p53.

C. D. Palani · J. F. Beck · J. Sonnemann
Department of Pediatric Hematology and Oncology,
University Children's Hospital Jena,
Jena, Germany

J. Sonnemann (✉)
Klinik für Kinder- und Jugendmedizin,
Friedrich-Schiller-Universität Jena,
Kochstr. 2,
07745 Jena, Germany
e-mail: juergen.sonnemann@med.uni-jena.de

Introduction

The transcription factor p53 is a powerful tumor suppressor that protects cells from neoplastic transformation by mediating apoptosis and cell cycle arrest [1]. p53 functions are under control of the product of a p53-inducible gene, MDM2, through a negative-feedback loop. In unstressed cells, MDM2 directly binds to p53, preventing its transactivation. Stress signals, such as DNA damage or oncogene activation, lead to the dissociation of the p53-MDM2 complex, unleashing the anticancer activities of p53.

p53 is the most commonly inactivated protein in human tumors [1]. Its inactivation may be the result of gene alterations: Approximately 50% of human cancers have mutations in the p53 gene. However, it may also be the result of an abrogated p53 signal transduction pathway; in this case, the function of p53 is effectively inhibited without requirement for mutations. In fact, of the 50% of human tumors with wild-type p53, many are thought to have compromised p53 function due to increased MDM2 levels [2]. Hence, restoration of p53 function through blocking the p53-MDM2 interaction appears to be an attractive strategy for the treatment of p53 wild-type cancers.

Agents targeting the p53-MDM2 interaction can indeed reactivate p53 and exert antineoplastic effects in tumors retaining wild-type p53 [3]. Of them, the imidazoline

compound nutlin-3 was the first reported to have anticancer activity in vivo [4]. Subsequent analyzes revealed that nutlin-3 was efficacious in several tumor models [5], and it has recently entered phase I clinical trials with patients suffering from advanced solid cancers [6] or hematologic malignancies [7].

Histone deacetylase inhibitors (HDACi), such as vorinostat (also known as suberoylanilide hydroxamic acid, SAHA), are another class of antitumor agents that hold promise to improve cancer therapy [8, 9]. They stimulate differentiation, inhibit proliferation and induce apoptosis in tumor cells while leaving normal cells relatively unscathed. HDACi function not only by mediating acetylation of histones but also of nonhistone proteins including p53 [10]. Their clinical potential is underscored by the fact that vorinostat and more recently romidepsin (also known as depsipeptide or FK228) have received approval by the US Food and Drug Administration for treatment of cutaneous T-cell lymphoma [11, 12].

Yet the greatest potential of HDACi may lie in their capability to augment the antineoplastic efficacy of other therapeutic regimens. In numerous preclinical studies, they have been demonstrated to synergize with a multitude of pharmacological and biological cancer therapeutics [13]. Likewise, the combination of nutlin-3 with other antitumor agents has been shown in vitro to have cooperative effects on cancer cells [3, 14]. However, a possible favorable interaction between HDACi and MDM2 inhibitors has not yet been studied, although this combination appears to be especially attractive, because the acetylation of p53 critically contributes to its activation [15]—in fact, acetylation has been found to be essential for p53-mediated apoptosis and growth arrest [16]—and HDACi have been shown to provoke p53 acetylation [17–21].

Therefore, we investigated whether nutlin-3 and HDACi belonging to four different structural classes—the hydroxamic acid vorinostat, the short chain fatty acid sodium butyrate (NaB), the benzamide MS-275, and the cyclic tetrapeptide apicidin—would cooperate in exerting cytotoxic activity against cancer cells. For comparison, we also tested the combination of nutlin-3 with the microtubule-targeted drug paclitaxel, for nutlin-3 has been reported to protect p53 wild-type cells from paclitaxel-induced cell killing [22, 23]. We found that nutlin-3 potently synergized with HDACi—but indeed antagonized paclitaxel—to induce cell death in p53 wild-type tumor cells.

Materials and methods

Reagents

Racemic nutlin-3, vorinostat, MS-275, apicidin and z-VAD-fmk were purchased from Alexis (Grünberg, Germany).

NaB was purchased from Sigma (Deisenhofen, Germany). Paclitaxel was purchased from Teva (Mörfelden-Walldorf, Germany).

Cell lines

p53 wild-type A549 and A2780 cells [24, 25] were obtained from ATCC (Manassas, VA, USA), and p53 null PC-3 cells [24] were obtained from the DSMZ (Braunschweig, Germany). A549 cells were maintained in Ham's F12K, and A2780 and PC-3 cells were maintained in RPMI 1640 (PAA, Cölbe, Germany). Media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate (PAA). Cells were cultivated at 37°C in a humidified 5% CO_2 incubator and routinely passaged when 90% confluent. Cell viability was determined by the trypan blue exclusion test. Cells were regularly inspected to be free of mycoplasma with the PCR mycoplasma detection kit from Applichem (Darmstadt, Germany).

Treatment of cells

The cells were plated at 150,000 cells/well in 6-well plates or 2,000 cells/well in 96-well plates and treated with nutlin-3 for 1 h or left untreated before application of HDACi or paclitaxel. The latter were added directly to the culture medium containing nutlin-3 without a medium change. Cells were then exposed to HDACi or paclitaxel for additional 24 h (caspase-3 activity, immunoblotting, quantitative PCR), 48 h (flow cytometric analyses) or 72 h (Alamar Blue assay). To inhibit the activation of caspases, z-VAD-fmk was applied 1 h before administration of nutlin-3.

Alamar Blue assay

The assays were done in triplicate in 96-well plates. At the end of the treatment period, 1/10 volume of Alamar Blue (Biosource, Solingen, Germany) solution was added and cells incubated at 37°C for an additional 3 h. The fluorescence was measured on a BMG Labtech (Offenburg, Germany) FLUOstar Omega using an excitation/emission wave length of 544/590 nm. Results are expressed as a percentage of fluorescence of untreated control cells.

