The histone deacetylase inhibitors suberoylanilide hydroxamic (Vorinostat) and valproic acid induce irreversible and MDR1-independent resistance in human colon cancer cells

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Abstract. Histone deacetylase (HDAC) inhibitors such as suberoylanilide hydroxamic acid (SAHA, Vorinostat), valproic acid (VPA), and FK228 are members of a relatively novel class of small molecular weight chemicals that have high anti-neoplastic activity. They cause growth inhibition and apoptosis specifically in tumor cells, and they act also as chemo- and radio-sensitizers. In the present study, the potential of SAHA and VPA to induce resistance was studied. To that aim HDAC inhibitor-resistant sublines were generated by stepwise exposure of colon tumor cells to increasing concentrations of these compounds. Clonogenic data demonstrated that the SAHA- and VPA-induced sublines were 2-fold resistant to these compounds. This resistance was non-reversible, as it was maintained even when the sublines were cultured in the absence of SAHA or VPA. The SAHA- and VPA-induced resistant sublines were also stably cross-resistant to VPA and SAHA, respectively, but retained sensitivity against non-HDAC inhibitor-type anticancer agents. The SAHA-induced resistance correlated with loss of the G_2/M checkpoint but it was not accompanied by reduced induction of the endogenous cell cycle inhibitors p21 and p27. Furthermore, SAHA-induced resistance was not due to reduced apoptosis, and it was neither dependent on MDR expression nor was it due to increased expression of HDAC1 and HDAC3. Taken together, these data demonstrate the potential of SAHA and VPA to induce resistance. This resistance was not dependent on MDR expression, did not involve MMR, and seemed to underlie a mechanism that differs from that underlying the previously observed FK228-induced resistance. The finding that SAHA and VPA induce only modest resistance despite continuous treatment and that the resistance is MDR-independent suggests a preference for these two drugs over FK228 for use in combination treatment with classic anticancer agents.

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Introduction

Enzymes modifying the activity of histones, such as histone acetyltransferases (HATs) and histone deacetylases (HDACs), are crucial to proliferation, apoptosis, development, angiogenesis, and carcinogenesis. The balance between these activities regulates the expression of genes controlling these processes, mainly by regulating the accessibility of DNA-interacting proteins for the DNA. HDAC-mediated silencing of tumor suppressor genes plays a role in cancer pathophysiology. HDACs are subdivided in four classes: class I (HDAC1, -2, -3, -8), class II (HDAC4, -5, -6, -7, -9, -10), class III (also referred to as sirtuins: SIRT1 through SIRT7), and class IV (HDAC11) (1-3).

Inhibitors of HDACs counteract the removal of acetyl-groups from histones and render the DNA available for DNA-interacting proteins. HDAC inhibitors have strong anticancer properties and many of them have moved forward into clinical trials, and Vorinostat (suberoylanilide hydroxamic, SAHA) has even been granted market approval for the indication of cutaneous T-cell lymphoma (4-7). HDAC inhibitors induce cell cycle checkpoint activation and apoptosis specifically in tumor cells (8-12). They also radio- and chemo-sensitize tumor cells (13,14). HDAC inhibitors have also been linked to some characteristics of DNA repair (15,16), suggesting that HDAC inhibitors can induce abnormal DNA structures that may be recognized by DNA repair proteins (17).

Recent studies have shown that the powerful HDAC inhibitor FK228 (depsipeptide) can induce reversible FK228 resistance in tumor cells by reversible induction of MDR1 (18,19), a multidrug resistance transporter that functions by extrusion of cytotoxic drugs from the cell and by mediating sequestration of the drugs into intracellular compartments, both leading to a reduction in effective intracellular drug concentrations (20). Expanding on this issue, we investigated whether the HDAC inhibitors SAHA and valproic acid (VPA), which are structurally unrelated to FK228, are potential inducers of HDAC inhibitor resistance in tumor cells and whether HDAC inhibitor-induced resistance is associated

with cross-resistance to non-HDAC inhibitor-type anticancer agents, inducible MDR1 expression, reduced expression of the HDAC-responsive gene p21, altered expression and/or acetylation status of HDACs, and impaired cell cycle checkpoint and apoptosis activation. To this aim and to determine a possible involvement of DNA mismatch repair (MMR), respective sublines of a pair of human adenocarcinoma cell lines either expressing the MMR protein MLH1 (MLH1-proficient HCT116ch3) or lacking MLH1 expression (MLH1-deficient HCT116ch2) were generated by stepwise exposure of these cell lines to increasing concentrations of HDAC inhibitors.

Our results identify SAHA and VPA as potential inducers of a non-reversible and MDR1-independent HDAC inhibitor resistance phenotype. This resistance seems to be different to that observed with FK228 and not dependent on the MMR-status of the tumor cells.

Materials and methods

Drugs and chemicals. Suberoylanilide hydroxamic acid (SAHA; Alexis Biochemicals, Lausen, Switzerland) and valproic acid (VPA; Sigma, Buchs, Switzerland) were purchased, as were cisplatin, docetaxel, and 6-thioguanine (Sigma). Temozolomide was a generous gift (Schering-Plough, Kenilworth, NJ). Stock solutions were prepared in DMSO (SAHA, temozolomide), in ethanol (docetaxel), or in H_2O (cisplatin, 6-thioguanine, VPA). All stock solutions were stored at -20˚C.

