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p53-dependent and p53-independent anticancer effects of different histone deacetylase inhibitors

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Background: Histone deacetylase inhibitors (HDACi) are promising antineoplastic agents, but their precise mechanisms of actions are not well understood. In particular, the relevance of p53 for HDACi-induced effects has not been fully elucidated. We investigated the anticancer effects of four structurally distinct HDACi, vorinostat, entinostat, apicidin and valproic acid, using isogenic HCT-116 colon cancer cell lines differing in p53 status.

Methods: Effects were assessed by MTT assay, flow-cytometric analyses of propidium iodide uptake, mitochondrial depolarisation and cell-cycle distribution, as well as by gene expression profiling.

Results: Vorinostat was equally effective in p53 wild-type and null cells, whereas entinostat was less effective in p53 null cells. Histone deacetylase inhibitors treatment suppressed the expression of MDM2 and increased the abundance of p53. Combination treatments showed that vorinostat enhanced the cytotoxic activity of TRAIL and bortezomib, independent of the cellular p53 status. Investigations into the effects of an inhibitor of the sirtuin class of HDAC, tenovin-1, revealed that tenovin-1-mediated cell death hinged on p53.

Conclusion: These results demonstrate that vorinostat activates p53, but does not require p53 for inducing its anticancer action. Yet they also demonstrate that entinostat-induced cytotoxic effects partially depend on p53, indicating that different HDACi have a different requirement for p53.

In recent years, the importance of epigenetics in human cancer development has grown increasingly clear (Baylin and Jones, 2011). Epigenetic mechanisms, such as DNA methylation and histone acetylation, have thus become a focus of intense interest for the development of antineoplastic agents (Arrowsmith *et al*, 2012). Among the first fruits of this quest are drugs that target histone deacetylases (HDACs), the HDAC inhibitors (HDACi) (Spiegel *et al*, 2012). Two HDACi, vorinostat (also known as suberoylanilide hydroxamic acid, SAHA) and romidepsin (also known as FK228 and depsipeptide), have been approved by the US Food and Drug Administration as second-line treatments for cutaneous T-cell lymphoma, and several others, for example, panobinostat, belinostat and entinostat, are currently in phase II or III development for diverse oncological indications (Arrowsmith *et al*, 2012).

Histone deacetylase inhibitors block, with distinct specificities, a subset of the 18 isozymes belonging to the HDAC superfamily (Arrowsmith *et al*, 2012; Spiegel *et al*, 2012). The latter is divided into five phylogenetic classes: class I (HDAC1, 2, 3 and 8), IIa (HDAC4, 5, 7 and 9), IIb (HDAC6 and 10), III (sirtuins 1–7; SIRT1–7) and IV (HDAC11). The class III enzymes, the sirtuins, are biochemically unrelated to the enzymes of the other classes. They require NAD^+ instead of Zn^{2+} as a cofactor for their catalytic activity, and they are not affected by HDACi (of

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note, as in the majority of reports, the acronym 'HDACi' here refers only to the inhibitors of the Zn^{2+}-dependent HDAC isozymes; sirtuin inhibitors are abbreviated as 'SIRTi'). HDACi elicit various antineoplastic effects: they stimulate differentiation, block proliferation, induce apoptosis, exert antiangiogenic and immune stimulatory activity, and, perhaps most importantly, augment the efficacy of other therapeutic regimens in tumour cells (Bots and Johnstone, 2009; Müller and Krämer, 2010; Spiegel *et al*, 2012). Histone deacetylase inhibitors function by increasing histone acetylation, but also by enhancing acetylation of non-histone proteins including p53 (Buchwald *et al*, 2009).

The outcome of anticancer chemotherapy can depend on the tumour's *p53* status (Soussi and Beroud, 2001). Tumour cells with mutated or deleted *p53* tend to be less responsive to several commonly used chemotherapeutic drugs, such as topoisomerase inhibitors, anthracyclines or alkylating agents (Lowe *et al*, 1993; Aas *et al*, 1996; O'Connor *et al*, 1997). Since *p53* is the most frequently mutated gene in human malignancies, therapies that do not depend on functional p53 are in general clinically preferable. Hence, it is of considerable relevance to define a drug's requirement for functional p53 to exert its antitumoural activity.

To which extent the anticancer effects of HDACi are influenced by the tumour's *p53* status has not been unequivocally resolved. The majority of works addressing this issue point to a largely p53-independent action of HDACi (Vrana *et al*, 1999; Huang *et al*, 2000; Ruefli *et al*, 2001; Yu *et al*, 2002; Insinga *et al*, 2005; Sonnemann *et al*, 2006; Lindemann *et al*, 2007; Ellis *et al*, 2009). Other studies, however, suggest an essential role of p53 in the response of tumour cells to HDACi treatment (Henderson *et al*, 2003; Joseph *et al*, 2005; Kim *et al*, 2006; Condorelli *et al*, 2008; Hacker *et al*, 2011; Bajbouj *et al*, 2012; Meng *et al*, 2012). These inconsistent observations may in part be due to methodological differences, but they may also be due to the use of different HDACi or divergent HDACi-induced effects examined.

To shed more light on this issue, we employed variants of colon cancer HCT-116 cells differing only in their *p53* status (wild type and null; henceforth referred to as p53+ and p53−, respectively) (Bunz *et al*, 1998). In this isogenic pair, we assessed the effects of four HDACi belonging to four different structural classes, the hydroxamic acid vorinostat, the benzamide entinostat (also known as MS-275), the cyclic tetrapeptide apicidin and the short-chain fatty acid valproic acid (VPA). These HDACi have different selectivities: vorinostat inhibits HDAC1, 2, 3, 6 and 8, entinostat inhibits HDAC1, 2 and 3, and apicidin and VPA inhibit HDAC1, 2, 3 and 8 (Bradner *et al*, 2010) (Supplementary Table 1). For comparison, we also assessed to which extent the *p53* status would alter the effects of two agents supposed to depend on p53, the sirtuin inhibitor tenovin-1 (Lain *et al*, 2008) and the topoisomerase II inhibitor etoposide (Lowe *et al*, 1993). We found that the single-agent responses to vorinostat, apicidin and VPA were largely independent of p53, while those to entinostat were partially and those to tenovin-1 were predominantly p53 dependent. We also found that vorinostat cooperated with various other agents to induce cell death in cancer cells irrespective of their *p53* status.

MATERIALS AND METHODS

Reagents. Vorinostat, entinostat, apicidin, etoposide, z-VAD-fmk, pifithrin-α and caffeic acid phenethyl ester (CAPE) were purchased from Enzo Life Sciences (Lörrach, Germany). Valproic acid was purchased from Sigma (Deisenhofen, Germany). Tenovin-1 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Bortezomib was purchased from LC Laboratories (Woburn, MA, USA). TRAIL was purchased from Peprotech (Hamburg, Germany). Obatoclax was a gift from Dr C Wichmann (Munich, Germany).

Cell culture. HCT-116 p53+ and p53− cells were a gift from Dr B Vogelstein (Baltimore, MD, USA). They were maintained in high-glucose DMEM with stable glutamine supplemented with 10% foetal calf serum, 100 units per ml penicillin G sodium and 100 $\mu g\,ml^{-1}$ streptomycin sulphate (PAA, Cölbe, Germany). 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 per well in 6-well plates (all assays except MTT assay) or 30 000 cells per well in 48-well plates (MTT assay) and treated with HDACi, tenovin-1 or etoposide for 24 h (caspase-3 assay, quantitative PCR and immunoblotting) or 48 h (flow-cytometric analyses and MTT assay). In the respective experiments, cells were pretreated with z-VAD-fmk, pifithrin-α, CAPE or obatoclax for 1 h. In the experiments with bortezomib and TRAIL, cells were pretreated with vorinostat for 4 h and then exposed to bortezomib or TRAIL for additional 48 h.

