MLH1 protects from resistance acquisition by the histone deacetylase inhibitor trichostatin A in colon tumor cells

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Abstract. The antineoplastic activity of HDAC inhibitors is an unquestionable property of these compounds, but recent studies in tumor cells have revealed the potential of HDAC inhibitors (e.g., suberoylanilide hydroxamic acid SAHA, valproic acid VPA) to cause acquisition of HDAC inhibitor resistance. We report that trichostatin A (TSA), an HDAC inhibitor structurally related to SAHA, causes the acquisition of multidrug resistance transporter-independent and irreversible 3-fold resistance to TSA in MLH1-deficient (absent MLH1 protein expression) but not in MLH1-proficient (expressing MLH1 protein) HCT116 colon tumor cells. This MLH1-deficient subline selected for TSA resistance by stepwise exposures to increasing TSA concentrations exhibited failure in the accumulation of acetylated histones, in p21 induction, and in apoptosis activation. These are cellular responses normally seen in tumor cells treated with HDAC inhibitors. Whereas the absence of acetyl-histone accumulation did not correlate with altered HDAC activity, the absence of apoptosis correlated with reduced expression of (pro-apoptotic) Bax. This TSA-resistant subline was cross-resistant to SAHA and VPA but not to 'classic' non-HDAC inhibitor-type anticancer agents such as docetaxel and doxorubicin. These herein presented results expand on a previous study reporting HDAC inhibitor resistance acquisition by SAHA which was independent of the MLH1 expression status. Taken together, the present study identifies TSA, besides SAHA and VPA, as another potential causative of HDAC inhibitor resistance acquisition specifically in MLH1-deficient HCT116 colon tumor cells, and it reveals a possible function of MLH1 protein in protecting colon tumor cells from resistance acquisition by TSA.

Introduction

Resistance to anticancer drugs is a relevant problem and limits the effectiveness of anticancer therapy. Drug resistance is multifactorial and multiple ways by which cancer cells can elude therapy have been described, including expression of efflux pumps, regulation of apoptosis, and drug detoxification. Acquired resistance is a particular problem, because tumors not only can become resistant to the drugs originally used to treat them, but may also become cross-resistant to other drugs with different mechanisms of action. Resistance development can occur with any chemotherapeutical agent and is not restricted to any specific tumor type.

We have previously shown that suberoylanilide hydroxamic acid (SAHA, vorinostat), a representative of the class of histone deacetylase (HDAC) inhibitors, can cause acquisition of irreversible and multidrug resistance-independent HDAC inhibitor-resistance in HCT116 colon tumor cells, with the corresponding losses of growth inhibition, of apoptosis, and of histone acetylation (1,2). HDAC inhibitors are a class of anticancer agents that act by epigenetically regulating gene expression through chromatin remodeling. HDAC inhibitors induce the acetylation of histones and many non-histone proteins involved in regulation of gene expression, cell migration, proliferation, cell cycle and cell death (3-7).

The observed SAHA-induced HDAC inhibitor resistance was found with HCT116 cells expressing MLH1 protein (MLH1-proficient) as well as in HCT116 cells lacking MLH1 expression (MLH1-deficient) (1). MLH1 is one out of at least 5 proteins crucial to DNA mismatch repair (MMR) function, a mechanism involved in DNA damage surveillance, elimination of biosynthetic errors arising during replication, and prevention of recombination between non-identical DNA sequences (8-10). Loss of MMR is another cause of drug resistance, in particular with platinum-derived chemotherapeutics, due to the inability to detect the DNA damage and to induce cell cycle arrest and apoptosis (11).

Recent studies (12-14) have shown that MLH1 expression is positively affected by trichostatin A (TSA), one of the first selective HDAC inhibitors identified and often considered as the model HDAC inhibitor (15). Furthermore, microsatellite instable (MSI) and HDAC-2-mutated tumor cells showed absence of histone hyperacetylation in response to TSA (16) and resistance to TSA-induced apoptosis (17). TSA has also been shown to slow the radiation-induced DNA damage repair process in part by suppressing *BRCA1* gene expression (18) and to be genotoxic due to the hyperacetylation of centromers leading to aneuploidy and DNA strand breaks (19) and leading to chromosome missegregation and multinucleation (20).

The present study was aimed at investigating: i) whether TSA, similar to SAHA, can cause HDAC inhibitor resistance

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in colon tumor cells, ii) whether the MLH1 expression status is relevant for resistance acquisition by TSA, and iii) whether TSA-resistant sublines are cross-resistant to 'classic' anticancer agents.