Cell culture and generation of HDAC inhibitor-resistant sublines. A pair of an MLH1-deficient human colorectal adenocarcinoma cell line (designated HCT116ch2, complemented with chromosome 2) and its MLH1-proficient counterpart (designated HCT116ch3, complemented with chromosome 3), which were derived from the MLH1-deficient parental human colorectal adenocarcinoma cell line HCT116 (American Type Culture Collection; ATCC CCL 247), were used. The characteristics of the cell lines (e.g. chromosome complementation) and the culturing conditions have been described previously (21-23). A HeLa cell line (provided by Dr G. Marra, Institute of Molecular Cancer Research, University of Zurich, Switzerland) was also used. When seeded sparsely on tissue culture plates, all the cell lines and sublines formed well-defined individual colonies.

Similar to the method described previously (18), the HDAC inhibitor-resistant cell sublines, hereafter designated as HCT116ch3/SAHA, HCT116ch2/SAHA, HCT116ch3/VPA, or HCT116ch2/VPA, respectively, were generated by stepwise exposures of the MLH1-proficient HCT116ch3 cell line and the MLH1-deficient HCT116ch2 cell line to increasing concentrations of either SAHA or VPA, starting with 1 μM for SAHA or 2.5 mM for VPA. A similar protocol was used to generate the HeLa/SAHA subline. The principle of selection was the clonal growth in the presence of increasing concentrations of the HDAC inhibitor, based on the idea that cells are altered by chronic HDAC inhibitor exposure in that they acquire new features in an irreversible fashion. Basically, cells (100,000) were plated in cell culture flasks and treated with SAHA or VPA 24 h after plating. After another 48 h, the HDAC inhibitor-containing medium was exchanged for inhibitor-free medium, followed by incubation for another 6 days to allow recovery of the surviving cells. These were then harvested by trypsinization, transferred into new flasks, and expanded to confluence. One fraction was stored at -80˚C (for protein analysis), the other (100,000 cells) was re-seeded in culture flasks and was subjected to treatment with SAHA or VPA 24 h later, to medium exchange, recovery and harvesting as described. This protocol was repeated (10 times for SAHA, 8 timed for VPA), and for each cycle, the concentrations of SAHA or VPA were incremented, resulting in a 6-fold total increment for SAHA (6 μM) and 60-fold for VPA (150 μM). The growth rates of the cell lines and the sublines were calculated from the doubling times from one passage to the subsequent and averaged for a period of two months. *MLH1* gene expression in the cell lines and sublines was determined by immunoblotting.

Drug sensitivity assay. In a typical clonogenic assay, 400 cells in medium were plated onto 60-mm cell culture dishes, followed by drug addition after 24 h. For a 24-h treatment the medium was replaced by drug-free medium 24 h after drug addition; for a continuous treatment (8 days) the drug-containing medium was maintained. Cells were incubated for 7 days to allow colony formation, fixed with 25% acetic acid in ethanol and stained with Giemsa. Colonies of at least 50 cells were scored. Each experiment was performed at least three times using triplicate cultures. The relative colony formation (% clonogenic survival) was plotted against the drug concentrations, and the IC_{50} concentrations were calculated by linear extrapolation.

Apoptosis and cell cycle analyses by flow cytometry. Analyses of apoptosis (TUNEL DNA fragmentation) and cell cycle were performed by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences; Allschwil, Switzerland) with CELLQuest software (BD Biosciences). Data analyses for apoptosis and cell cycle distribution were performed on linear PI histograms using the mathematical software ModFit LT 2.0 (Verity Software House; Topsham, ME, USA). For sample preparation, cells were grown to 70% confluence in 60-mm dishes and treated with the concentration of SAHA which reduced clonogenic survival by at least 95%. At the times indicated, adherent and floating cells were collected and prepared for apoptosis and cell cycle analysis as described previously (23).

Immunoblot analysis. After cells had grown to 70% confluence in 60-mm dishes, they were treated with SAHA or TSA and collected 3, 7, or 14 h later, washed in PBS, and prepared for immunoblot analysis performed following standard protocols for PAGE gel electrophoresis. Protein (20 μg) was separated using 10% SDS-PAGE, followed by blotting onto a polyvinylidene difluoride membrane (Amersham Biosciences, Otelfingen, Switzerland). Expression of MLH1 protein was detected by the mouse antibody (550838; BD Biosciences Pharmingen, Basel, Switzerland) and the anti-mouse secondary horseradish peroxidase-conjugated antibody (M15345; Transduction Laboratories, Lexington, KY). p21 and p27 proteins were detected by the rabbit antibody (05-345; Upstate,