Flow-cytometric analysis of cell death and mitochondrial transmembrane potential ($\Delta\psi m$). 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 $\mu g\,ml^{-1}$ PI in PBS at 4 °C in the dark. $\Delta\psi_m$ was assessed by determining the accumulation of 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] (Molecular Probes, Eugene, OR, USA) in the mitochondrial matrix. Before harvesting, cells were incubated with 50 nM $DiOC_6(3)$ at 37 °C for 30 min. In both read-outs, 10 000 cells were analysed in each sample on a BD FACSCanto II (Becton Dickinson, Heidelberg, Germany); data were gated to exclude debris.

MTT assay. At the end of the treatment periods, MTT reagent was added to a final concentration of 0.5 $mg\,ml^{-1}$. After incubation at 37 °C for additional 2 h, the supernatant was removed and the water-insoluble product formazan was dissolved in 2-propanol. Formazan staining was determined by measuring the absorbance at 596 nm using a BMG Labtech FLUOstar Optima plate reader (BMG Labtech, Ortenberg, Germany).

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 $\mu g\,ml^{-1}$ Ac-DEVD-AMC at 37 °C for 2 h. The release of AMC was analysed on a BMG Labtech FLUOstar Omega (BMG Labtech) 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.

Flow-cytometric analysis of DNA content. To measure DNA content, ethanol-fixed cells were analysed for PI incorporation into DNA. After harvesting, cells were washed twice with PBS and fixed in 70% ethanol at −20 °C overnight. After centrifugation, cells were resuspended in PBS containing 1% glucose, 50 $\mu g\,ml^{-1}$ RNase A (Roche, Mannheim, Germany) and 50 $\mu g\,ml^{-1}$ PI and incubated in the dark at room temperature for 30 min. Flow-cytometric analysis was performed on the FACSCanto II. In all, 20 000 cells were analysed in each sample; data were gated to exclude debris. The different cell-cycle phases were quantified using the FACSDiva (Becton Dickinson) software.

Quantitative real-time RT–PCR. Total RNA was isolated using the Peqgold Total RNA Kit including DNase digestion (Peqlab, Erlangen, Germany). RNA was transcribed into cDNA using Omniscript (Qiagen, Hilden, Germany). Quantitative PCR for p53, MDM2, MDM4 and p21 was performed using the Applied Biosystems (Darmstadt, Germany) 7900HT Real-Time PCR system. Expression levels were normalised to β-2-microglobulin. Reactions were done in duplicate using Applied Biosystems Gene Expression Assays (p53: Hs01034249_m1; MDM2: Hs99999008_m1; MDM4: Hs00159092_m1; p21: Hs00355782_m1; β-2-microglobulin: Hs00187842_m1) and Universal PCR Master Mix. All procedures were performed according to the manufacturers' protocols. The relative gene expressions were calculated by the $2^{(-\Delta\Delta Ct)}$ method.

Immunoblotting. After harvesting by scraping, cells were resuspended in 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS supplemented with a protease inhibitor cocktail and 0.5 mM PMSF, 0.1% NaF, 1 mM Na_3VO_4, 20 mM glycerophosphate, 0.5 μM trichostatin A and 10 mM nicotinamide followed by brief sonification. In all, 10–20 μg of total cellular protein per lane was separated by standard SDS–PAGE on 13% gels and electrophoretically transferred onto PVDF membrane. After blocking in 100 mM Tris–HCl (pH 8.0), 450 mM NaCl, 5% dry milk and 0.05% Tween-20, proteins were immunodetected using antibodies against p53 (dilution 1 : 50 000), BCL2 (1 : 2500), MCL1 (1 : 5000), p53-inducible gene 3 (PIG3; 1 : 5000), p21 (1 : 1000), BAX (1 : 1000) (Santa Cruz Biotechnology, Heidelberg, Germany), acetylated (K382) p53 (1 : 1000; Cell Signaling Technology, Danvers, MA, USA), MDM2 (1 : 1000; Calbiochem, Darmstadt, Germany), cleaved PARP-1 (1 : 5000; BD Pharmingen, Heidelberg, Germany) and acetylated histone H4 (1 : 10 000; Upstate Biotechnology, Lake Placid, NY, USA). Equal loading of protein was verified by using either anti-HSP90 (1 : 10 000) or anti-β-actin (1 : 50 000) antibodies (Santa Cruz Biotechnology). Peroxidase-conjugated anti-mouse or anti-rabbit IgG (1 : 5000; Pierce, Rockford, IL, USA) was used as secondary antibodies followed by detection of specific signals using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Densitometric quantification

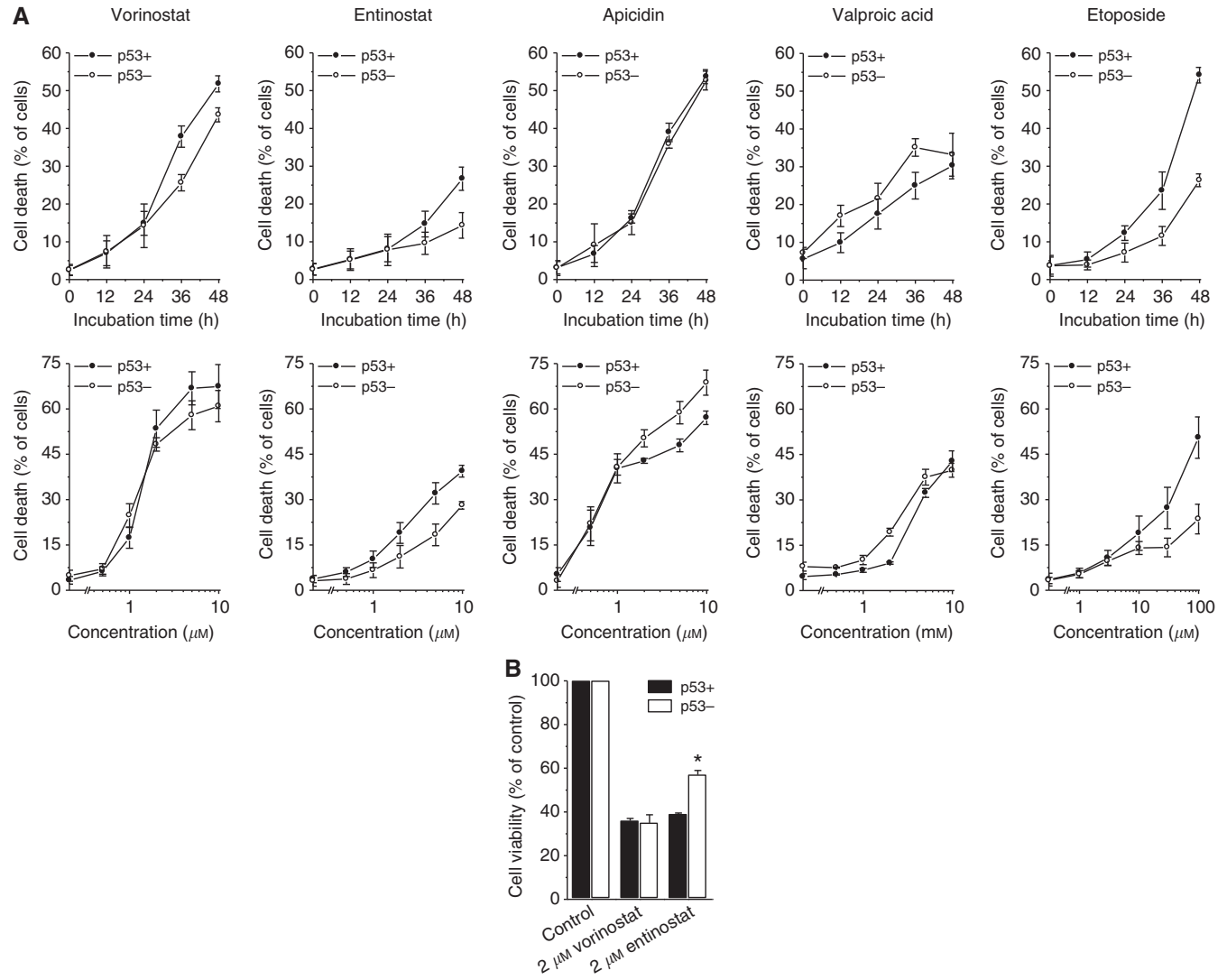


Figure 1. Antineoplastic effects of HDACi in HCT-116 p53+ and p53− cells. Cells were exposed to HDACi or etoposide for 24 h (**E**) or 48 h (**A–D**, **F**); in the time-course experiments (**A**, **C**), cells were exposed to 5 μM of vorinostat, entinostat and apicidin, 5 mM VPA or 100 μM etoposide. z-VAD-fmk was applied 1 h before treatment with HDACi (**D**, **F**). (**A**, **D**) Cell death was determined by flow-cytometric analysis of PI uptake. (**B**) Cell viability was determined by MTT assay. (**C**, **D**) $\Delta\psi_m$ was determined by flow-cytometric analysis of $DiOC_6(3)$ staining. (**E**) Caspase-3 activity was determined using the fluorogenic substrate Ac-DEVD-AMC, relative caspase-3 activities are the ratio of treated cells to untreated cells. (**F**) Cell-cycle profiles were determined by flow cytometry. Means ± s.e.m. of each three separate measurements are shown.