Flow cytometric analysis of cell death

Cell death was assessed by determining the integrity of the cell membrane by flow cytometric analysis of propidium iodide (PI) uptake. After harvesting, cells were incubated for 5 min in 2 μg/ml PI in PBS at 4°C in the dark and PI uptake was measured on a BD (Heidelberg, Germany) FACSCanto II. 10,000 cells were analyzed in each sample;

Fig. 1 Nutlin-3 and HDACi cooperate in affecting cell viability in A549 cells. One hour after administration of nutlin-3, cells were exposed to HDACi for another 72 h. Cell viability was assessed by Alamar Blue assay. Means ± SEM of each 3 separate experiments are shown
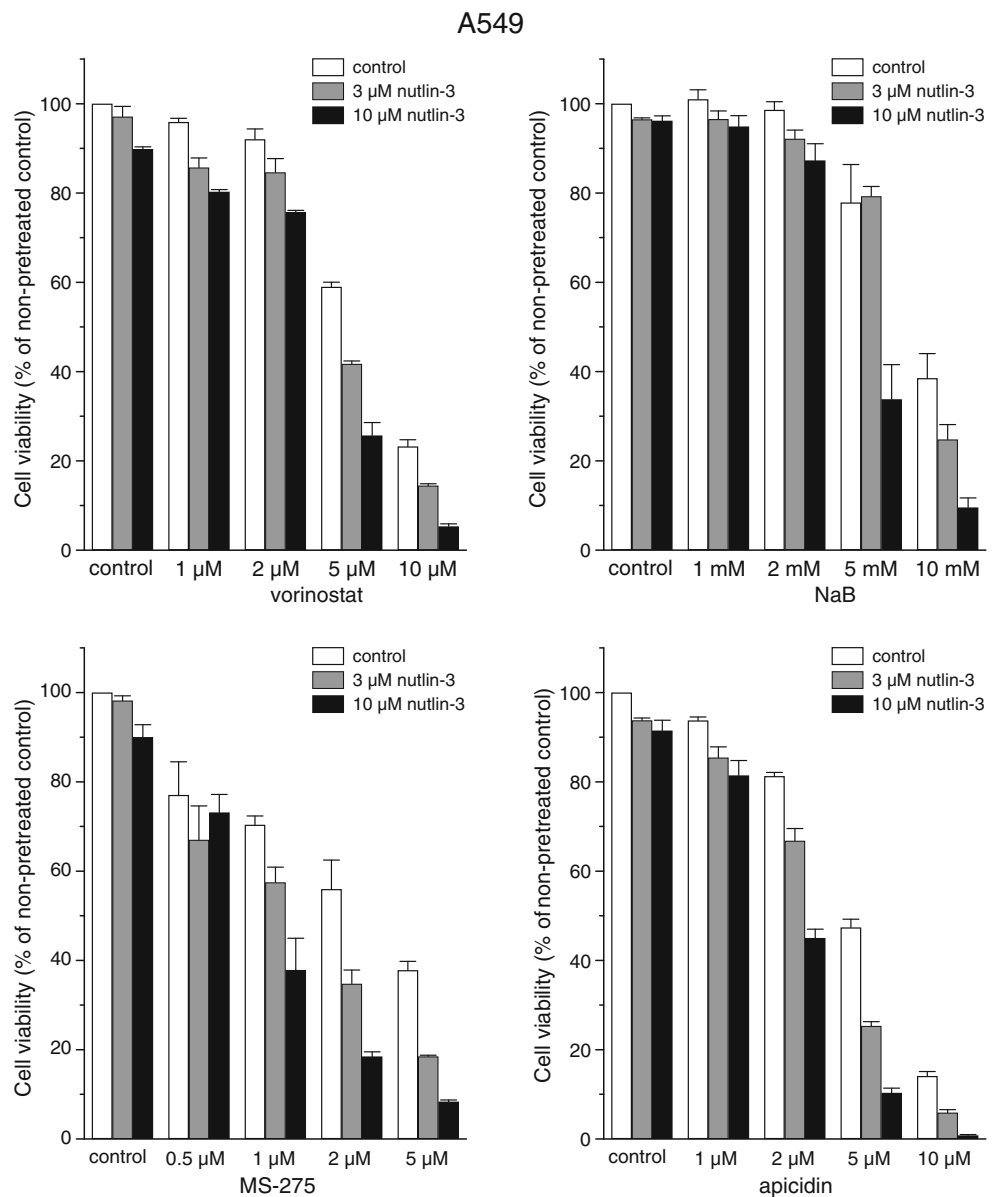
data were gated to exclude debris. The results from the assays were analyzed by the combination index (CI) method according to Chou and Talalay [26] using Calcusyn software from Biosoft (Cambridge, UK). CI values of <1, = 1 and >1 indicate synergism, additivism and antagonism, respectively.

Flow cytometric analysis of mitochondrial transmembrane potential ($\Delta\psi_m$)

$\Delta\psi_m$ was determined by assessing the accumulation of the cationic lipophilic fluorochrome 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] in the mitochondrial matrix. At the end of the treatment period, cells were incubated with 50 nM $DiOC_6(3)$ (Molecular Probes, Eugene, OR, USA) at 37°C for 30 min before harvesting. After washing, 10,000

cells were analyzed using the FACSCanto II. Data were gated to exclude debris.

Caspase-3 activity

Caspase-3 activity was measured using the fluorogenic substrate Ac-DEVD-AMC (Bachem, Weil am Rhein, Germany). After harvesting, cells were lysed in 10 mM Tris-HCl, 10 mM $NaH_2PO_4/NaHPO_4$ (pH 7.5), 130 mM NaCl, 1% Triton-X-100, and 10 mM $Na_4P_2O_7$ and then incubated with 20 mM Hepes (pH 7.5), 10% glycerol, 2 mM DTT, and 25 μg/ml Ac-DEVD-AMC at 37°C for 2 h. The release of AMC was analyzed on the FLUOstar Omega using an excitation/emission wavelength of 355/ 460 nm. Relative caspase-3 activities were calculated as a ratio of emission of treated cells to untreated cells.