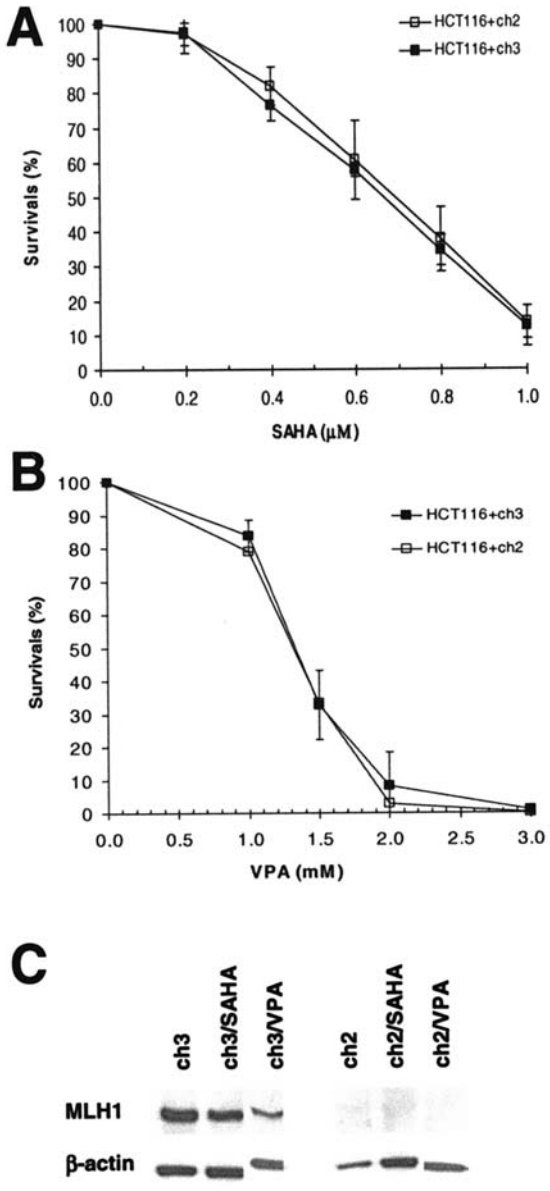

Figure 1. Clonogenic survival of MLH1-proficient HCT116ch3 (■) and MLH1-deficient HCT116ch2 (□) human colorectal adenocarcinoma cells after an 8-day treatment with the HDAC inhibitors SAHA (A) and VPA (B). Each point is the mean ± SD of three independent experiments performed in triplicate cultures. (C) The presence or absence of MLH1 expression in the HCT116ch3 (ch3) or HCT116ch2 (ch2) cells, respectively, and in the corresponding HDAC inhibitor-induced sublines (ch3/SAHA, ch3/VPA, ch2/SAHA, ch2/VPA). ß-actin is the sample loading control.

Lucerna Chem AG, Lucerne, Switzerland) and the rabbit antibody (2552; Cell Signaling, BioConcept, Allschwil, Switzerland), respectively. p53 and MDR1 were detected by the respective rabbit antibodies (sc-6243, sc-13131; Santa Cruz Biotechnology Inc., LabForce AG, Nunningen, Switzerland). HDAC1, -3, -4, -5, -6, and -7 were detected by rabbit antibodies (Kit 9928; Cell Signaling), acetyl-histone H3 (Lys9) and acetyl-histone H4 (Lys8) by the respective rabbit antibodies (6971, 2594; Cell Signaling). The anti-rabbit secondary horseradish peroxidase-conjugated antibody (7074; Cell Signaling) was used. As a sample loading control, the mouse antibody against ß-actin (A5441; Sigma) was used. All the complexes were visualized by enhanced chemiluminescence (Amersham Biosciences). Quantitative analysis of the complexes (intensity on the autoradiograph) was performed by densitometry (normalized against ß-actin) using the Scion Image 4.01 Win software (Scion Corporation, Frederick, MD).

Table I. Comparison of the cell doubling time.

Cell lines	Doubling time (h)[a]
HCT116+ch3	21.4±1.1
HCT116+ch3/SAHA	23.4±0.5
HCT116+ch3/VPA	23.7±0.5
HCT116+ch2	21.6±0.9
HCT116+ch2/SAHA	22.4±1.3
HCT116+ch2/VPA	23.8±1.1

[a]The doubling times of the cell lines and the respective sublines were calculated from one passage to the subsequent and averaged for a period of two months. The values represent the mean ± SD of 5 independent data sets.

Statistical analysis. The mean ± SD values were calculated for all data sets. A p value <0.05 was considered statistically significant (paired, two-tailed Student's t test).

Results

Resistance induction by SAHA and VPA. Clonogenic data revealed that the MLH1-deficient cell line (HCT116ch2) was as sensitive as the MLH1-proficient cell line (HCT116ch3) to a continuous (8 days) treatment with SAHA or VPA (Fig. 1A and B). For the generation of the SAHA- and VPA-induced sublines, the respective IC_{90} concentrations were determined, being 1 μM for SAHA and 2.5 μM for VPA. Expression of MLH1 protein was present in both sublines derived from the HCT116ch3 cell line and was absent in those derived from the HCT116ch2 cell line (Fig. 1C). The growth rates of the HDAC inhibitor-induced sublines were similar to those of the non-induced cell lines (Table I).

It was determined whether these HDAC inhibitor-induced sublines are on the one hand resistant to the agent by which they were induced and on the other cross-resistant to VPA, a carboxylate HDAC inhibitor which is structurally different from SAHA, a member of the hydroxamic acids class of HDAC inhibitors. The results (Fig. 2; Table II) showed that both the MLH1-proficient and the MLH1-deficient SAHA-induced sublines were 2-fold ($p<0.01$) resistant to SAHA (Fig. 2A) and also 2-fold ($p<0.01$) cross-resistant to VPA (Fig. 2B). Likewise, VPA treatment also induced a 2-fold ($p<0.01$) resistance to VPA (Fig. 2C) as well as a 2-fold ($p<0.01$) cross-resistance to SAHA (Fig. 2D), irrespective of the MLH1 status of the cells.

To consider a possible contribution of the complemented chromosomes, resistance induction by SAHA was also investigated for the parental HCT116 cell line. The results showed that 8 cycles of SAHA treatment also induced a 2-fold ($p<0.01$) resistance with this cell line. The respective IC_{50}