Materials and methods

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

Cell culture and generation of TSA-resistant sublines. The following cell lines were used. The parental HCT116 human colon tumor cell line (American Type Culture Collection; ATCC CCL 247), which lacks expression of MLH1 protein due to a mutation in the coding region of the *MLH1* gene and hence is deficient in DNA mismatch repair function; the MLH1-proficient HT-29 colon tumor cell line and the (MLH1-proficient) HeLa cervical tumor cell line; a pair of the quasi-isogenic MLH1-deficient HCT116ch2 cell line (complemented with chromosome 2) and the MLH1-proficient HCT116ch3 counterpart cell line (complemented with chromosome 3 housing a wild-type copy of the *MLH1* gene, thus rendering it MLH1-proficient), which were derived from the parental MLH1-deficient HCT116 cell line. The characteristics of the cell lines (e.g., chromosome complementation) and the culturing conditions have been described previously (1,21). When seeded sparsely on tissue culture plates, all the cell lines and sublines formed well-defined individual colonies.

Analogous to the protocol described previously (1), the following sublines, hereafter designated as HCT116/TSA, HT-29/TSA, HeLa/TSA, HCT116ch2/TSA, and HCT116ch3/TSA, were generated by stepwise exposures of the respective cell lines to increasing concentrations of TSA, starting with 50 nM TSA for HCT116, HCT116ch2, HCT116ch3, and HeLa or with 250 nM TSA for HT-29. Basically, 200,000 cells were plated in culture flasks and treated with TSA after 24 h. After another 48 h, the medium was exchanged for TSA-free medium, followed by incubation to allow recovery of the surviving cells. These were harvested by trypsinization, transferred into flasks, and expanded to confluence. One fraction was stored at -80˚C, the other (200,000 cells) was re-seeded in culture flasks and subjected to treatment with TSA 24 h later, to medium exchange, to recovery and to harvesting as described. This protocol was repeated 7 times for each cell line, except for the HT-29 cell line (5 times: at the subsequent step 6 but cells did not manage to expand further). For each cycle, the TSA concentration was incremented, resulting in a 40-fold total increment (last cycle: 2 μM TSA) for each cell line, except for HeLa (20-fold) and HT-29 (4-fold). Further increases in the selection pressure beyond these TSA concentrations failed to produce sufficient surving cells for further cell culture expansion.

The principle of selection was clonal growth in the presence of increasing TSA concentrations, on the basis that cells are altered by chronic TSA exposure in a way they acquire new features in an irreversible fashion. The growth rates of the cell lines and the respective sublines were calculated from the doubling times from one passage to the subsequent, averaged over a period of two months, and compared to one another. The level of resistance was determined right after the cells had been expanded to confluence after the last cycle by the clonogenic assay (these are the IC_{50} values presented throughout this report) and was periodically monitored against the parental cell line. The level of resistance was stable over a period of five months even when cells were cultured in the absence of the selection pressure of TSA.

Drug sensitivity and apoptosis assays. Drug sensitivity was determined by the clonogenic assay as described previously (1,2). Apoptosis induced by TSA was assessed by immunoblot analysis (see below) on the basis of the proteolytic cleavage of the PARP-1 precursor. The reduction in the level of the 116-kDa PARP-1 full-length precursor and the reciprocal accumulation of its 86-kDa cleaved fragment is a measure for ongoing apoptosis.

Immunoblot analysis. Cells grown to 70% confluence in 60-mm dishes were treated with TSA. Floating and adherent cells were collected by trypsinization 12 , 24, 36, and 48 h after addition of 350 nM TSA. They were prepared for immunoblot analysis performed following standard protocols for PAGE gel electrophoresis. Protein (20 μg) was separated using SDS-PAGE, followed by blotting onto a PVDF membrane (Amersham Biosciences, Otelfingen, Switzerland). Expression of MLH1 was detected by the mouse antibody (550838; BD Biosciences Pharmingen, Basel, Switzerland). Acetyl-histone H3 (Lys9) and acetyl-histone H4 (Lys8) were detected by the respective rabbit antibodies (6971, 2594; Cell Signaling; BioConcept, Allschwil, Switzerland), as were the rabbit antibodies directed against acetyl-tubulin (T-6793; Sigma), p21 (2947; Cell Signaling), PARP-1 (9542; Cell Signaling), HDAC-1, HDAC-3, HDAC-6 (2062, 2632, 2662; Cell Signaling), HDAC-2 (05-814; Upstate, Lake Placid, NY), the multidrug resistance transporters MDR and MRP-1 (sc-13131, sc-18835; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Bax (2272; Cell Signaling), Bcl-xL (2262; Cell Signaling), thioredoxin (2285; Cell Signaling), and the sample loading control α/ß-tubulin (2148; Cell Signaling) or ß-actin (A-5541; Sigma). The anti-rabbit horseradish peroxidase-conjugated antibody (7074; Cell Signaling) or the anti-mouse horseradish peroxidase-conjugated antibody (M15345; Transduction Laboratories, Lexington, KY) were used as the secondary antibodies. Complexes were visualized by enhanced chemiluninescence and autoradiography.