Fig. 2 Nutlin-3 and HDACi cooperate in inducing cell death and $\Delta\psi_m$ loss in A549 cells. One hour after administration of nutlin-3, cells were exposed to HDACi for another 48 h. **a**, Cell death was determined by flow cytometric analysis of PI uptake. **b**, $\Delta\psi_m$ was assessed by flow cytometric analysis of $DiOC_6(3)$ staining. Means ± SEM of each 3 separate experiments are shown
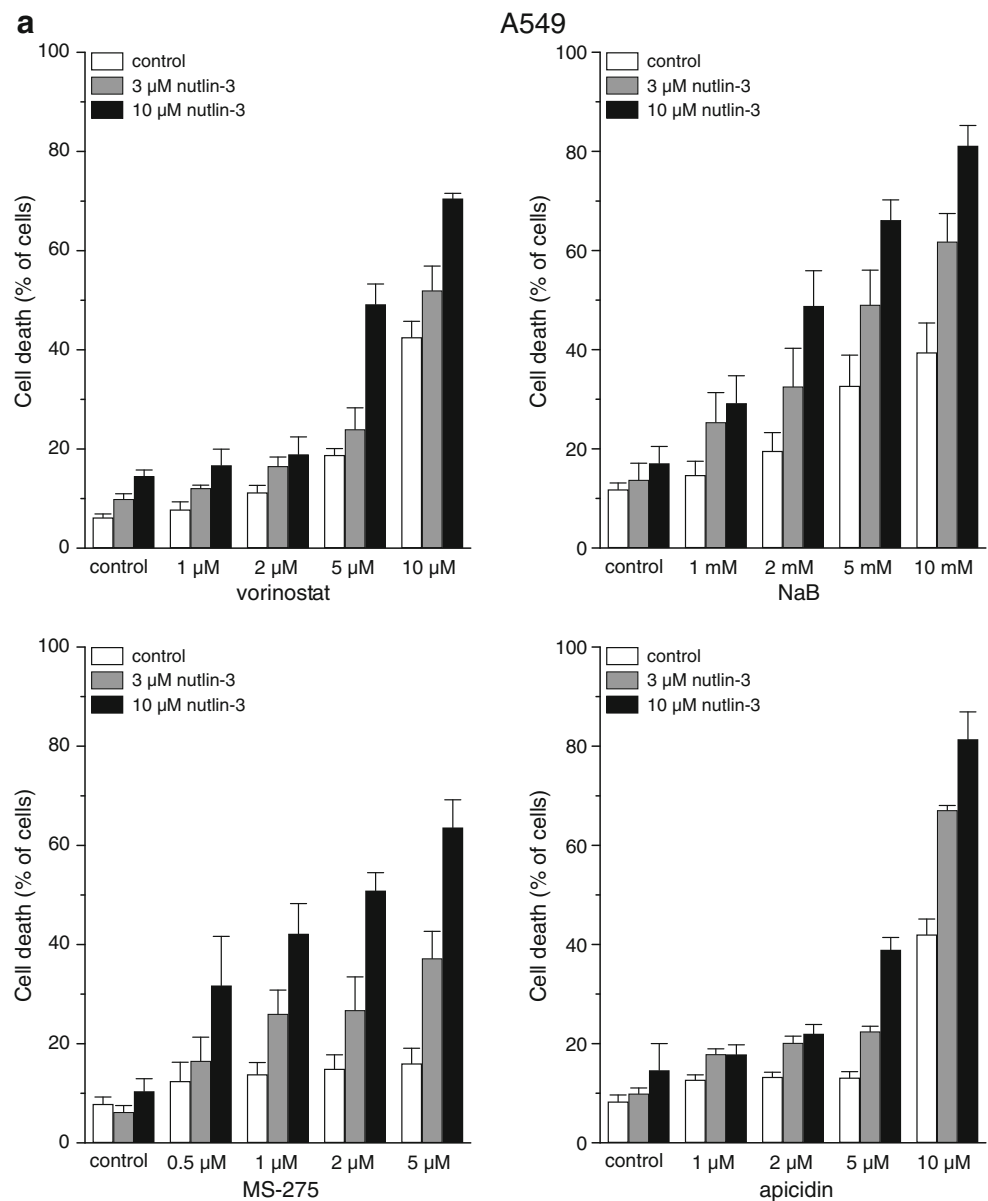

Immunoblotting

After harvesting, cells were lysed on ice for 15 min in 40 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany) followed by brief sonication. Protein concentration was assayed using bicinchoninic acid (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. 60–90 μg of total cellular protein per lane were separated by standard SDS-PAGE on 10% gel and electrophoretically transferred to nitrocellulose membrane (Whatman, Dassel, Germany). After blocking in 100 mM Tris-HCl (pH 8.0), 450 mM NaCl, 5% dry milk and 0.05% Tween 20, p53 was immunodetected using mouse anti-p53 DO-2 monoclonal antibody (dilution 1:500; sc-53394, Santa Cruz Biotechnology, Heidelberg,

Germany) and acetyl-p53 (Lys373/382) was immunodetected using rabbit anti-AcK373/382-p53 polyclonal antibody (dilution 1:500; 06-758, Upstate, Temecula, CA, USA). Equal loading of protein was verified by detection of GAPDH using mouse anti-GAPDH monoclonal antibody (dilution 1:10,000; Biodesign International, Saco, ME, USA). Peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (dilution 1:25,000; Dianova, Hamburg, Germany) were used as secondary antibodies followed by enhanced chemiluminescence detection (GE Healthcare, Freiburg, Germany) of specific signals.

Quantitative real-time RT-PCR

Total RNA was isolated using Peqgold Total RNA Kit including DNase digestion (Peqlab, Erlangen, Germany).