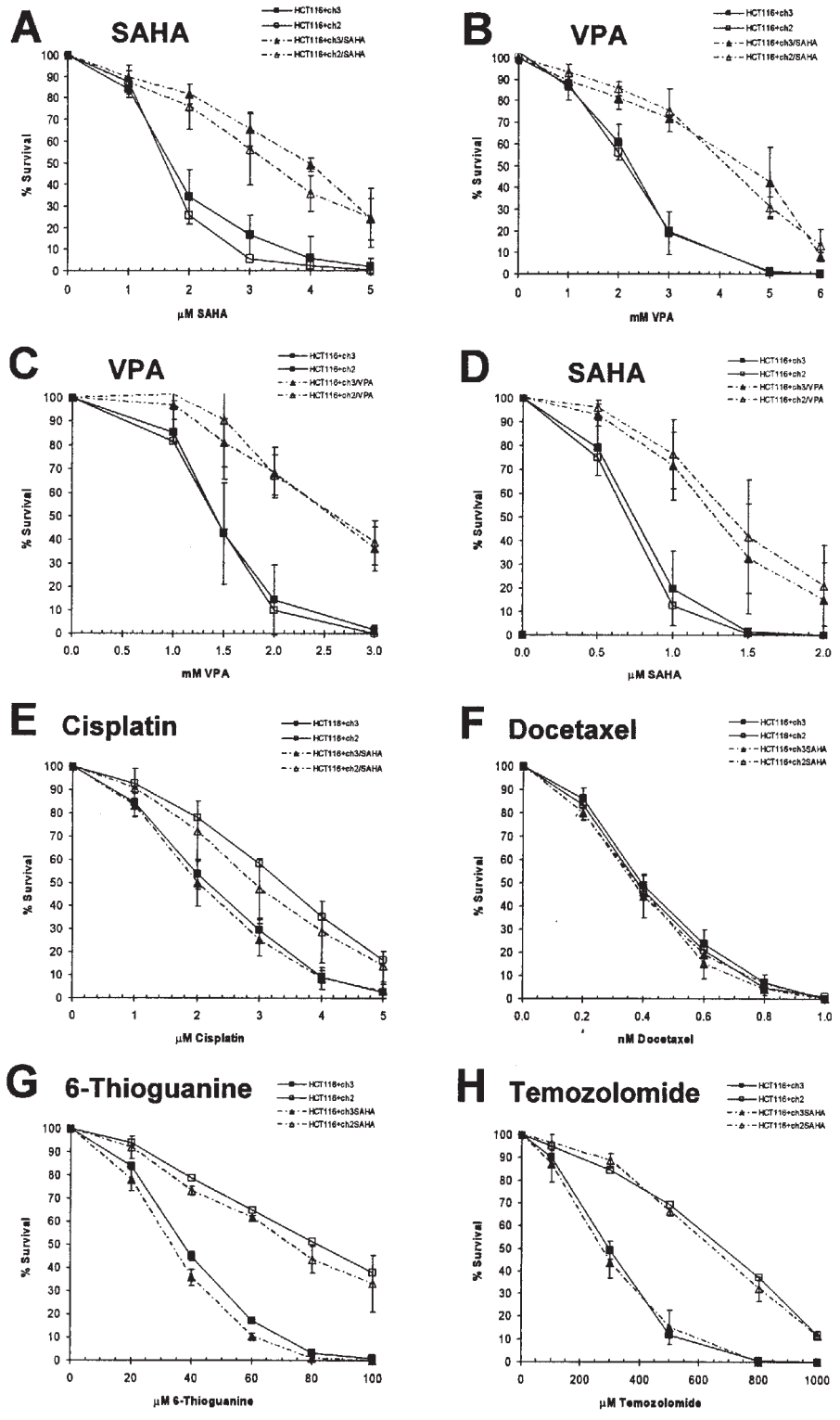

Figure 2. Clonogenic survival after an 8-day treatment with HDAC inhibitors for the MLH1-proficient HCT116ch3 (■) and MLH1-deficient HCT116ch2 (□) cell lines (straight lines) and the respective SAHA-induced (A, B) or VPA-induced (C, D) sublines (dashed lines). (A, C) Resistance to the HDAC inhibitors themselves; (B, D) cross-resistance of SAHA-induced sublines to VPA. Shown is also the cross-resistance of SAHA-induced sublines to the non-HDAC inhibitor-type antitumor agents cisplatin (E), docetaxel (F), 6-thioguanine (G), and temozolomide (H). Each point is the mean ± SD of 4 independent experiments performed in triplicate cultures.

values were 0.6±0.1 μM (HCT116) vs. 1.3±0.3 μM (HCT116/SAHA). Notably, SAHA (80-fold increment, 7 cycles) failed to induce resistance in HeLa cells. The respective IC_{50} values were similar: 1.1±0.1 μM (HeLa) vs. 1.3±0.1 μM (HeLa/SAHA).

It is noteworthy that all the HDAC inhibitor-induced sublines maintained resistance for at least 6 months (>30 passages, maximum period of time tested) even when cultured in HDAC inhibitor-free medium. In addition, the SAHA-resistant sublines were not cross-resistant to non-HDAC inhibitor-type anti-

Table II. IC_{50} values for the MLH1-proficient HCT116ch3 cell line and the MLH1-deficient HCT116ch2 cell line and the respective sublines derived from stepwise exposure to SAHA.

Inhibitor	HCT116ch3	HCT116ch3/SAHA	Fold change[a]	HCT116ch2	HCT116ch2/SAHA	Fold change[a]
SAHA (μM)	1.7±0.1	3.9±0.2	2.3[b]	1.6±0.1	3.2±0.6	2.0[b]
VPA (mM)	2.1±0.3	4.7±1.3	2.2[b]	2.1±0.2	4.2±0.5	2.0[b]
	HCT116ch3	HCT116ch3/VPA	Fold change[a]	HCT116ch2	HCT116ch2/VPA	Fold change[a]
VPA (mM)	1.4±0.2	2.6±0.3	1.9[b]	1.4±0.2	2.6±0.3	1.9[b]
SAHA (μM)	0.7±0.1	1.3±0.3	1.9[b]	0.7±0.1	1.4±0.3	2.0[b]

[a]Fold change is referred to as resistance or cross-resistance and is defined as the ratio of the IC_{50} values for the sublines and those for the non-induced cell lines. [b]$p<0.01$.