HDAC-2 immunoprecipitation and determination of HDAC activity. HDAC-2 was immunoprecipitated according to the manufacturer's protocols from nuclear extracts of the TSA-sensitive MLH1-proficient HCT116ch3 cell line, MLH1-deficient HCT116ch2 cell line, MLH1-proficient HCT116ch3/TSA subline, and the TSA-resistant HCT116ch2/TSA subline using Protein A agarose beads (16-266; Upstate) and

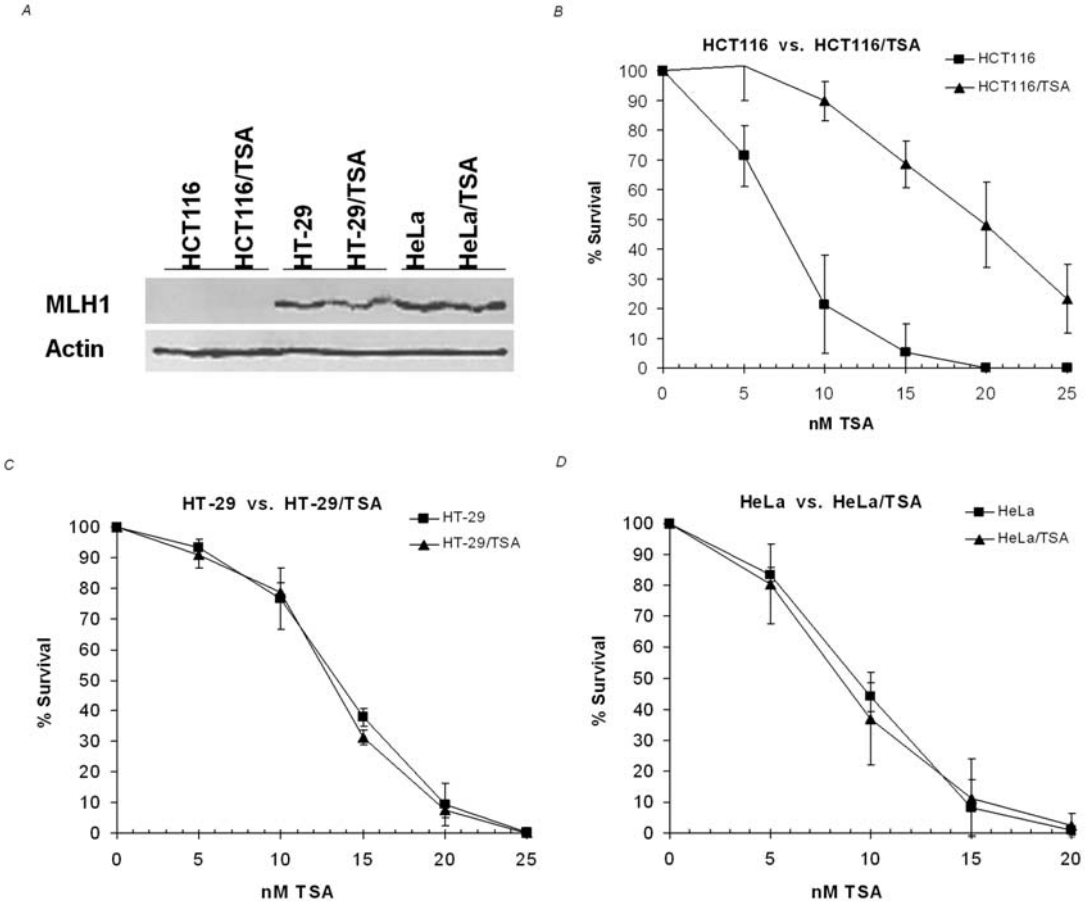

Figure 1. Expression of MLH1 protein and clonogenic survival in response to TSA treatment for the colon tumor or cervical tumor cell lines and the respective sublines generated by stepwise exposures of these cell lines to increasing concentrations of TSA. A, Presence or absence of MLH1 expression in the cell lines and the respective sublines (actin is sample loading control). B, MLH1-deficient HCT116 colon tumor cell line (square) and MLH1-deficient HCT116/TSA subline (triangle). C, MLH1-proficient HT-29 colon tumor cell line (square) and MLH1-proficient HT-29/TSA subline (triangle). D, MLH1-proficient HeLa cervical tumor cell line (square) and MLH1-proficient HeLa/TSA subline (triangle). Each point is the mean ± SD of 2 independent experiments performed in triplicate cultures.