Fig. 2 (continued)
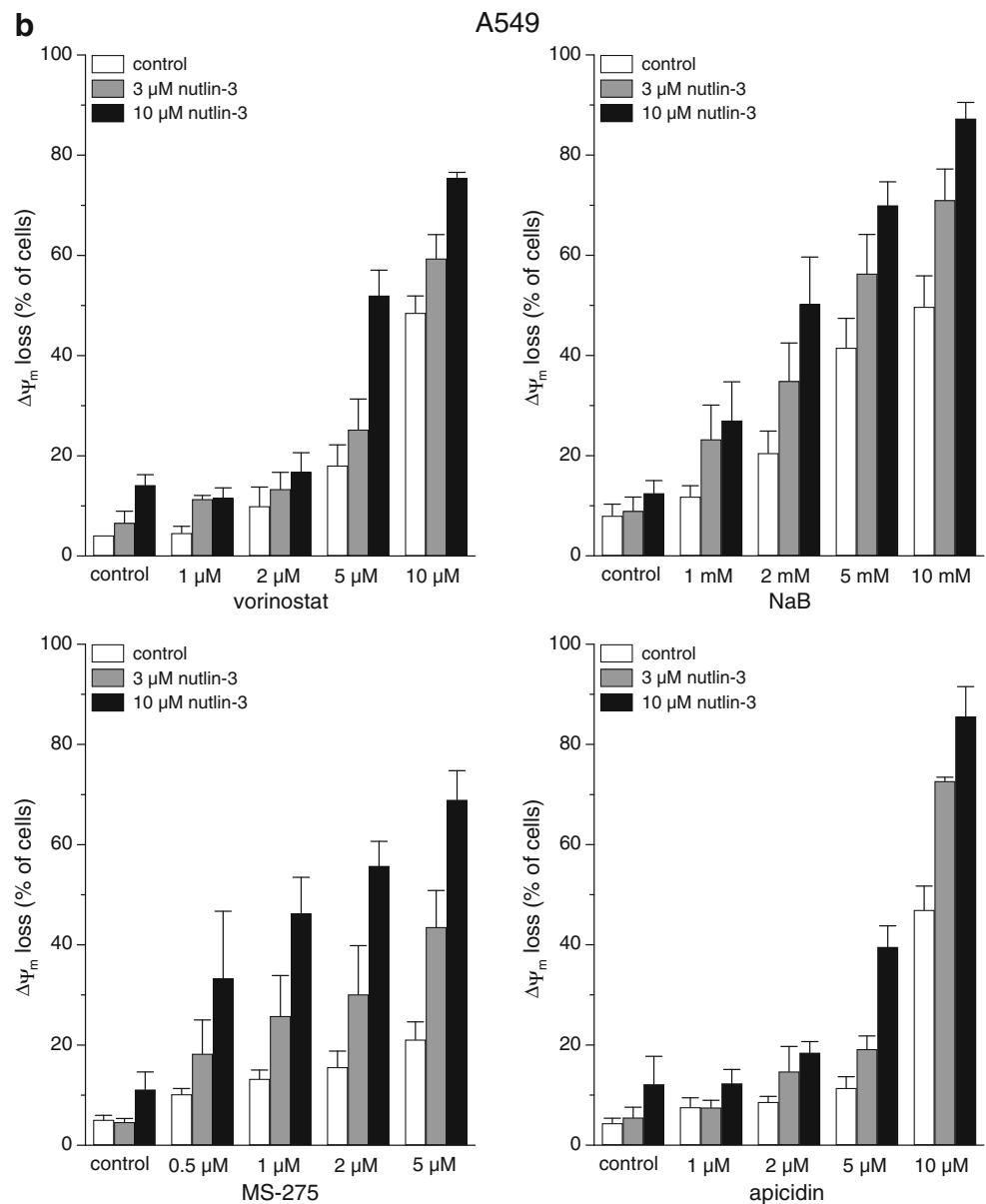
Table 1 Combination index values for nutlin-3 plus vorinostat in A549 cells

Nutlin-3 (μM)	Vorinostat (μM)	CI
3	1	1.030
3	2	0.858
3	5	1.045
3	10	0.509
10	1	0.956
10	2	0.968
10	5	0.295
10	10	0.212

Based on data from Fig. 2a, CI values were calculated using the Chou-Talalay method

Table 2 Combination index values for nutlin-3 plus NaB in A549 cells

Nutlin-3 (μM)	NaB (mM)	CI
3	1	0.344
3	2	0.357
3	5	0.279
3	10	0.236
10	1	0.267
10	2	0.114
10	5	0.086
10	10	0.046

Based on data from Fig. 2a, CI values were calculated using the Chou-Talalay method

Table 3 Combination index values for nutlin-3 plus MS-275 in A549 cells

Nutlin-3 (μM)	MS-275 (μM)	CI
3	0.5	0.178
3	1	0.030
3	2	0.028
3	5	0.009
10	0.5	0.052
10	1	0.020
10	2	0.009
10	5	0.003

Based on data from Fig. 2a, CI values were calculated using the Chou-Talalay method

RNA was transcribed into cDNA using Omniscript (Qiagen, Hilden, Germany). Quantitative PCR for MDM2 and MDM4 was performed using the 7900HT Fast Real-Time PCR system (Applied Biosystems, Darmstadt, Germany). Expression levels were normalized to β-2-microglobulin. Reactions were done in duplicate using Applied Biosystems Taqman Gene Expression Assays (MDM2: Hs99999008_m1; MDM4: Hs00159092_m1; β-2-microglobulin: Hs00187842_m1) and Universal PCR Master Mix. All procedures were carried out according to the manufacturers' protocols. The relative MDM2 and MDM4 expression was calculated by the $2^{(-\Delta\Delta Ct)}$ method [27].

Statistical analysis

Statistical significance of differences between experimental groups was determined using the paired two-tailed Student's t test.

Results

Nutlin-3 and HDACi synergize to induce cell death in p53 wild-type A549 cells

To assess a possible favorable interaction between nutlin-3 and HDACi, we initially monitored cell viability in the p53 wild-type lung cancer cell line A549 using Alamar Blue assay. Figure 1 shows that the four HDACi tested (vorinostat, NaB, MS-275, apicidin) reduced cell viability in a concentration-dependent manner. In contrast, cells were only marginally sensitive to nutlin-3 alone, in concordance with previous reports on its effects on A549 cells [5, 23]. However, in conjunction with HDACi, nutlin-3 caused a marked additional decrease in cell viability. For example, 5 μM vorino-stat reduced cell viability by 41% and 10 μM nutlin-3 reduced cell viability by 10%, while the combination of both compounds reduced cell viability by 74%.

The Alamar Blue assay is a convenient method to indirectly measure the number of viable cells, but it is not capable of distinguishing between effects on proliferation and cell death. Thus, to establish whether nutlin-3 and HDACi cooperated in eliciting cell death, we determined the latter by flow cytometric analysis of PI uptake. These measurements revealed a cooperative induction of cell death after combined treatment with nutlin-3 and HDACi (Fig. 2a). For instance, when administered individually, 10 μM nutlin-3 and 5 μM vorinostat elicited cell death in 15% or 19% of cells, respectively. When applied together, the agents evoked cell death in 49% of cells. To test for synergy, we analyzed these data by the CI method (CI <1 is indicative for a synergistic interaction; [26]). The calculated CI values indicated synergism for the combinations of nutlin-3 with vorinostat or apicidin at most concentrations, and for the combinations of nutlin-3 with NaB or MS-275 at all concentrations applied (Tables 1, 2, 3 and 4).

p53 is a potent inducer of apoptosis and as such it predominantly triggers the mitochondrial pathway of apoptosis [1]. The latter is also the major pathway for HDACi to elicit cell death [28]. We therefore assessed whether nutlin-3 and HDACi could interact at the mitochondrial level. Since this apoptotic pathway involves a perturbation of $\Delta\psi_m$, we determined $\Delta\psi_m$ dissipation by flow cytometric analysis of $DiOC_6(3)$ staining. As presented in Fig. 2b, the results reflect those of the cell death assay: nutlin-3 cooperated with all HDACi applied to induce decay of $\Delta\psi_m$.