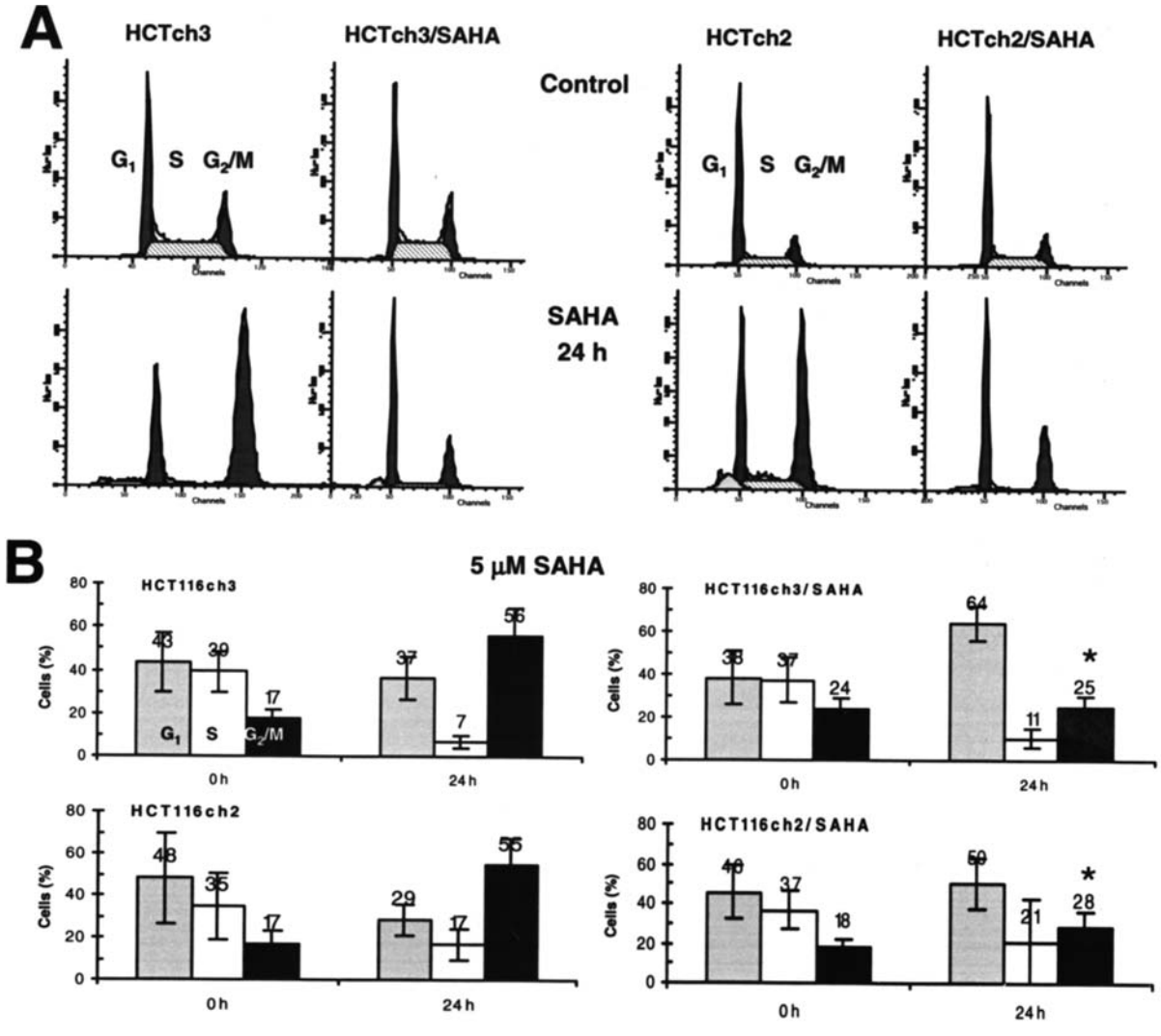

Figure 3. G_2/M cell cycle checkpoint induction in the SAHA-induced sublines and the corresponding non-induced cell lines in response to a 24-h exposure to 5 μM SAHA. (A) Representative cell cycle phase distribution profiles of the DNA content obtained by flow cytometry for the MLH1-proficient (HCTch3) and the MLH1-deficient (HCTch2) HCT116 cell lines and the respective sublines induced by SAHA (HCTch3/SAHA, HCTch2/SAHA). X-axis (channels): position of cells accumulated in G_1, S, or G_2/M. Y-axis; number of events per channel. (B) Quantitative presentation of primary flow cytometry data captured 24 h post-treatment of the cells with 5 μM SAHA. The changes in the proportion of cells accumulated at the G_2/M (black) transition, in the S phase (white), and at the G_1/S transition (grey) are presented. Each point is the mean ± SD of 3 independent experiments. *$p<0.05$.

cancer agents such as cisplatin, docetaxel, 6-thioguanine, and temozolomide, regardless of the MLH1 status of the cells (Fig. 2E-H).