the immunoprecipitation-qualified HDAC-2 antibody (05-814; Upstate). Nuclear extracts were produced using the TransFactor Extraction kit according to the manufacturer's protocol (631921; Clontech, Takara Bio Europe, Saint-Germain-en-Laye, France). Protein concentrations were determined by the BCA Protein Assay kit (23227; Perbio Science, Lausanne, Switzerland).

The HDAC enzymatic activity was determined in nuclear cell extracts using the colorimetric HDAC activity assay kit (ab1432; Abcam) or the fluorometric HDAC assay kit (17-356; Upstate). Measurements were made with a SpectraFluor Plus Reader (TECAN AG, Switzerland). The assays, including all standard assays, were performed according to the protocols provided by the manufacturers. The activity assays were performed in two independent settings under no-limiting assay conditions. Enzymatic activities were standardized (expressed as optical density or counts per amount protein).

Statistical analysis. The mean ± SD values were calculated. A $p<0.05$ is considered statistically significant (paired, two-tailed Student's t-test).

Results

TSA causes resistance acquisition in the MLH1-deficient HCT116 colon tumor cell line but not in the MLH1-proficient HT-29 colon and the HeLa cervical tumor cell lines. The potential of TSA to cause resistance acquisition in two colon tumor cell lines, i.e., HCT116 and HT-29, was determined. Clonogenic assay data showed that stepwise exposures to increasing concentrations of TSA caused a 3-fold resistance to TSA in the MLH1-deficient (not expressing MLH1 protein) HCT116 colon tumor cell line but not in the MLH1-proficient (expressing MLH1 protein) HT-29 colon tumor cell line and in the MLH1-proficient HeLa cervical tumor cell line (Fig. 1 and Table I). From this, it seemed that TSA caused resistance acquisition only when MLH1 protein was absent. To confirm this, quasi-isogenic cell lines (derived from the parental MLH1-deficient HCT116 cell line) either expressing MLH1 protein or lacking MLH1 expression were subject to the TSA resistance selection protocol (details described in Materials and methods). This protocol produced a 2-fold TSA-resistant subline (HCT116ch2/TSA) only with the MLH1-deficient (HCT116ch2) but not in the MLH1-proficient (HCT116ch3) cell line (Fig. 2 and Table I). The presence or absence of MLH1 in all the cell lines and the respective sublines was confirmed by immunoblot analysis. The growth rates of the cell lines and the respective sublines were not different: 22.6±0.9 h (HCT116) vs. 23.1±1.2 h (HCT116/TSA), 21.4±1.1 h (HCT116ch3) vs. 22.4±0.9 h (HCT116ch3/TSA), 21.6±0.9 h (HCT116ch2) vs. 23.3±0.9 h (HCT116ch2/TSA).

Table I. Sensitivity of the cell lines and the respective sublines to TSA as expressed by the IC_{50} values determined by the clonogenic assay.

Cell line	Subline	Resistance[a] (p-values)
HCT116	HCT116/TSA	
7.5±1.4 nM[b]	20.6±1.4 nM	2.75 (p<0.001)
HT-29	HT-29/TSA	
9.3±0.6 nM	8.7±1.6 nM	0.94 (p=0.673)
HeLa	HeLa/TSA	
13.9±1.3 nM	13.4±0.8 nM	0.96 (p=0.595)
HCT116ch2	HCT116ch2/TSA	
8.1±0.4 nM	15.3±2.8 nM	1.89 (p<0.005)
HCT116ch3	HCT116ch3/TSA	
9.4±3.4 nM	7.4±0.8 nM	0.79 (p=0.273)

[a]Fold resistance, expressed as the ratio of the IC_{50} values of the sublines and the respective cell lines; [b]mean ± SD of 4 independent data sets.

TSA-mediated resistance acquisition in the MLH1-deficient sublines can thus not be explained by differential growth rates. In addition, acquired TSA resistance was irreversible, as the TSA-resistant sublines maintained resistance for 5 months of continuous culturing in the absence of the TSA selection pressure in the culture medium (30 passages). TSA causes acquisition of irreversible resistance in MLH1-deficient but not in MLH1-proficient HCT116 colon tumor cells.