Nutlin-3 protects A549 cells from paclitaxel-induced cytotoxic effects

Nutlin-3 has been reported to confer protection against paclitaxel [22, 23]. For comparison to the observed synergistic interaction of nutlin-3 and HDACi, we thus examined the combination of nutlin-3 and paclitaxel in A549 cells and determined cell death by PI uptake and

Table 4 Combination index values for nutlin-3 plus apicidin in A549 cells

Nutlin-3 (μM)	Apicidin (μM)	CI
3	1	0.484
3	2	0.615
3	5	1.087
3	10	0.079
10	1	0.854
10	2	0.682
10	5	0.301
10	10	0.022

Based on data from Fig. 2a, CI values were calculated using the Chou-Talalay method

Fig. 3 Nutlin-3 protects A549 cells from cytotoxicity of paclitaxel. One hour after administration of nutlin-3, cells were exposed to paclitaxel for another 48 h. Cell death was determined by flow cytometric analysis of PI uptake, and $\Delta\psi_m$ was assessed by flow cytometric analysis of $DiOC_6(3)$ staining. Means ± SEM of each 3 separate experiments are shown
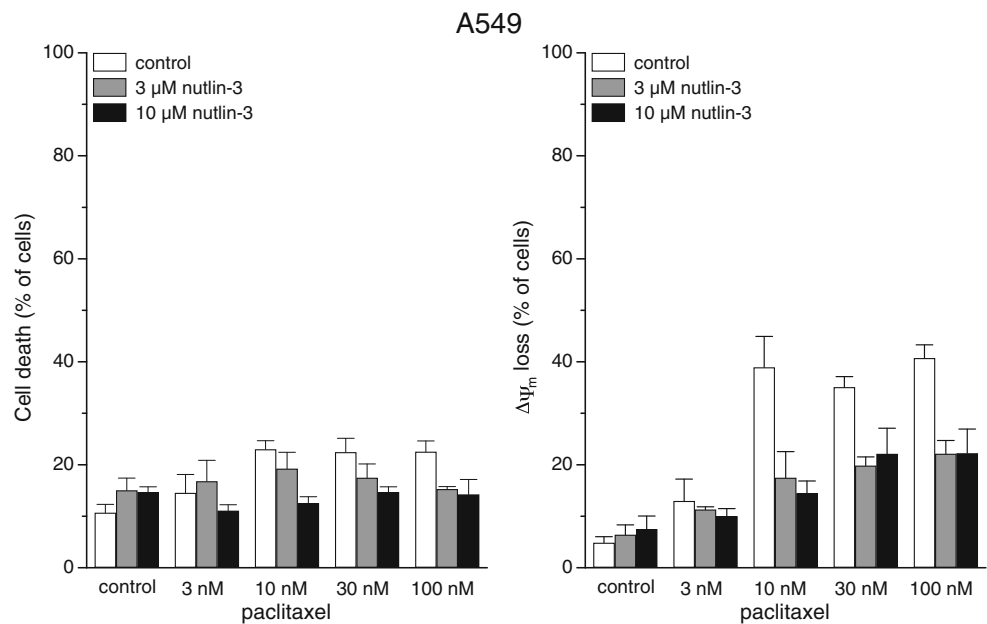
$\Delta\psi_m$ loss by $DiOC_6(3)$ staining. Both the assays revealed that nutlin-3 indeed protected the cells from the cytotoxicity of paclitaxel (Fig. 3). The CI analysis of the cell death results demonstrated a strong antagonism for this drug combination (Table 5).

Nutlin-3 and vorinostat synergize to induce cell death in p53 wild-type A2780 cells

To confirm the synergistic activity of nutlin-3 and HDACi in another cell line with wild-type p53, we engaged A2780 ovarian cancer cells. Figure 4a shows that nutlin-3 and the HDACi representatively used, vorinostat, also exerted a cooperative cytotoxic activity in A2780 cells, as judged by assessing cell death and $\Delta\psi_m$ dissipation. The cell death data were analyzed by the CI method, evidencing a synergistic effect at all concentrations except one (Table 6).

Table 5 Combination index values for nutlin-3 plus paclitaxel in A549 cells

Nutlin-3 (µM)	Paclitaxel (nM)	CI
3	3	242
3	10	227,000
3	30	1701
3	100	77
10	3	33
10	10	40
10	30	33
10	100	136

Based on data from Fig. 3 (cell death), CI values were calculated using the Chou-Talalay method

To explore whether the synergistic action of nutlin-3 and HDACi involved caspases, we determined the activity of caspase-3 and we applied the pan-caspase inhibitor z-VAD-fmk in the PI uptake analysis. As presented in Fig. 4b, nutlin-3 and vorinostat cooperated in triggering caspase-3 activity. In line with this result, z-VAD-fmk reduced cell death induced by the combination of nutlin-3 and vorinostat (Fig. 4c).

Nutlin-3 and vorinostat do not synergize in p53 null PC-3 cells

Nutlin-3 functions by activating wild-type p53. To validate that the observed synergistic effect of the nutlin-3/HDACi combination was p53-dependent, we employed p53 null PC-3 prostate cancer cells. Figure 5 shows that vorinostat induced both cell death and $\Delta\psi_m$ decay in a dose-dependent fashion. In contrast, nutlin-3 had no effect, and the cytotoxicity of vorinostat in combination with nutlin-3 did not exceed that of vorinostat alone.