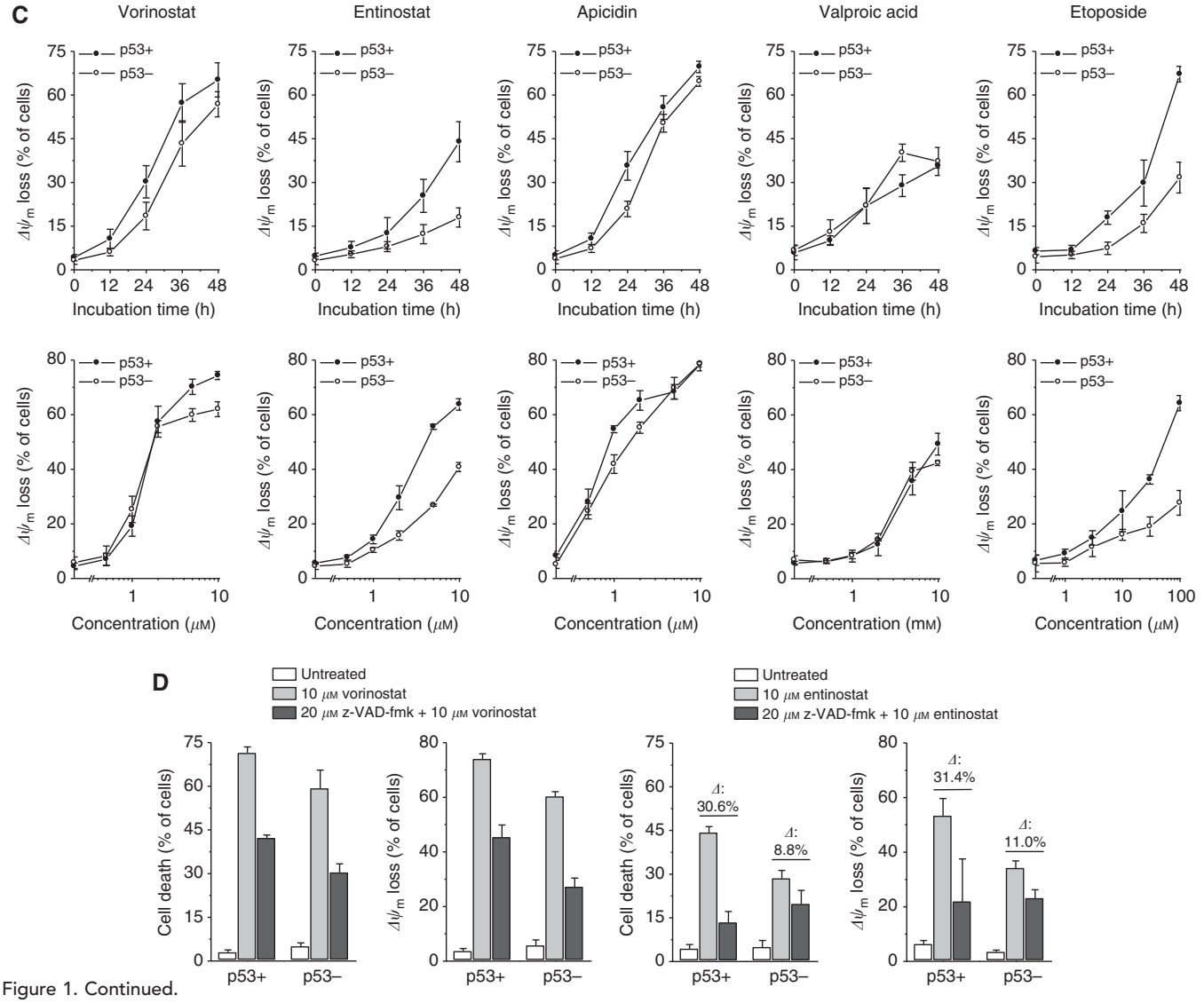


Figure 1. Continued.

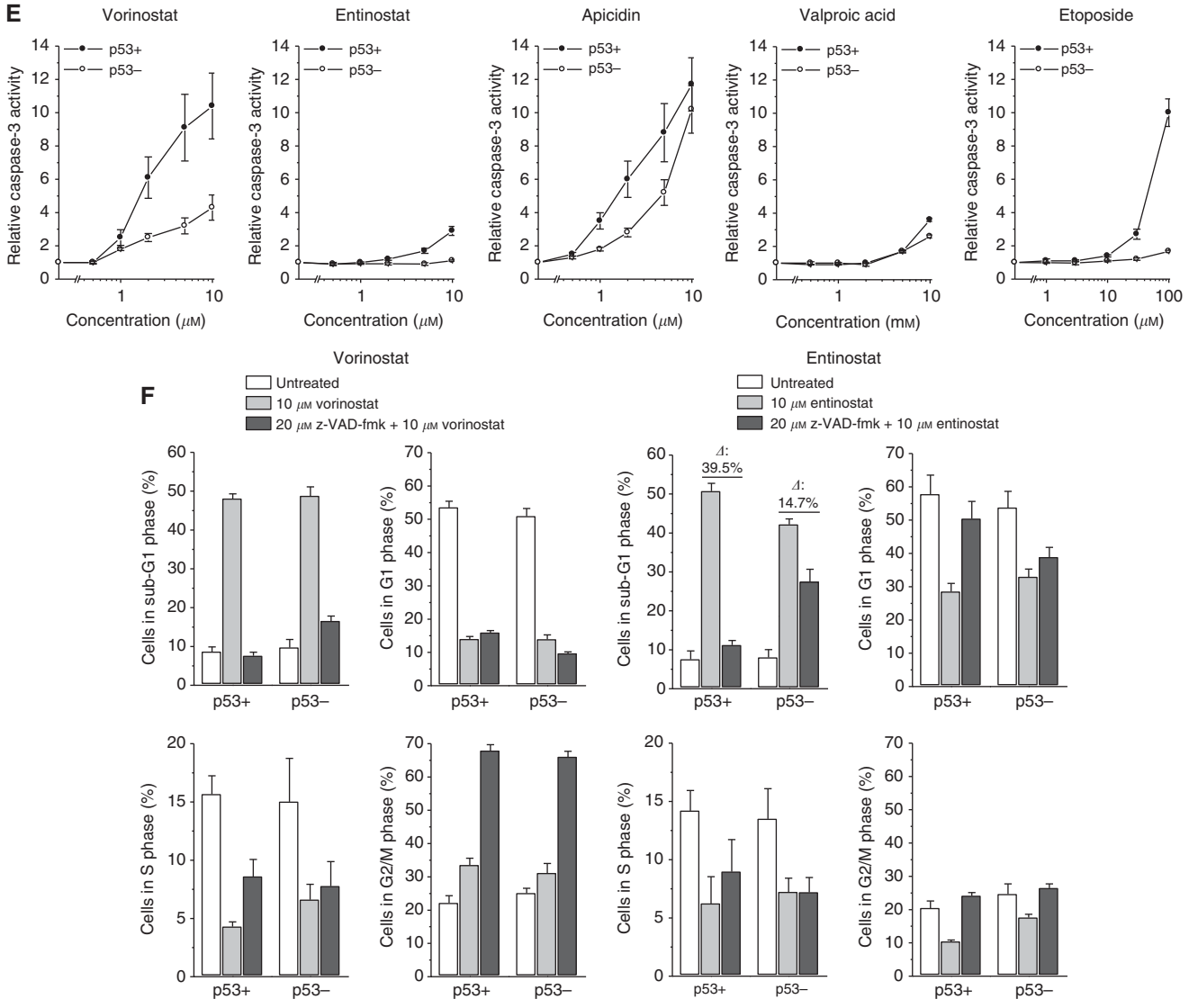
of MCL1 normalised to HSP90 band intensities was done with ImageJ 1.47 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Statistical significance of differences between experimental groups was determined using two-tailed Student's *t* test ($^*P<0.05$, $^{**}P<0.01$).