The TSA-resistant, MLH1-deficient subline fails to accumulate acetylated histones and to induce p21 in response to TSA. As accumulation of acetylated histones and non-histone proteins (e.g., tubulin) is a typical response to HDAC inhibitors (22,23), it was determined whether the TSA-resistant, MLH1-deficient HCT116ch2/TSA subline lacks this response. Immunoblot analysis showed that 350 nM TSA produced acetylation of histone H3 and histone H4 in all three TSA-sensitive cell lines, i.e., the MLH1-proficient HCT116ch3 and HCT116ch3/TSA cells as well as the MLH1-deficient HCT116ch2 cells, but not in the TSA-resistant, MLH1-deficient HCT116ch2/TSA subline (Fig. 3A). TSA-mediated acetylation of tubulin was detected in all four samples (Fig. 3B). Induction of the endogenous cell cycle inhibitor p21 is another typical response to HDAC inhibitors (24). It was determined whether acquired TSA resistance in MLH1-deficient HCT116ch2/TSA cells correlated with loss of p21 induction. The results showed (Fig. 3B) that 350 nM TSA did not induce p21 in these cells, whereas it did in all three TSA-sensitive cell lines. Acquired TSA-resistance correlates with loss of histone acetylation and of p21 induction.

TSA-mediated resistance acquisition in the MLH1-deficient subline is associated with loss of apoptosis. It was determined by immunoblot analysis whether loss of apoptosis contributed to acquired TSA resistance in the MLH1-deficient subline. The data showed that PARP-1 cleavage was strongly reduced in the TSA-resistant subline as compared to all three TSA-sensitive cell lines by 350 nM TSA (Fig. 4), indicating that loss of apoptosis accounted for acquired TSA resistance in the MLH1-deficient subline.

TSA-mediated resistance acquisition in the MLH1-deficient subline is not associated with expression of multidrug resistance transporters or altered expression of HDACs but with reduced expression of Bax. One reason for acquired TSA resistance and failure in histone acetylation and in p21 and apoptosis induction may lie in the reduced availability of TSA in the cells, possibly by expression of the multidrug resistance transporters MDR and MRP-1. Immunoblot data

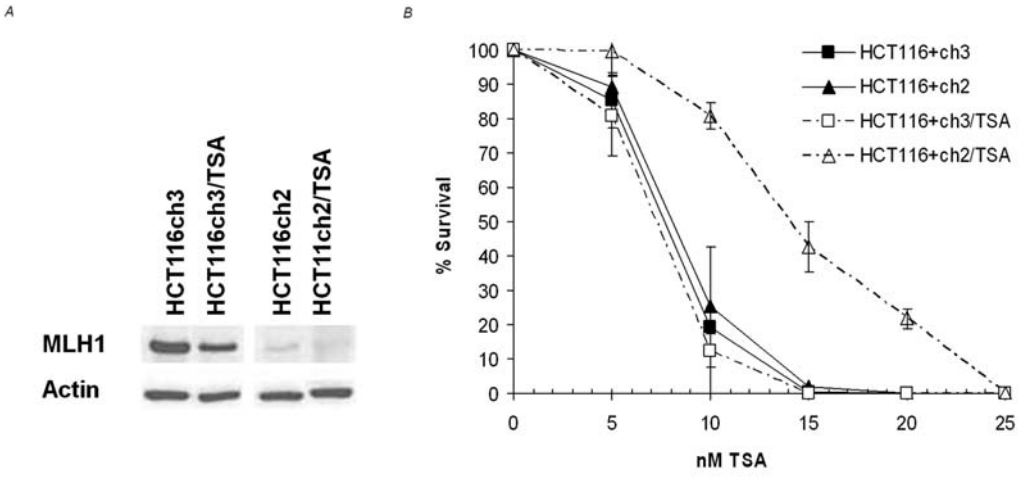

Figure 2. Expression of MLH1 protein (A) and clonogenic survival in response to TSA treatment (B) for the chromosome 3-complemented MLH1-proficient HCT116ch3 (black square) and the chromosome 2-complemented MLH1-deficient HCT116ch2 (black triangle) colon tumor cell lines and the respective sublines HCT116ch3/TSA (white square) and HCT116ch2/TSA (white triangle) generated as described by stepwise exposures of these cell lines to increasing concentrations of TSA. Actin is sample loading control. Each point is the mean ± SD of 3 independent experiments performed in triplicate cultures.