Vorinostat induces p53 hyperacetylation as well as downregulation of MDM2 and MDM4 gene expression

Because HDACi have been observed to activate p53 by acetylation [17–21], we tested whether the synergistic activity of nutlin-3/HDACi was associated with an increase in acetyl-p53. To determine the acetylation status of p53, we used an antibody specific for p53 acetylation at lysine residues 373 and 382. For comparison, we also assessed the abundance of total p53. As expected, nutlin-3 treatment raised total p53, both in the absence and in the presence of vorinostat (Fig. 6a). With respect to acetyl-p53 in A549

Fig. 4 Nutlin-3 and vorinostat cooperate in inducing cell death, $\Delta\psi_m$ loss and caspase-3 activity in A2780 cells. One hour after administration of nutlin-3, cells were exposed to vorinostat for another 24 h (caspase-3 assay) or 48 h (flow cytometric analyzes). **a**, Cell death was determined by flow cytometric analysis of PI uptake, and $\Delta\psi_m$ was assessed by flow cytometric analysis of $DiOC_6(3)$ staining. **b**, Caspase-3 activity was measured using the fluorogenic substrate Ac-DEVD-AFC; relative caspase-3 activities are the ratio of treated cells to untreated cells. **c**, z-VAD-fmk was applied 1 h before treatment with nutlin-3. Means ± SEM of each 3 separate experiments are shown (***$p < 0.005$)
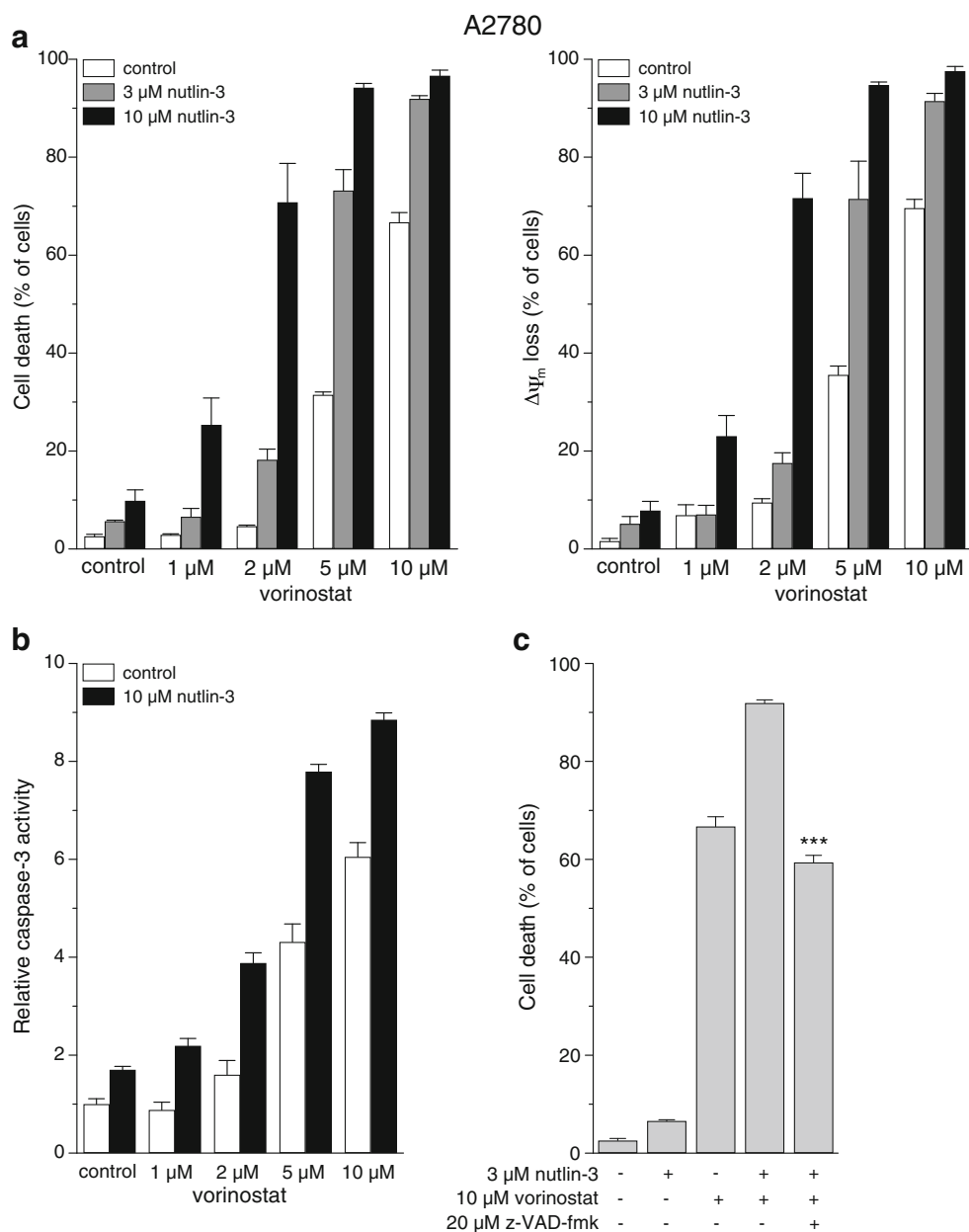

**Table 6** Combination index values for nutlin-3 plus vorinostat in A2780 cells

Nutlin-3 (μM)	Vorinostat (μM)	CI
3	1	1.254
3	2	0.656
3	5	0.395
3	10	0.375
10	1	0.338
10	2	0.170
10	5	0.155
10	10	0.231

Based on data from Fig. 4a (cell death), CI values were calculated using the Chou-Talalay method

cells, we made the noteworthy observation that nutlin-3 treatment—though enhancing total p53—did not alter the abundance of acetylated p53. However, cotreatment with vorinostat resulted in a considerable increase in acetyl-p53. In A2780 cells, nutlin-3 enhanced acetyl-p53, which was further enhanced by cotreatment with vorinostat.