RESULTS

Antitumour effects of HDACi on HCT-116 p53+ and p53− cells. To investigate the effects induced by the four HDACi vorinostat, entinostat, apicidin and VPA in comparison with the cytostatic drug etoposide in HCT-116 p53+ and p53− colon cancer cells, we first determined cell death by flow-cytometric analysis of PI uptake. Throughout the figures, p53+ cells are depicted by closed symbols and p53− cells by open symbols. We assessed whether these compounds time and dose dependently affected the tumour cells. Figure 1A shows that all agents elicited cell death in a time- and dose-dependent manner in both p53+ and p53− cells. However, the agents differed in their efficacy regarding the cells' *p53* status: the extent of vorinostat-, apicidin- and VPA-induced cell death was practically independent of p53, whereas entinostat and etoposide were less effective in p53− cells. While we had expected etoposide to act in a p53-dependent manner (Lowe *et al*, 1993), we were surprised to discover the difference between the HDACi. Thus, to verify these data, we compared the effects of vorinostat and entinostat on cell viability by MTT assay. As presented in Figure 1B, the results of the MTT assay reflect those of the PI uptake measurements: vorinostat was as effective in p53− cells as in p53+ cells, whereas entinostat had a lesser effect on the viability of p53− cells. To further corroborate these data, we studied the action of the five drugs on $\Delta\psi_m$ by flow-cytometric analysis of $DiOC_6(3)$ staining. In all cases, the results matched those of the other assays: $\Delta\psi_m$ dissipation induced by vorinostat, apicidin and VPA did not depend on p53, whilst entinostat and etoposide provoked a smaller decay of $\Delta\psi_m$ in p53− cells (Figure 1C).

Like other antineoplastic agents, HDACi are supposed to trigger cell death through the induction of apoptosis (Spiegel *et al*, 2012). Because p53 has a key function in the apoptotic response (Vousden and Prives, 2009), we investigated its contribution to HDACi-evoked apoptosis. To begin with, we determined the effect of z-VAD-fmk on PI uptake and $DiOC_6(3)$ staining analyses. z-VAD-fmk is a broad-spectrum caspase inhibitor that serves to distinguish caspase-dependent from caspase-independent cell death (Chipuk and Green, 2005). Figure 1D shows that z-VAD-fmk had divergent impact on the action of vorinostat and entinostat: it diminished vorinostat-induced cell death and $\Delta\psi_m$ loss to roughly the same extent in p53+ and p53− cells,



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but reduced the effects of entinostat to a much lesser degree in p53 − cells (z-VAD-fmk alone had no effect; see Figure 2A and C). Second, we measured caspase-3 activity. All five agents activated caspase-3 in p53 + cells, albeit to different extents (Figure 1E). In contrast, entinostat and etoposide had no effect on caspase-3 activity in p53 − cells. Third, we assessed cells for apoptosis by staining the nuclei of ethanol-fixed cells with PI and determining the DNA content by flow cytometry; the sub-G1 fraction of cells with fragmented DNA $<2n$ is indicative of apoptosis. As demonstrated in Figure 1F, vorinostat- and entinostat-mediated apoptosis amounted to approximately the same level in p53 + and p53 − cells. Yet again, z-VAD-fmk showed different effectiveness dependent on the HDACi applied: it protected cells from vorinostat-triggered apoptosis irrespective of the *p53* status, while it was significantly less effective in p53 − cells in preventing entinostat-induced apoptosis. Interestingly, with the inhibition of caspase activity, vorinostat treatment produced a pronounced G2/M arrest—which was largely concealed in the absence of z-VAD-fmk—in both p53 + and p53 − cells (Figure 1F; Supplementary Figure 1).

Antitumour effects of the SIRTi tenovin-1 on HCT-116 p53 + and p53 − cells. So far, our analyses have revealed p53-dependent and -independent effects of different HDACi. Further, we investigated an SIRTi, because the class III HDAC isoforms, the sirtuins, have also recently emerged as promising targets for cancer therapy (Roth and Chen, 2013). An inhibitor of SIRT1 and SIRT2, tenovin-1, has been shown to affect a variety of cancer cell lines in a p53-dependent manner (Lain *et al*, 2008). To compare tenovin-1 with HDACi, we assessed its effects with the above methods. Figure 2A illustrates that the SIRTi was effective in inducing cell death, $\Delta\psi_m$ dissipation and caspase-3 activity in p53 + cells only. Different from its effect on HDACi (see Figure 1D), z-VAD-fmk entirely abolished tenovin-1's cytotoxic action. We further assessed the requirement of p53 for tenovin-1-mediated effects by using the p53 inhibitor pifithrin-α (Komarov *et al*, 1999): pifithrin-α alleviated tenovin-1-induced—but did not affect vorinostat-induced—cell death, substantiating that the antineoplastic activity of tenovin-1 hinged on p53, whereas that of vorinostat did not (Figure 2B). Cell-cycle analysis confirmed these findings and, moreover, revealed that tenovin-1 produced a G1 cell-cycle arrest in p53 + cells when apoptosis was blocked by z-VAD-fmk (Figure 2C; Supplementary Figure 2). Hence, the SIRTi tenovin-1 and the HDACi vorinostat exert distinct effects on cell-cycle control in HCT-116 cells.

Effects of HDACi and tenovin-1 on gene expression in HCT-116 p53 + and p53 − cells. The tumour suppressor p53 operates