SAHA-induced sublines and G_2/M checkpoint activation. It was determined whether loss of cell cycle checkpoint activation accounted for the observed SAHA-induced resistance. Flow

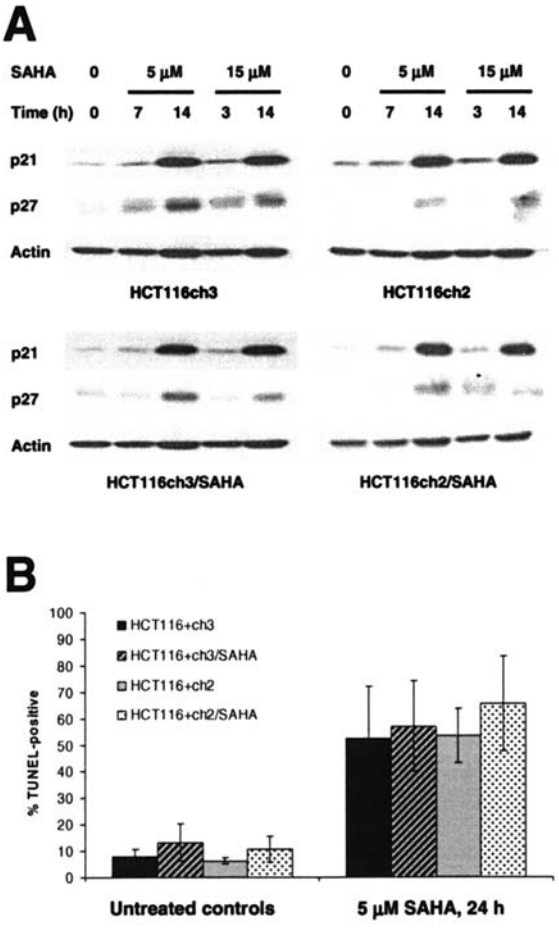

Figure 4. (A) Induction of p21 and p27 by SAHA in untreated control samples (no SAHA) and in samples captured 7 or 14 h after treatment with 5 or 15 μM SAHA. Actin is the sample loading control. Representative of two independent experiments. (B) Induction of apoptosis (DNA fragmentation) presented as the percentage of TUNEL-positive (apoptotic) cells as a function of treatment with 15 μM SAHA (captured 24 h post treatment). Mean ± SD of 3 independent data sets.

cytometry data analysis (Fig. 3) demonstrated that 5 μM SAHA (reduced clonogenic survival by >95%) produced an arrest at the G_2/M transition of the cell cycle in both the MLH1-proficient HCT116ch3 and the MLH1-deficient HCT116ch2 cell lines. This, however, was not observed for the respective SAHA-induced sublines, HCT116ch3/SAHA and HCT116ch2/SAHA. This demonstrated that the ability to induce the G_2/M checkpoint was substantially (2-fold, $p<0.05$) reduced in the SAHA-induced sublines as compared to the non-induced cell lines, indicating that loss of this checkpoint contributes to the SAHA-induced resistance observed for these sublines.

SAHA-induced resistance and regulation of p21 or p27. It was determined whether the SAHA-induced resistance was accompanied by reduced induction of p21 and/or p27, two endogenous cell cycle inhibitors induced by HDAC inhibitors (24-26). Immunoblot data showed that SAHA strongly induced p21 and p27 expression in all samples, i.e. in both sublines (MLH1-proficient and MLH1-deficient) and in the respective sublines (Fig. 4A), demonstrating that SAHA-induced resistance and loss of the G_2/M checkpoint were not due to loss of p21 and p27 induction in the resistant sublines.

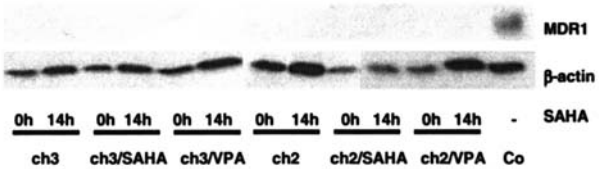

Figure 5. Expression of MDR1 as a function of a 14-h treatment with 15 μM SAHA in the non-induced cell lines and the respective SAHA- or VPA-induced sublines. A control lysate (sc-2284; Santa Cruz Biotechnology Inc.) was used as a positive control for MDR1 (Co).

SAHA-induced resistance and apoptosis. DNA fragmentation data (Fig. 4B) showed that susceptibility to SAHA-imposed apoptosis was nearly the same for the SAHA-induced (resistant) sublines and for the non-induced (sensitive) cell lines, regardless of the presence or absence of MLH1 expression in the cells, demonstrating that SAHA-induced resistance did not correlate with loss of apoptosis.

SAHA-induced resistance and MDR1 expression. The recently reported resistance to FK228 has been attributed to the inducible and reversible expression of MDR1 (18,19). In contrast, our observations (Fig. 5) showed that MDR1 was not expressed in any of the cell lines or the respective SAHA-induced sublines (untreated or treated with 15 μM SAHA for 14 h), demonstrating that SAHA-induced resistance was not associated with induction of MDR1. Expression of MDR1 was also not detected in the VPA-induced sublines.

SAHA-induced resistance and HDAC1 and HDAC3 expression. HDAC inhibitors cause hyperacetylation of histones and it was thus determined whether the SAHA-induced, SAHA-resistant sublines displayed hypoacetylation of the histones H2B, H3, and H4 (relative to the non-induced cell lines). The immunoblot results are shown (Fig. 6A). SAHA produced acetylation of H3 and H4 in both non-induced cell lines (i.e. MLH1-proficient and MLH1-deficient) as well as in the respective SAHA-induced sublines, demonstrating that SAHA exerts its effect in a target-specific manner. For the MLH1-proficient setting, SAHA produced a higher acetylation of H3 and H4 in the SAHA-induced, resistant subline (HCT116ch3/SAHA) than in the non-induced cell line (HCT116ch3), indicating that SAHA-induced resistance in MLH1-expressing cells was not accompanied by hypoacetylation. This was different for the MLH1-deficient setting. First, the level of acetylated H4 in the SAHA-induced subline (HCT116ch2/SAHA) was similar to that in the non-induced cell line (HCT116ch2). Second, the level of acetylated H3 in the SAHA-induced subline was lower than in the respective non-induced cell line. This demonstrated that SAHA-induced resistance was accompanied by hypoacetylation of only H3 and only in MLH1-deficient cells. In addition, detection of acetylated histone H2B was poor.