MDM2 and MDM4 (also known as MDMX) are the main negative regulators of p53 function [2]. We thus wondered whether the enhancement of nutlin-3-induced apoptosis by HDACi could be correlated with an effect of HDACi on MDM2 and/or MDM4 gene expression. The regulation of MDM2 and MDM4 expression differs in that the former is induced by p53—that way producing a negative-feedback loop—while the latter is not [2]. In consistence, we found

Fig. 5 Nutlin-3 and vorinostat do not cooperate in PC-3 cells. One hour after administration of nutlin-3, cells were exposed to vorinostat for another 48 h. Cell death was determined by flow cytometric analysis of PI uptake, and $\Delta\psi_m$ was assessed by flow cytometric analysis of $DiOC_6(3)$ staining. Means ± SEM of each 3 separate experiments are shown
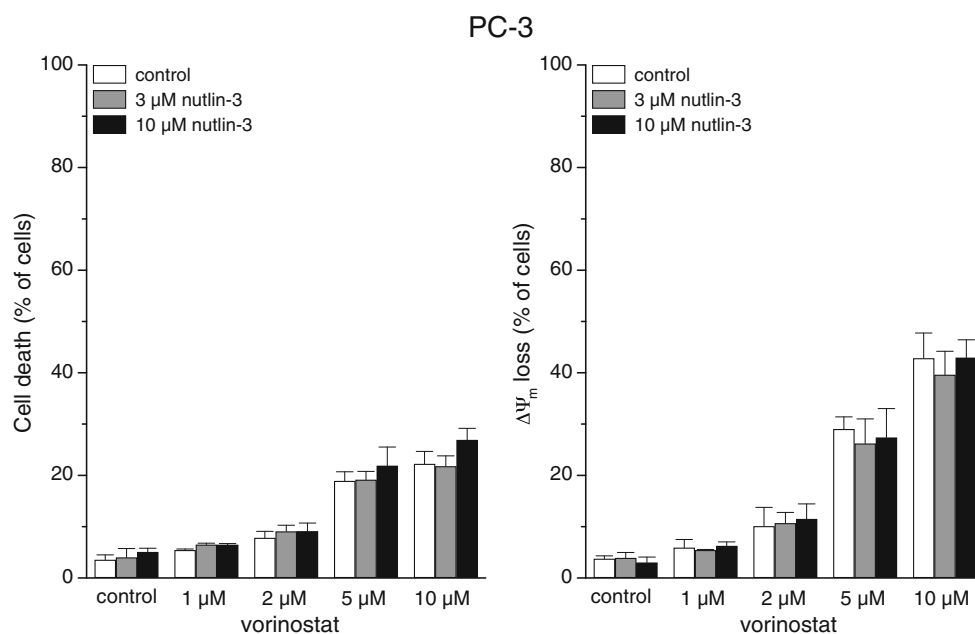
nutlin-3 to induce the gene expression of MDM2, but not of MDM4, as determined by real-time RT-PCR (Fig. 6b). Interestingly, vorinostat significantly reduced the constitutive gene expression of both MDM2 and MDM4 as well as the nutlin-3-elevated gene expression of MDM2.

Discussion

Our study is the first one to investigate the combined effect of nutlin-3 and HDACi on cancer cells. In this study, we have found that nutlin-3 cooperated with four compounds representative for four different structural classes of HDACi to affect p53 wild-type A549 and A2780, but not p53 null PC-3 carcinoma cells. We have shown that nutlin-3 and HDACi interacted to promote cell death, and CI analysis indicated that this interaction was synergistic. These results are in concordance with a report on the cooperative induction of p53 target genes by nutlin-3 treatment together with knockdown of HDAC2 [29].

Like the majority of anticancer drugs, nutlin-3 and HDACi elicit cell death through the induction of apoptosis [2, 28]. Our findings suggest that the coadministration of nutlin-3 and HDACi resulted in the collaborative initiation of apoptosis. We noted that nutlin-3 in conjunction with HDACi cooperatively induced $\Delta\psi_m$ dissipation and caspase-3 activation, both features characteristic of apoptosis. Furthermore, the use of the polycaspase inhibitor z-VAD-fmk revealed that caspase activity was to some extent required for nutlin-3/HDACi-mediated cell death. In a study employing ten randomly selected cancer cell lines, nutlin-3 has been reported to promote cell cycle arrest rather than apoptosis [5]. Nutlin-3 was found to induce G1

and/or G2/M arrest in all the cell lines, but effective apoptosis in only some of them, with A549 being the least responsive one. In agreement, we also found A549 as well as A2780 cells to be hardly sensitive to nutlin-3 alone. Therefore, our data argue that the cotreatment with HDACi turns the nutlin-3 effect from cell cycle arrest into apoptosis.

Which mechanism may account for the observed synergistic activity of nutlin-3 and HDACi? To shed light on this issue, we determined p53 acetylation and MDM2 and MDM4 gene expression. Our findings are compatible with the following explanations: (i) HDACi may interact synergistically with nutlin-3 by mediating hyperacetylation of p53. Acetylation has been shown to be required for p53 activation [16]. Although we expectedly found nutlin-3 to enhance the abundance of total p53, we failed to detect an effect of nutlin-3 on the abundance of acetylated p53 in A549 cells. However, nutlin-3 in combination with vorinostat produced a strong rise of acetyl-p53. These observations point to the following scenario: Nutlin-3 treatment increases total, nonacetylated—potentially less active—p53. HDACi treatment, in turn, induces acetylation—and hyperactivation—of p53.—(ii) HDACi may enhance the antitumor action of nutlin-3 by diminishing nutlin-3-induced MDM2 expression. MDM2 is upregulated by p53 activation in a feedback loop that negatively controls p53 activity [1]. Thus, by inducing its target MDM2, nutlin-3 could potentially limit its efficacy. We have found that vorinostat reduced the induction of MDM2 gene expression by nutlin-3.—(iii) HDACi may second nutlin-3 to elicit cancer cell death by downregulating MDM4 expression. MDM4 is the second main negative regulator of p53, which is structurally homologues, but functionally not redundant

a

p53 (DO-2)

A549

A2780

| nutlin-3 (µM) | 0 | 3 | 10 | 0 | 3 | 10 |
| vorinostat (µM) | 0 | 0 | 0 | 10 | 10 | 10 |

acetyl-p53 (Lys373/382)