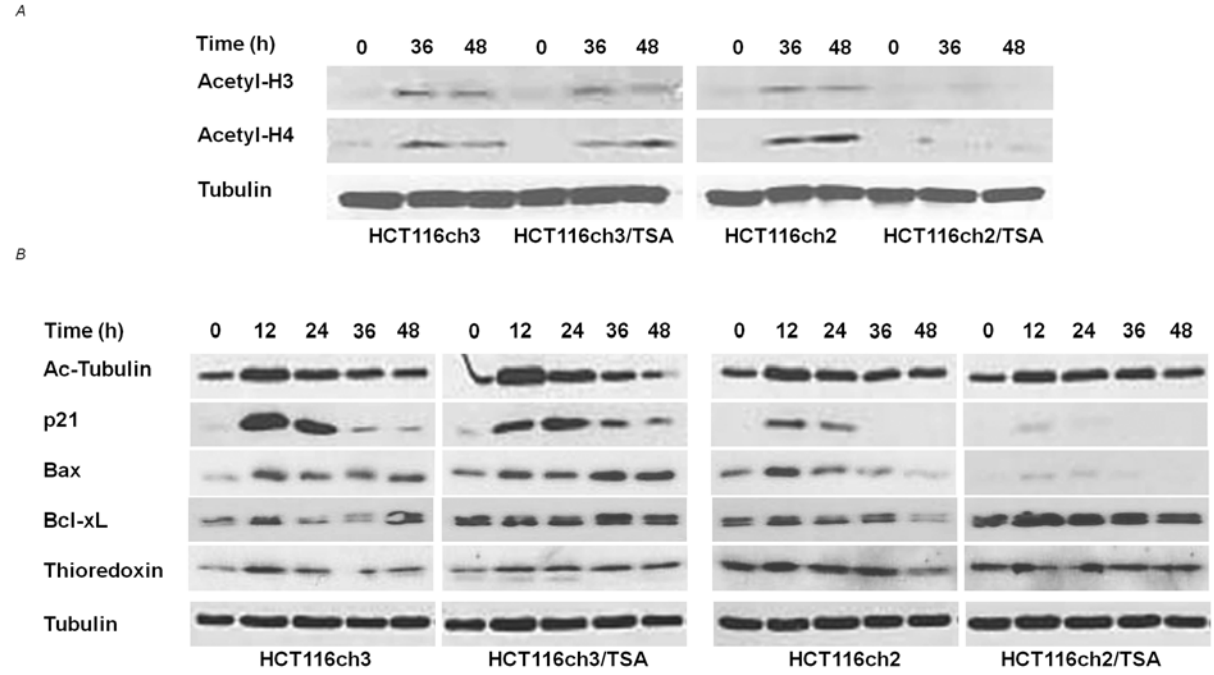

Figure 3. The acetylation of the histones H3 and H4 (A) and the acetylation of the non-histone protein tubulin, the induction of p21, and the expression of Bax, Bcl-xL, and thioredoxin (B) in response to 350 nM TSA in the MLH1-proficient HCT116ch3 and the MLH1-deficient HCT116ch2 colon tumor cell lines and their respective HCT116ch3/TSA and HCT116ch2/TSA sublines. Representative of 2 independent data sets (tubulin is the sample loading control).

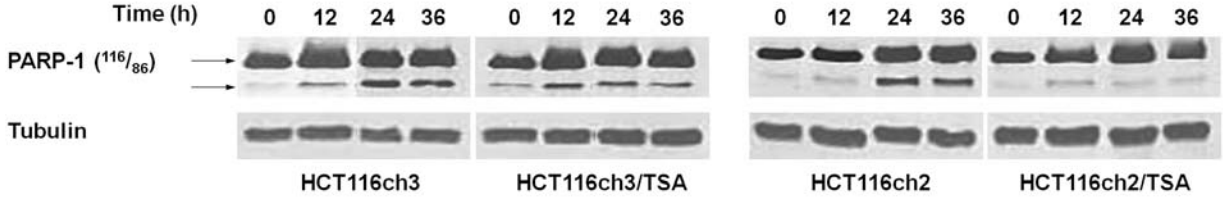

Figure 4. TSA-mediated apoptosis, represented as the cleavage of the 116-kDa PARP-1 full length precursor into the 86-kDa cleaved fragment, in response to treatment with 350 nM TSA in the MLH1-proficient HCT116ch3 and the MLH1-deficient HCT116ch2 colon tumor cell lines and the respective HCT116ch3/TSA and HCT116ch2/TSA sublines. Representative of 2 independent data sets with tubulin as the sample loading control.

(Fig. 5A) showed that these transporters were not present in untreated cells nor was their expression induced by TSA in the TSA-resistant subline.