It was reasoned that SAHA-induced resistance could be due to increased levels of HDACs present in the respective sublines. This was not the case, as the SAHA-induced sublines expressed levels of HDAC1, -3, -6, and -7 that were similar to those expressed in the respective non-induced cell lines (Fig. 6B). Expression of HDAC4 and HDAC5 was not

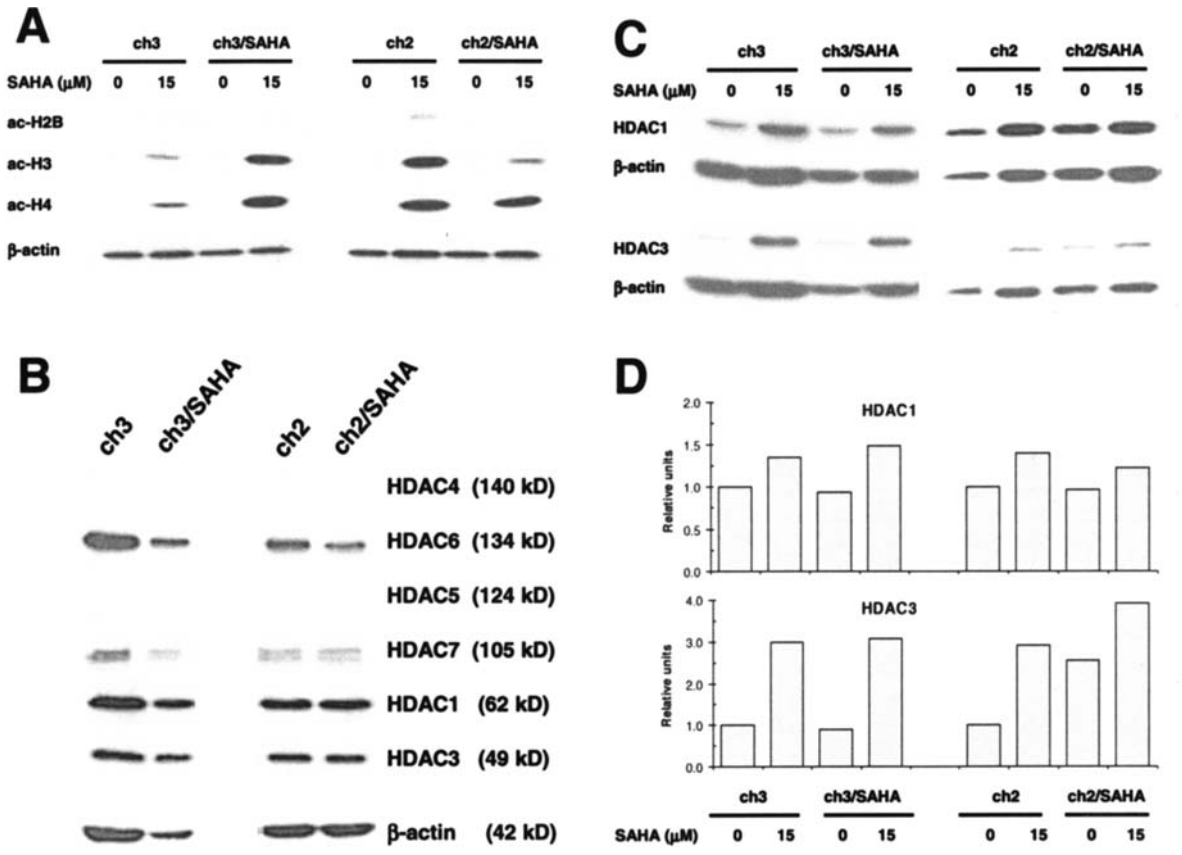

Figure 6. (A) Acetylation of histones H2B, H3, and H4. (B) Expression of HDACs in the non-induced cell lines and the SAHA-induced sublines. (C) Expression of HDACs as a function of treatment with 15 μM SAHA in the non-induced cell lines and the respective SAHA-induced sublines captured 14 h post treatment. (D) Quantitative presentation of the changes in HDAC1 and HDAC3 expression (relative to untreated controls and normalized against ß-actin loading control).

detected. In addition, SAHA produced increases in the levels of HDAC1 and HDAC3 in all the cell lines and the respective sublines relative to untreated samples (Fig. 6C) and to a comparable extent (Fig. 6D). This demonstrated that resistance to SAHA was not accompanied by elevated expression of HDAC1 and HDAC3 in these sublines.

Discussion

We observed the following: i) that HDAC inhibitors SAHA and VPA induced HDAC inhibitor (cross-) resistance which was not associated with cross-resistance to some none-HDAC inhibitor-type anticancer agents; ii) that this type of HDAC inhibitor-induced resistance was stable, MDR1-independent, and not associated with elevated expression of HDAC1 and HDAC3; iii) that the SAHA-induced resistance correlated with defective activation of the G_2/M checkpoint but not with loss of p21 and p27 induction and apoptosis; and iv) that MLH1 was irrelevant for the cytotoxic effect of SAHA and VPA. We may conclude that a novel mechanism of induced drug resistance, which is different to that observed with FK228, underlies the herein described resistance induced by these HDAC inhibitors.

The antineoplastic activity of HDAC inhibitors is an unquestionable property of these compounds. However, our observations with SAHA and VPA, together with those reported for FK228 (18,19), may shed some light on another less well-described aspect of HDAC inhibitors, namely their potential to induce resistance in tumor cells. In general terms, resistance induction may mean that existing cells are altered by HDAC inhibitor exposure in that they acquire new features in an irreversible fashion that renders them resistant (clonal growth in the presence of an HDAC inhibitor) or it might mean selection of those cells that are *a priori* more resistant to cell killing by HDAC inhibitors. The former principle was the basis of our experimental design, although a possible contribution of the latter cannot be ruled out. Despite some shared findings, our observations differ from those reported for FK228 in several ways, indicating that different mechanisms may underlie these resistance phenomena.