A549

A2780

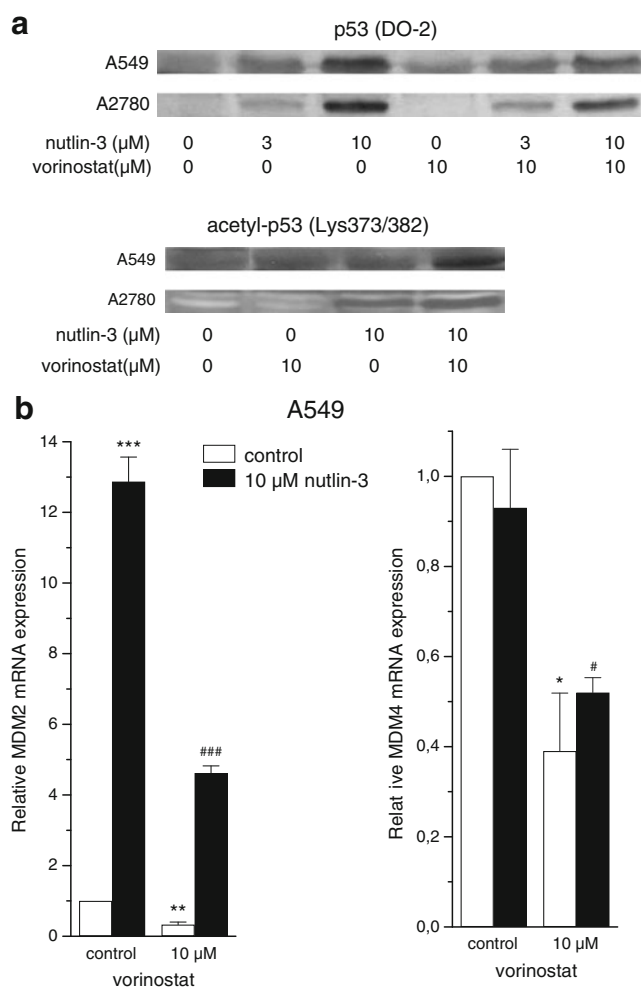| nutlin-3 (µM) | 0 | 0 | 10 | 10 |
| vorinostat (µM) | 0 | 10 | 0 | 10 |

b

Fig. 6 Vorinostat induces p53 hyperacetylation and downregulation of MDM2 and MDM4 gene expression. One hour after administration of nutlin-3, cells were exposed to vorinostat for another 24 h. **a**, The acetylation status of p53 was analyzed by immunoblotting using an anti-acetyl-p53 (Lys373/382) antibody; the expression of total p53 was determined using the anti-p53 DO-2 antibody. **b**, MDM2 and MDM4 mRNA expression levels were determined by real-time RT-PCR and normalized to β-2-microglobulin mRNA levels. Means ± SEM of each 3 separate experiments are shown (nutlin-3- or vorinostat-treated vs. untreated: $*p<0.05$, $**p<0.01$, $***p<0.005$; nutlin-3/vorinostat-treated vs. nutlin-3-treated: $^{\#}p<0.05$, $^{\#\#\#}p<0.005$)

to MDM2. Unfortunately, nutlin-3 as well as other MDM2-targeting agents fail to also effectively target MDM4 [2]. Rather, MDM4 overexpression endows cancer cells with resistance to nutlin-3 [30–32]. Consistently, simultaneous targeting of MDM2 and MDM4 has been shown to cause effective apoptosis of tumor cells overexpressing MDM2 and MDM4 [33]. We have observed that vorinostat reduced MDM4 gene expression.

These explanations are by no means mutually exclusive, but they imply that HDACi enhance nutlin-3-induced apoptosis by exerting p53-dependent and p53-independent effects. On the one hand, HDACi increase p53 acetylation,

which supposedly propels p53 to induce its target genes. HDACi-mediated p53 acetylation has indeed been observed to stimulate the expression of p53 targets [18]. On the other, HDACi suppress gene expression of MDM2, an effect that can best be explained by assuming that HDACi exert p53-independent effects on p53 targets. In fact, HDACi have repeatedly been shown to induce the expression of the p53 target p21 in a p53-independent fashion [34, 35]. Consequently, it is also conceivable that HDACi have a p53-independent effect on the regulation of other—possibly proapoptotic—p53 targets, thereby lowering the threshold for nutlin-3-induced apoptosis.

From a clinical perspective, two aspects need to be taken into consideration for nutlin-3 combination regimens. First, evidently, nutlin-3 has to cooperate with the agent it is combined with. Nutlin-3 has been found to synergize with e.g. the genotoxic cytostatics doxorubicin, chlorambucil, cisplatin, etoposide and topotecan [36–38], the apoptosis-inducing cytokine TRAIL [39], the proteasome inhibitor bortezomib [40] and the BCR/ABL kinase inhibitor imatinib [41], but it has also been found to antagonize paclitaxel [22, 23], the antimetabolites gemcitabine and cytarabine [42], and the polo-like kinase 1 inhibitor BI-2536 [43]. Thus, potential nutlin-3 combination therapies have to be carefully evaluated to ensure an efficacious application (of note, the protecting effect of nutlin-3 may be exploited for the treatment of patients with p53 mutant tumors, a concept known as cyclotherapy [2]). Our study shows that HDACi are a class of drugs that synergize with nutlin-3, and at the same time it confirms that nutlin-3 protects from paclitaxel-mediated cytotoxic effects. Second, nutlin-3 may best be combined with agents that elicit cancer cell death independent of p53. Nutlin-3 is a very effective activator of p53 and, thus, may put heavy selection pressure on tumor cells for loss of p53 function to generate treatment resistance. The development of nutlin-3 resistance may be avoided by cotargeting p53-independent pathways. HDACi have been shown, both in vitro and in vivo, to induce apoptosis p53-independently [35, 44–46], and our data presented here also demonstrate the susceptibility of p53 null PC-3 cells to vorinostat-induced cell death. Moreover, support for the general usefulness of HDACi in avoiding drug resistance comes from a recent work, in which it has been shown that coexposure to HDACi prevented the development of resistance against cisplatin, the EGFR kinase inhibitor erlotinib and the RAF kinase inhibitor AZ628 [47].

Activation of the p53 tumor pathway, mediated by agents such as nutlin-3, is a promising strategy for anticancer therapy. However, the antineoplastic activity of nutlin-3 applied as single agent may be insufficient. For instance, in an orthotopic retinoblastoma model, nutlin-3 alone failed to decrease tumor growth, while its combination with topotecan produced a remarkable anticancer effect

[38]. Hence, nutlin-3 may have to be combined with other treatment regimens to achieve a successful outcome. Herein, we have described that the antineoplastic efficacy of nutlin-3 can be considerably enhanced by cotreatment with HDACi. This effect was observed with all four structurally diverse compounds evaluated, suggesting an HDACi class effect. In conclusion, our in vitro findings provide a rationale for an in vivo exploration into the therapeutic potential of this drug combination.

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