Altered expression of apoptosis regulatory proteins may also account for acquired TSA resistance. Immunoblot analysis (Fig. 3B) showed that expression of the pro-apototic Bax was decreased in TSA-resistant MLH1-deficient HCT116ch2/TSA subline as compared to the TSA-sensitive HCT116ch2 cell line, while these expression levels in the TSA-sensitive HCT116ch3/TSA subline and the HCT116ch3 cell line were comparable. The expression levels of anti-apoptotic Bcl-xL were similar in all four samples, as were those of thioredoxin, a protein that scavenges reactive oxygen species that are produced in response to HDAC inhibitors.

Overexpression of one or more HDAC, i.e., the targets of HDAC inhibitors, or altered HDAC activity may contribute to acquired TSA resistance. However, the class I HDAC-1, HDAC-2, and HDAC-3, and the class II HDAC-6 expression was comparable in all four samples and the respective expression levels were not affected by 350 nM TSA in the four cell lines (Fig. 5B). Overall HDAC activity was comparable in all four samples, and TSA reduced the overall activity in all four samples to a similar extent (Fig. 6). The values for a 50% activity reduction by TSA in the samples were 7.1±0.6 nM (HCT116ch3), 7.7±1.8 nM (HCT116ch3/TSA), 6.9±1.5 nM (HCT116ch2), and 6.5±1.3 nM (HCT116ch2/TSA). Likewise, the basal enzymatic activity of individual HDAC-2 [inactivating mutations of which have been reported to confer apoptosis resistance (16,17)] in the TSA-resistant sample was not different from those in the TSA-sensitive samples, and the HDAC-2 activities in the four samples were reduced by TSA to a similar extent. The values for a 50% activity reduction by TSA in the samples were 18.8±0.6 nM (HCT116ch3), 18.8±0.4 nM (HCT116ch3/TSA), 16.5±0.7 nM (HCT116ch2), and 16.3±0.4 nM (HCT116ch2/TSA).

Acquired TSA-resistance in the MLH1-deficient HCT116ch2/TSA subline correlates with reduced expression of pro-apoptotic Bax, but is not associated with expression of the MDR and MRP-1 neither with overexpression of HDAC-1, HDAC-2, HDAC-3, HDAC-6, and thioredoxin, nor with altered HDAC activity.