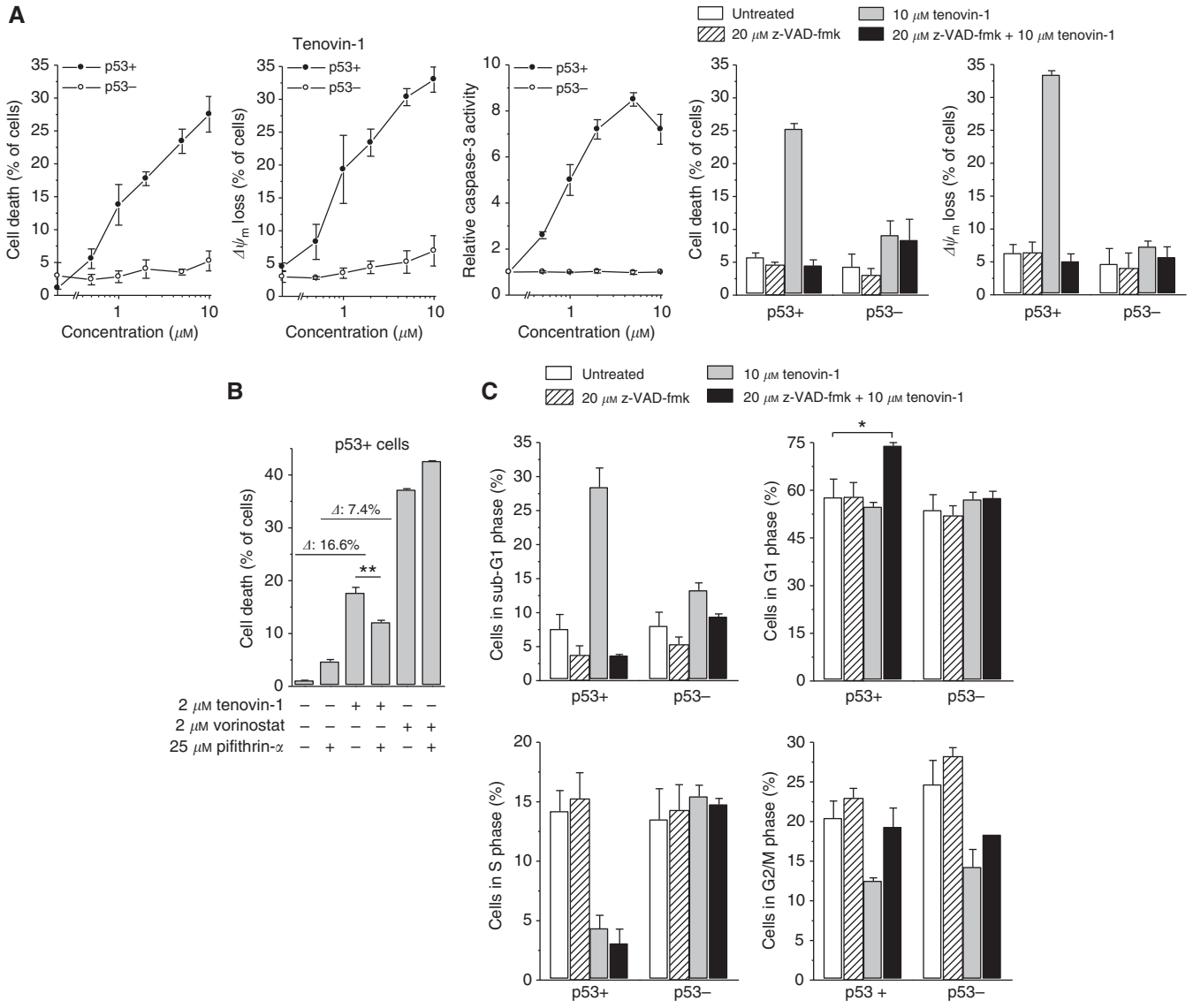

Figure 2. Antineoplastic effects of tenovin-1 in HCT-116 p53 + and p53 − cells. Cells were exposed to tenovin-1 for 24 h (caspase-3 activity) or 48 h (other read-outs). z-VAD-fmk was applied 1 h before treatment with tenovin-1 (**A**, **C**). (**A**) Cell death and $\Delta\psi_m$ were determined by flow-cytometric analyses of PI uptake or $DiOC_6(3)$ staining, respectively; caspase-3 activity was determined using the fluorogenic substrate Ac-DEVD-AMC, relative caspase-3 activities are the ratio of treated cells to untreated cells. (**B**) Pifithrin-α was applied 1 h before treatment with tenovin-1 or vorinostat; cell death was determined by flow-cytometric analysis of PI uptake. (**C**) Cell-cycle profiles were determined by flow cytometry. Means ± s.e.m. of each three separate measurements are shown. $^*P<0.05$; $^{**}P<0.01$.

predominantly by altering transcriptional programmes, though it can also exert transcription-independent activities (Vousden and Prives, 2009). Likewise, HDACi are supposed to function, at least in part, through their ability to modify gene expression (Bots and Johnstone, 2009; Müller and Krämer, 2010; Spiegel *et al*, 2012). To investigate a potential interplay of p53 and HDACi at the level of transcription, we determined the mRNA expression levels of p53 itself as well as of MDM2, MDM4 and p21 by real-time RT–PCR. MDM2 and MDM4 (also known as MDMX) are key negative regulators of p53; *MDM2* is a transcriptional target of p53 while *MDM4* is not (Vousden and Prives, 2009). MDM2 and MDM4 additionally differ in their modes of action: MDM2 primarily acts as an E3 ligase that ubiquitylates p53 for proteasomal degradation, while MDM4 has no ubiquitin ligase activity but acts by inhibiting p53's transactivation function and by seconding MDM2's action (Wade *et al*, 2013). Figure 3A shows that vorinostat, entinostat and apicidin suppressed the expression of *p53*, whereas tenovin-1 and etoposide left it unaffected. *MDM2* expression was downregulated by HDACi in p53 + and p53 − cells, but upregulated by tenovin-1 and etoposide in p53 + cells. All five agents decreased the expression of *MDM4*. *CDKN1A*, the gene encoding the cyclin-dependent kinase inhibitory protein p21, is a prominent transcriptional target of p53 (Vousden and Prives, 2009), whose expression can be activated by HDACi independent of p53 (Spiegel *et al*, 2012) (see also Figure 3B). In contrast, tenovin-1 and etoposide induced *CDKN1A* expression exclusively in p53 + cells.

To verify the mRNA expression data and to gain more insight into p53-dependent and -independent effects of HDACi and SIRTi, we employed western blot analyses (Figure 3B). Tenovin-1 strongly induced p53 expression, but, strikingly, also vorinostat and entinostat elevated p53 levels. All three agents raised the abundance of acetylated p53. MDM2 levels were decreased by HDACi, but increased by tenovin-1. p21 was induced by HDACi and tenovin-1 in p53 + cells, but only by HDACi in p53 − cells. Another expression target of p53, the proapoptotic BCL2 family member BAX (Vousden and Prives, 2009), was slightly enhanced