First, the SAHA- and VPA-induced resistance phenotype is likely to be stable, as it was maintained for at least 6 months (maximum period of observation) even in the absence of the drugs in the culture medium, and unlikely to be due to differential growth rates of the HDAC inhibitor-induced sublines. In contrast, FK228-induced resistance required the presence of FK228 and correlated with slower growth rates (19).

Second, our results demonstrate that the SAHA- and VPA-induced resistance is not dependent on the MDR1 transporter. This is consistent with the findings that SAHA is not substrate for MDR1 (9,18) and also explains why we did not observe cross-resistance to 'classic' anticancer agents such as cisplatin and docetaxel which are substrates for MDR1 (20). This is opposed to the FK228-induced resistance that correlated with

reversible MDR1 induction and cross-resistance to doxorubicin and etoposide but not to SAHA and TSA (18,19). We also found cross-resistance among members of structurally different classes of HDAC inhibitors (e.g. the hydroxamic acid-like SAHA versus the carboxylate-like VPA). It therefore seems that mechanisms of resistance induction other than those mediated by MDR1 are involved, and this may also explain the rather low magnitude of resistance (2-fold) for SAHA and VPA, as compared to the high degree of resistance (up to 1,700-fold) reported for FK228 (18). The finding that MDR1 is not involved in SAHA- and VPA-induced resistance suggests that, despite their potential to induce modest resistance, these HDAC inhibitors can be used in combination with other antitumoral agents that are substrates for MDR1. In addition, it remains to be seen whether this low-level resistance is sufficient to accumulate a stably resistant subpopulation of tumor cells and therefore to impair tumor treatment in *in vivo* models, although this has been shown for the 2-fold cisplatin resistance in MMR-deficient cells (27).

Candidate mechanisms of resistance may include differential induction of cell cycle checkpoints and apoptosis, overexpression of HDACs, loss of HDAC expression due to mutations, or even 'off-target' (i.e. HDAC-independent) effects. Our particular interest was directed towards SAHA, as it is considered one of the most potent and promising HDAC inhibitors and is the first of its class to be granted market approval. Our observations indeed demonstrated that SAHA-induced resistance was accompanied by loss of the G_2/M checkpoint, meaning that this defective checkpoint may provide these cells with a growth advantage over the SAHA-sensitive cells. The involvement of HDAC inhibitors in G_2/M checkpoint control has been shown previously (26,28-30). Other studies have shown that HDAC inhibitors regulate the G_1/S checkpoint, and this is via induction of p21, an HDAC inhibitor-inducible endogenous cell cycle inhibitor (24,31). Our observations indicate that neither the loss of the G_1/S checkpoint nor the failure to induce p21 accounted for SAHA-induced resistance. In addition, loss of the G_2/M checkpoint was not accompanied by reduced induction of p27, a protein also involved in regulation of HDAC inhibitor-mediated checkpoint control (26,32) or by altered p53 expression. Furthermore, SAHA-imposed apoptosis (8) was observed in the non-induced (sensitive) cell lines as well as in the SAHA-induced (resistant) sublines, indicating the SAHA resistance in these cells did not correlate with loss of apoptosis.

It was reasoned that resistance to SAHA was due to an increased expression of its specific targets. Consistent with a previous study reporting HDAC inhibitor-imposed increases in HDAC levels (33), SAHA-imposed increases in HDAC1 and HDAC3 expression were found, but these increases were not more pronounced in the resistant sublines, suggesting that resistance may not be caused by increased expression of HDACs. Likewise, the acetylation status of histones H3 and H4 was not generally lower in the resistant sublines, meaning that SAHA specifically affected its targets and that resistance may not be explained by the failure of SAHA to inhibit HDAC activity.

It has been suggested that HDAC inhibitors may induce abnormal DNA structures that can be recognized by DNA repair proteins (16,17). Recently, HDAC inhibitor resistance associated with loss of HDAC2 protein due to a truncating mutation in the *HDAC2* gene was reported in MLH1-deficient, microsatellite-instable tumor cells (34). Therefore, we have addressed the issue of whether the lack of MLH1 expression reduces the cytotoxic effect of HDAC inhibitors, and our results showed that this was not the case. We also questioned whether the absence of MLH1 is a determinant for resistance induction. Our observations showed that this was not the case with SAHA and VPA. In addition, the presence of the extra chromosomes within the cells used in this study is unlikely to be critical for resistance induction by these HDAC inhibitors, as we observed SAHA-induced resistance also for the parental HCT116 cell line.

Although inconclusive, our data suggest a novel mechanism of HDAC inhibitor-induced resistance that is not due to MDR1 expression, elevated expression of HDAC1 and HDAC3, and reduced apoptosis. It is unclear whether SAHA-induced resistance is a cell line- or tissue-specific phenomenon (it was not seen with HeLa cells), and therefore it may thus not be generalizable. In addition, the molecular bases of the underlying mechanisms of this possibly novel type of HDAC inhibitor induced resistance are still unknown and more detailed studies are required to identify them. These include microarray studies, the use of a larger set of cell lines, and the consideration of other cellular drug detoxification systems. The possibility should also be considered that this low-fold resistance may be ascribed to subtle, chronic treatment-induced differences in gene expression profiles that may be hard to identify experimentally rather than being clearly ascribed to a few single factors.

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