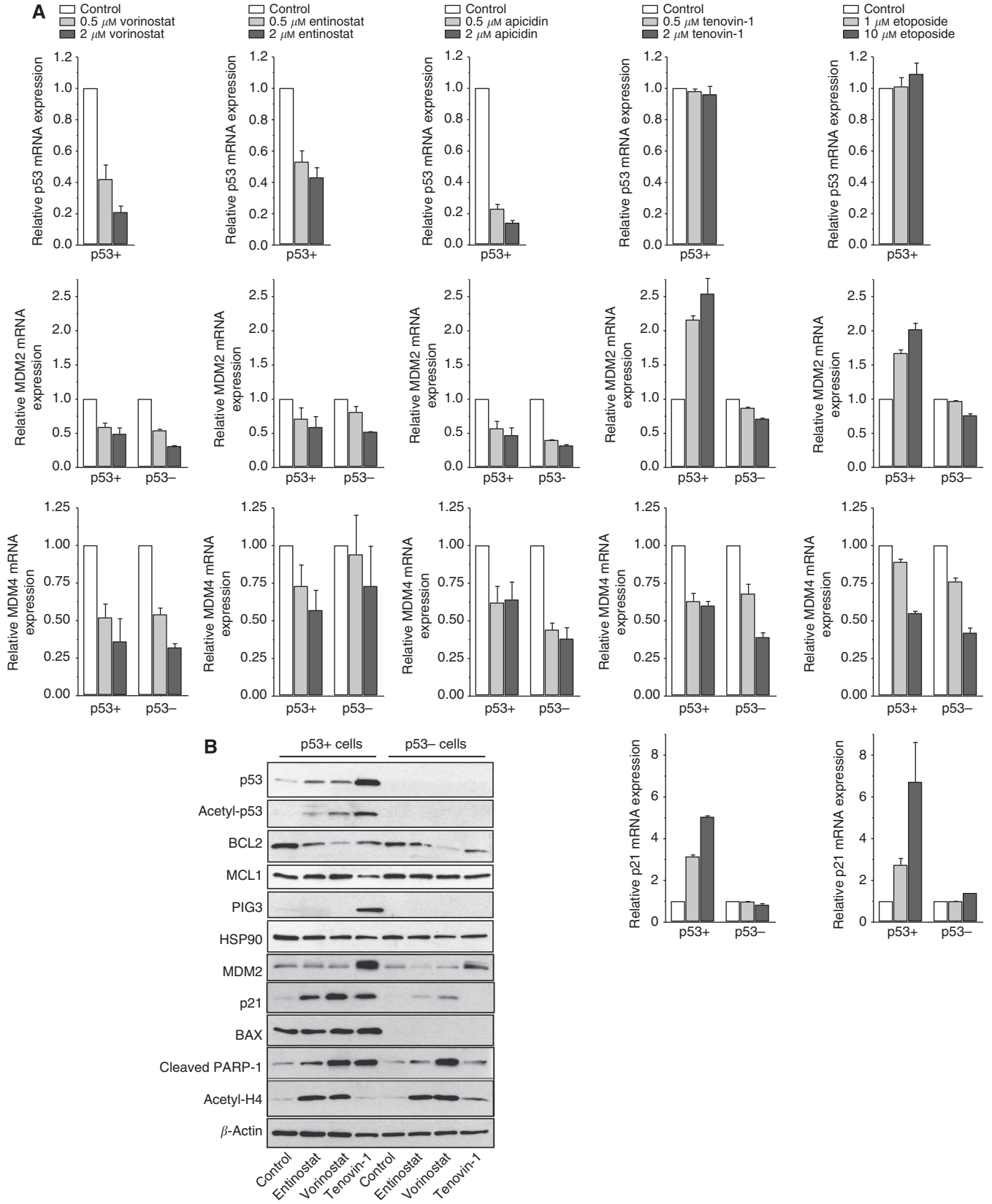

Figure 3. Effects of HDACi and tenovin-1 on gene expression in HCT-116 p53 + and p53 − cells. Cells were exposed to drugs for 24 h. (**A**) mRNA expression levels were determined by real-time RT–PCR and normalised to β-2-microglobulin mRNA levels. Means ± s.e.m. of each two separate measurements are shown. (**B**) Cells were treated with 2 μM HDACi or 10 μM tenovin-1. Protein expression levels were determined by western blotting.

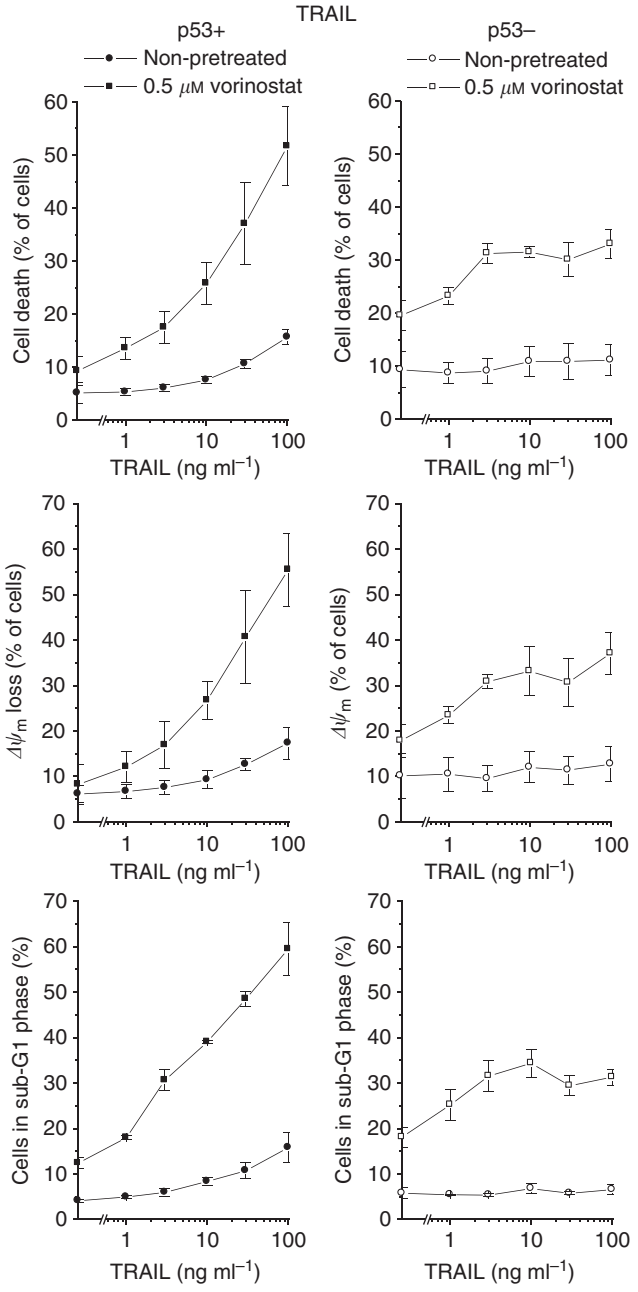

Figure 4. Antineoplastic effects of vorinostat in combination with TRAIL in HCT-116 p53+ and p53− cells. Four hours after administration of vorinostat, cells were exposed to TRAIL for 48 h. Cell death and $\Delta\psi_m$ were determined by flow-cytometric analyses of PI uptake or $DiOC_6(3)$ staining, respectively. Cells in sub-G1 phase were determined by flow-cytometric cell-cycle analysis. Means ± s.e.m. of each three separate measurements are shown.

by tenovin-1 in p53+ cells, but was not detectable in p53− cells. To elucidate a possible involvement of antiapoptotic proteins whose expression has been demonstrated to be repressed by HDACi (Spiegel *et al*, 2012), we determined BCL2 and MCL1 levels. BCL2 was downregulated by HDACi and tenovin-1 in a p53-independent manner. Unexpectedly, MCL1 showed a slight trend towards upregulation in p53+ cells; the densitometric quantification of the corresponding bands (of three independent assessments) revealed that MCL1 was elevated by 37% (vorinostat), 26% (entinostat) and 3% (tenovin-1). In p53− cells, HDACi caused an insignificant downregulation of MCL1 (vorinostat: −12%, entinostat: −13%) while tenovin-1 caused a significant one (−38%, $P = 0.0038$). Another direct transactivation target of p53, PIG3 (Polyak *et al*, 1997), was clearly induced by tenovin-1 in p53+ cells only.

In addition, as a further measure for apoptosis, we determined the cleavage of PARP-1 by detecting the 89-kDa cleavage product. Consistent with the other apoptosis read-outs, treatment with HDACi and tenovin-1 provoked the appearance of the PARP-1 fragment in p53+ cells, while only vorinostat caused substantial PARP-1 cleavage in p53− cells (Figure 3B). To authenticate that HDACi inhibit HDAC activity, we analysed the acetylation status of histone H4. Vorinostat and entinostat induced pronounced histone H4 hyperacetylation, whereas tenovin-1 did not.

Effects of vorinostat in combination with other anticancer agents in HCT-116 p53+ and p53− cells. The greatest potential of HDACi may lie in their ability to cooperate with other antineoplastic treatment modalities (Bots and Johnstone, 2009; Spiegel *et al*, 2012). However, the role of p53 in the response of tumour cells to HDACi combination treatments has not yet been resolved.

TRAIL. TRAIL is an attractive candidate for cancer treatment because it targets tumour cells while sparing normal cells. Yet, many tumours fail to respond to TRAIL monotherapy and, thus, require cotreatment with sensitising agents in order for TRAIL to exert antineoplastic activity (Ashkenazi, 2008). Histone deacetylase inhibitors have been established as potent TRAIL sensitisers (Bots and Johnstone, 2009; Spiegel *et al*, 2012). Cell killing by TRAIL does in general not depend on p53, but on BAX (Deng *et al*, 2002; LeBlanc *et al*, 2002). Because HCT-116 p53− cells did not express BAX (see Figure 3B), these cells were likely to be resistant to TRAIL. Indeed, p53− cells showed no response to TRAIL, as judged by determining cell death, $\Delta\psi_m$ loss and DNA fragmentation (Figure 4). However, vorinostat sensitised both p53+ and p53− cells to TRAIL-induced apoptosis, though the sensitising effect was less pronounced in the latter.

Proteasome inhibitor bortezomib. Histone deacetylase inhibitors synergise with proteasome inhibitors, such as bortezomib, and phase II and phase III clinical trials of vorinostat in combination with bortezomib have been initiated (Bots and Johnstone, 2009). As with HDACi, the role of p53 in the response of cancer cells to bortezomib is controversial. For instance, in HCT-116 cells, bortezomib-induced apoptosis has been reported to both depend and not depend on p53 (Ding *et al*, 2007; Pandit and Gartel, 2011). Here, we explored whether the vorinostat/bortezomib combination effect depended on p53 by determining cell death and $\Delta\psi_m$ loss. Figure 5A shows that bortezomib elicited a concentration-dependent effect in p53+ and p53− cells, which was, however, weaker in the latter. Vorinostat enhanced bortezomib-induced cell death and $\Delta\psi_m$ dissipation irrespective of the cells' *p53* status.

NF-κB inhibitor CAPE. Bortezomib is an inhibitor of the transcription factor NF-κB, which can induce a number of tumour-promoting effects. It is unclear to which extent bortezomib's antineoplastic effects stem from suppression of NF-κB activity (Chaturvedi *et al*, 2011). Therefore, we tested whether the observed cooperative action of vorinostat and bortezomib could be mimicked by the combination of vorinostat with the NF-κB inhibitor CAPE (Schneider and Krämer, 2011). As presented in Figure 5B, the combination of vorinostat and CAPE was equally effective in p53+ and p53− cells.

Obatoclax. Histone deacetylase inhibitors have been shown to reduce the expression of the antiapoptotic BCL2 family member MCL1 (Spiegel *et al*, 2012). To our surprise, we found that vorinostat and entinostat somewhat, albeit insignificantly,

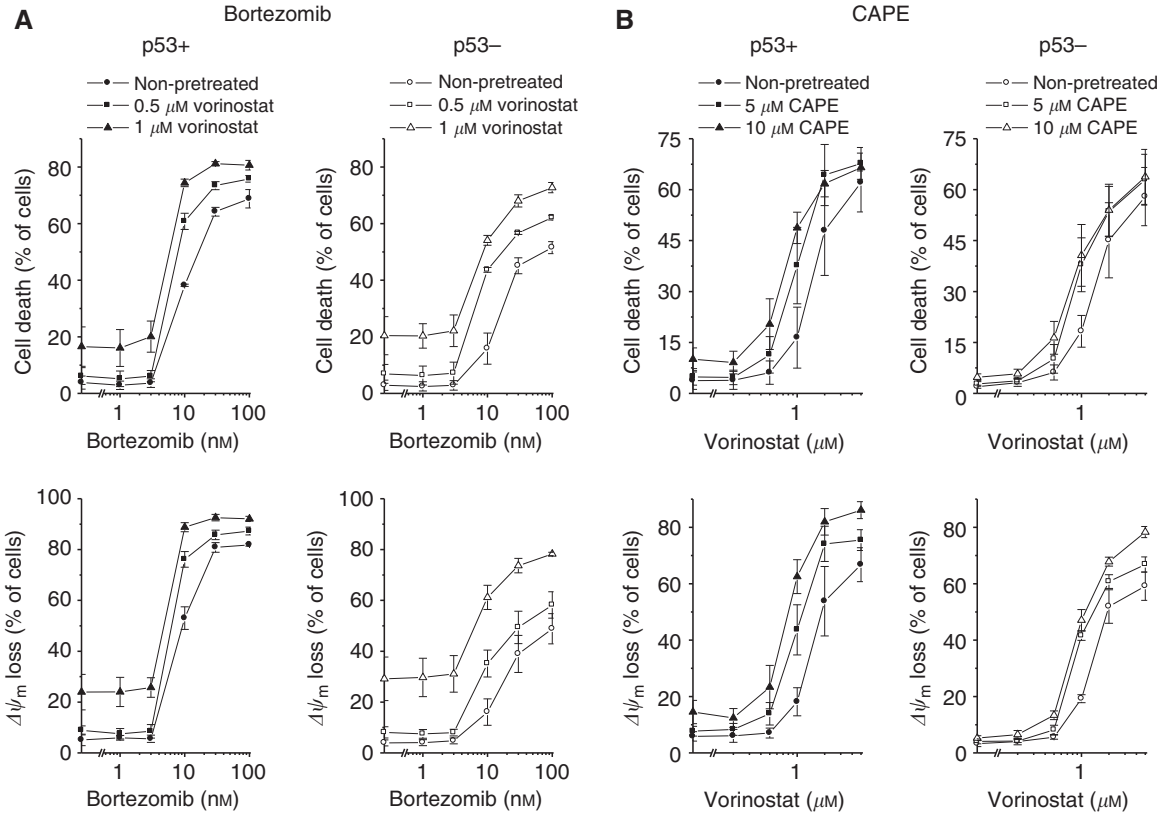

Figure 5. Antineoplastic effects of vorinostat in combination with bortezomib or CAPE in HCT-116 p53+ and p53− cells. (**A**) Four hours after administration of vorinostat, cells were exposed to bortezomib for 48 h. (**B**) One hour after administration of CAPE, cells were exposed to vorinostat for 48 h. Cell death and $\Delta\psi_m$ were determined by flow-cytometric analyses of PI uptake or $DiOC_6(3)$ staining, respectively. Means ± s.e.m. of each three separate measurements are shown.

increased MCL1 levels in p53+ cells (see Figure 3B). Hence, by inducing MCL1, HDACi could potentially, by themselves, limit their efficacy. If this was the case, then inhibition of MCL1 should enhance the anticancer action of HDACi. To test this hypothesis, we applied the MCL1 inhibitor obatoclax (Nguyen *et al*, 2007). Figure 6 shows that vorinostat was similarly effective in the presence and in the absence of obatoclax, suggesting that MCL1 is not crucial for HDACi-induced anticancer effects.

DISCUSSION

In this study, we delineated p53-dependent and -independent activities of HDACi and, in part, resolved the controversies (Vrana *et al*, 1999; Huang *et al*, 2000; Ruefli *et al*, 2001; Yu *et al*, 2002; Henderson *et al*, 2003; Insinga *et al*, 2005; Joseph *et al*, 2005; Kim *et al*, 2006; Sonnemann *et al*, 2006; Lindemann *et al*, 2007; Condorelli *et al*, 2008; Ellis *et al*, 2009; Hacker *et al*, 2011; Bajbouj *et al*, 2012; Meng *et al*, 2012) over the impact of p53 on HDACi-driven effects. As general bottom line, we have shown that HDACi accomplish the ultimate goal of cancer therapy, the demise of malignant cells, independent of the tumour's *p53* status. In addition, a number of distinctive features regarding the molecular effects of HDACi—and SIRTi—emerged from our study.

We noted that p53 was of variable significance for the biological effects of different HDACi: vorinostat, apicidin and VPA were practically as effective in p53− as in p53+ cells, whereas entinostat was less effective in the former. This finding suggests that HDACi with narrower specificity are more likely to depend on functional p53 (see Supplementary Table 1). It also suggests that the inconsistencies in previous studies stemmed from the particular HDACi investigated, though it is also possible that p53 is of different relevance in different model systems. Interestingly, vorinostat and entinostat differed in another respect: only vorinostat elicited a strong G2/M arrest when cells were protected from apoptosis by the pan-caspase inhibitor z-VAD-fmk (in both p53+ and p53− cells). Hence, vorinostat is capable of provoking growth arrest when its apoptosis-promoting activity is blocked. Taken together, these observations demonstrate that vorinostat can exert anticancer activities in the absence of both, functional p53 and intact caspases, in line with previous findings (Ellis *et al*, 2009).

As to caspases, it is worth noting that all four HDACi induced lesser caspase-3 activation in p53− cells, which, however, in case of vorinostat, apicidin and VPA, was not accompanied by lesser cell death. This finding shows that HDACi-triggered caspase activity does hinge, to some extent, on p53, though cell killing by vorinostat, apicidin and VPA does not. Therefore, studies that rest predominantly on the analysis of caspase activation may be misleading with regard to relevance of p53 for the antineoplastic action of HDACi. In this context, it should also be noted that z-VAD-fmk protected cells only incompletely from cell death, pointing to a caspase-independent mechanism of cell death induced by vorinostat and entinostat. Strikingly, the partially p53-dependent agent entinostat was more susceptible than vorinostat to inhibition by z-VAD-fmk in p53+ cells. On the other hand, in p53− cells, entinostat was much less prevented by z-VAD-fmk from mediating cell death and, consistently, failed to induce detectable caspase-3 activity and substantial PARP-1 cleavage. From these observations, it can be concluded that entinostat promotes mainly caspase-dependent cell death in p53+ cells, whereas, in p53− cells, caspase inhibition switches the mode

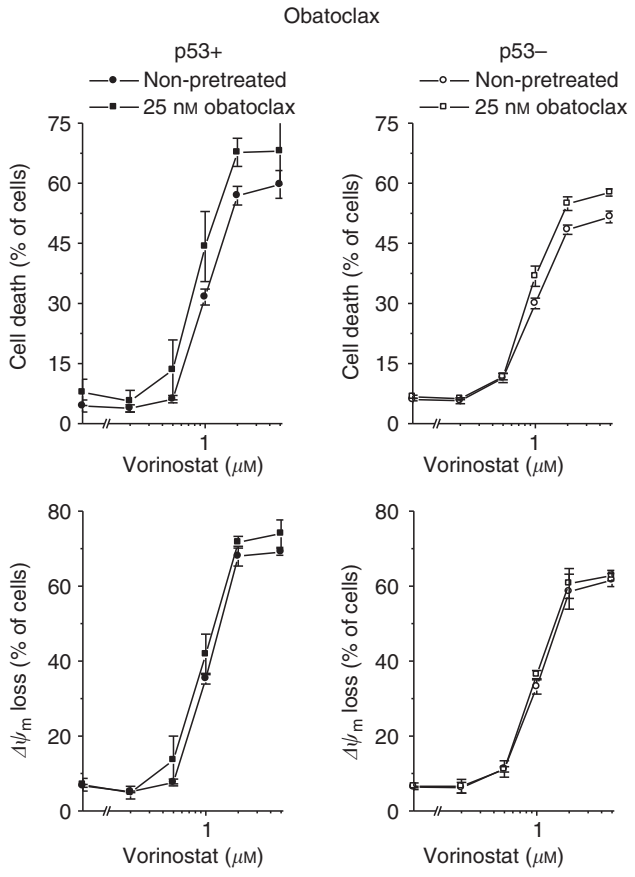

Figure 6. Antineoplastic effects of vorinostat in combination with obatoclax in HCT-116 p53+ and p53− cells. One hour after administration of obatoclax, cells were exposed to vorinostat for 48 h. Cell death and $\Delta\psi_m$ were determined by flow-cytometric analyses of PI uptake or $DiOC_6(3)$ staining, respectively. Means ± s.e.m. of each three separate measurements are shown.

of cell death to a mainly non-caspase-dependent one. It can also be concluded that the p53-dependent fraction of cell death is particularly sensitive to caspase inhibition.

The latter consideration is also in line with the finding that cell death induced by the SIRTi tenovin-1 depended on p53 and could be blocked by z-VAD-fmk. The seven sirtuins are presently emerging as promising drug targets for various diseases, yet they appear to have ambiguous roles in cancerogenesis (Roth and Chen, 2013). In particular SIRT1, the most widely studied sirtuin isoform, may operate as either tumour promoter or tumour suppressor: on the one hand, SIRT1 can repress p53 function via deacetylation, on the other hand, it can also facilitate DNA repair (Fang and Nicholl, 2011). Here, we have shown that tenovin-1 acted antineoplastically against HCT-116 p53+ cells. We found that tenovin-1 elicited cell death, in keeping with the study that discovered tenovins as potential antitumour agents (Lain *et al*, 2008). Furthermore, we found that tenovin-1 caused a G1 cell-cycle arrest when cell death was prevented by z-VAD-fmk, a feature that has not been previously described. Our protein expression analysis demonstrated that tenovin-1 strongly elevated the abundance of p53 and acetylated p53 and induced three key targets of p53, that is, MDM2, p21 and PIG3, in p53+ cells. The observation that tenovin-1 treatment simultaneously increased p53, acetylated p53 and the intrinsic p53 inhibitor MDM2 (mRNA and protein) points to the following scenario: MDM2 is induced by p53 activation in a feedback loop that normally serves to negatively control p53 activity (Vousden and Prives, 2009). However, MDM2 interacts only weakly with acetylated p53 and thus inefficiently destabilises p53 (Poyurovsky *et al*, 2010). All told, our data indicate that tenovin-1 exerts anticancer effects via p53. Yet, we also found that tenovin-1 diminished BCL2 expression in p53+ and p53− cells, showing that tenovin-1 can act on gene expression in a p53-independent manner, though its cell-killing action had the requirement for p53.

The PCR and western blotting analyses confirm the notion that HDACi can modulate gene expression in a p53-independent manner. For instance, HDACi reduced the expression of the antiapoptotic protein BCL2 independently of the cellular *p53* status. Likewise, in consistence with previous reports (Spiegel *et al*, 2012), vorinostat and entinostat upregulated p21, a major transcriptional target of p53 (Vousden and Prives, 2009), in p53+ and p53− cells. It should be taken into account, however, that the induction of p21 was less pronounced in p53− cells, in accordance with data collected in a study on lung cancer cells (Zhao *et al*, 2006). In contrast, the p21-inducing activity of tenovin-1 and etoposide was restricted to p53+ cells. More interestingly, we observed a considerable effect of HDACi on the expression of p53 itself and of the two p53 negative regulators, MDM2 and MDM4. At the transcriptional level, vorinostat, entinostat and apicidin downregulated the expression of *p53*, *MDM2* and *MDM4*. It is of note that *MDM2* downregulation also occurred in p53− cells, evidencing that this effect was not the consequence of p53 downregulation. At the protein level, vorinostat and entinostat blocked MDM2 expression, but induced p53. The implication is therefore that the downregulation of MDM2 overrides the downregulation of p53 mRNA, and the net result of these opposing effects is the increase in p53 abundance. We have previously observed that HDACi suppress MDM2 expression also in other cancer cell lines (Brandl *et al*, 2012; Palani *et al*, 2012), suggesting that this is a general effect of HDACi. Hence, HDACi can activate p53 by attenuating MDM2. In addition, it is equally possible that HDACi- and SIRTi-mediated acetylation of p53 weakens the p53–MDM2 interaction, that way stabilising p53 (Zhao *et al*, 2006; Poyurovsky *et al*, 2010; Brandl *et al*, 2012; Gu and Zhu, 2012). This consideration appears to particularly apply to tenovin-1 treatment, which increased the abundance of both p53 and MDM2. At the level of transcription, tenovin-1 had no effect on *p53* expression, whereas vorinostat, entinostat and apicidin suppressed it. These observations suggest that class I HDACs are necessary for expression of the p53 gene. Further studies are underway to address the interplay of HDAC activity, p53 acetylation and MDM2 expression in the control of p53 expression. To sum up this point, HDACi can activate p53, but they do not necessarily require p53 for inhibiting cancer cell growth and survival.

Likewise, vorinostat does not require p53 to enhance the anticancer action of other agents, at least with regard to the agents tested in this study. Vorinostat cooperated with the apoptosis-inducing cytokine TRAIL, the proteasome inhibitor bortezomib and the NF-κB inhibitor CAPE, in killing both p53+ and p53− cells. Notably, vorinostat enhanced the cytotoxic activity of bortezomib equally effective in p53− cells, which were less responsive to bortezomib alone, and it sensitised p53− cells to TRAIL-mediated cell death. TRAIL is generally conceived to elicit cell death independent of p53 (Ashkenazi, 2008). Yet we found p53− cells to be resistant to TRAIL, which is most likely attributable to the lack of BAX expression in these cells (see Figure 3B). Indeed, BAX-deficient HCT-116 cells have been shown to be unresponsive to TRAIL-induced apoptosis (Deng *et al*, 2002; LeBlanc *et al*, 2002). Our data suggest that vorinostat treatment partially overcame this type of resistance.

In summary, the present study substantiates the general usefulness of HDACi—applied as single agents or in conjunction

with other antineoplastic regimens—for the treatment of p53-deficient tumours. Yet it also demonstrates that the action of some HDACi may be partially influenced by p53. Thus, p53 independence of HDACi cannot *a priori* be taken for granted. Furthermore, it shows that HDACi suppress MDM2 expression and induce accumulation of p53, a feature that might have confounded conclusions on the relevance of p53 for HDACi-mediated anticancer effects in previous studies on this subject.

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CONFLICT OF INTEREST

There authors declare no conflict of interest.

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