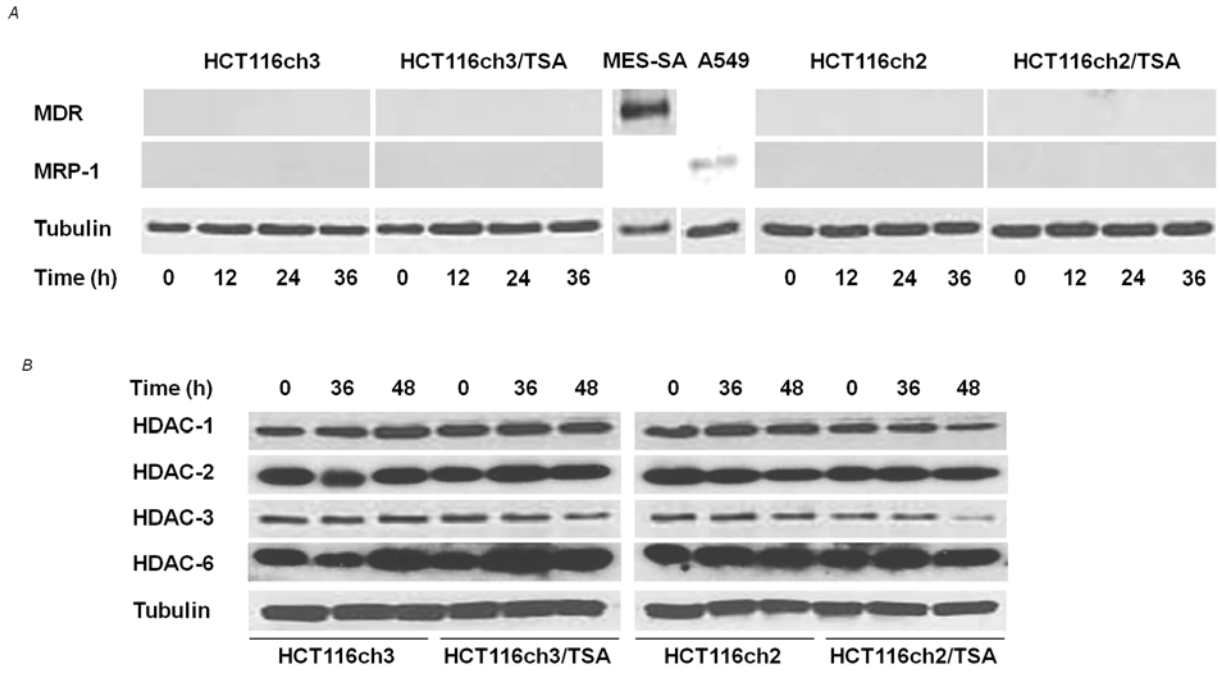

Figure 5. Expression of the multidrug resistance transporters MDR and MRP-1 (A) and of the histone deacetylases HDAC-1, HDAC-2, HDAC-3, and HDAC-6 (B) in response to 350 nM TSA in the MLH1-proficient HCT116ch3 and the MLH1-deficient HCT116ch2 colon tumor cell lines and the respective HCT116ch3/TSA and HCT116ch2/TSA sublines. Representative of two independent data sets. Positive control lysates for MRP-1 (A549, sc-2413) and MDR (MES-SA, sc-2284) were loaded (center lanes in A). Tubulin is sample loading control.

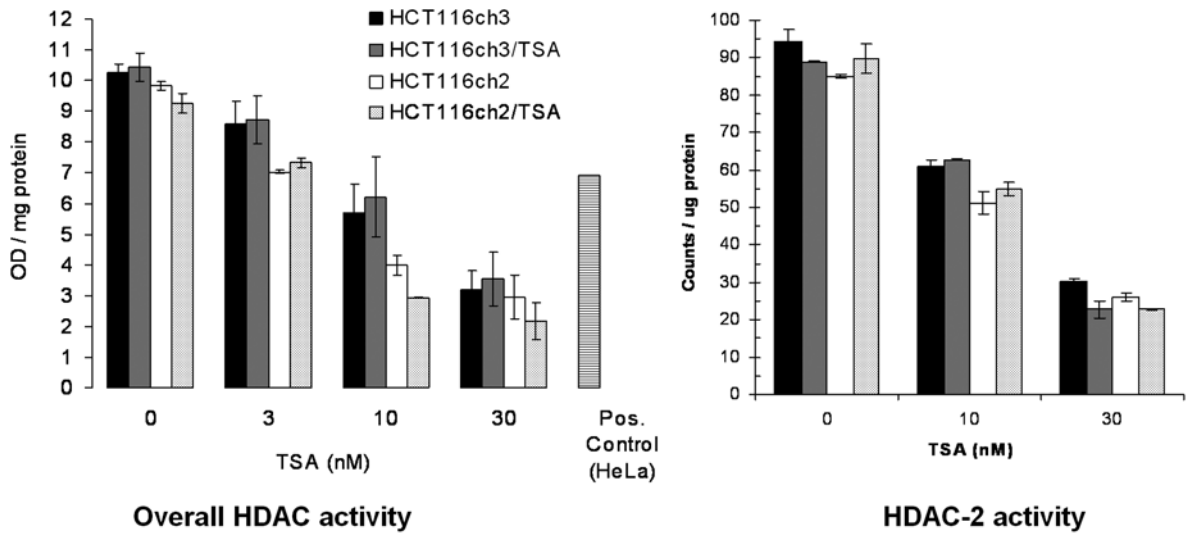

Figure 6. Overall HDAC activity expressed as optical density (OD) per mg of nuclear extract protein (left panel; colorimetric HDAC activity assay) and individual HDAC-2 activity expressed as counts per μg HDAC-2 immunoprecipitated from nuclear extracts (right panel, fluorometric HDAC assay) obtained from the MLH1-proficient HCT116ch3 and the MLH1-deficient HCT116ch2 colon tumor cell lines and the respective HCT116ch3/TSA and HCT116ch2/TSA sublines treated with various TSA concentrations. Also shown is a positive (HeLa nuclear extract) assay control. Mean ± SD of 2 independent data sets.

The TSA-induced resistant subline is cross-resistant to other HDAC-inhibitors but not to non-HDAC inhibitor-type anticancer agents. The potential cross-resistance of the TSA-resistant MLH1-deficient HCT116ch2/TSA subline to other HDAC inhibitors or to non-HDAC inhibitor-type anticancer agents was determined. It was found (Fig. 7 and Table II) that the TSA-resistant, MLH1-deficient subline was cross-resistant to the HDAC inhibitors SAHA and VPA. In contrast, the TSA-resistant, MLH1-deficient subline was as sensitive to the non-HDAC inhibitor-type docetaxel, doxorubicin, 6-thioguanine, and temozolomide as its TSA-sensitive counterpart HCT116ch2. The MLH1-proficient HCT116ch3/TSA subline and HCT116ch3 cell line were comparably sensitive to these agents. The TSA-resistant MLH1-deficient HCT116ch2/TSA

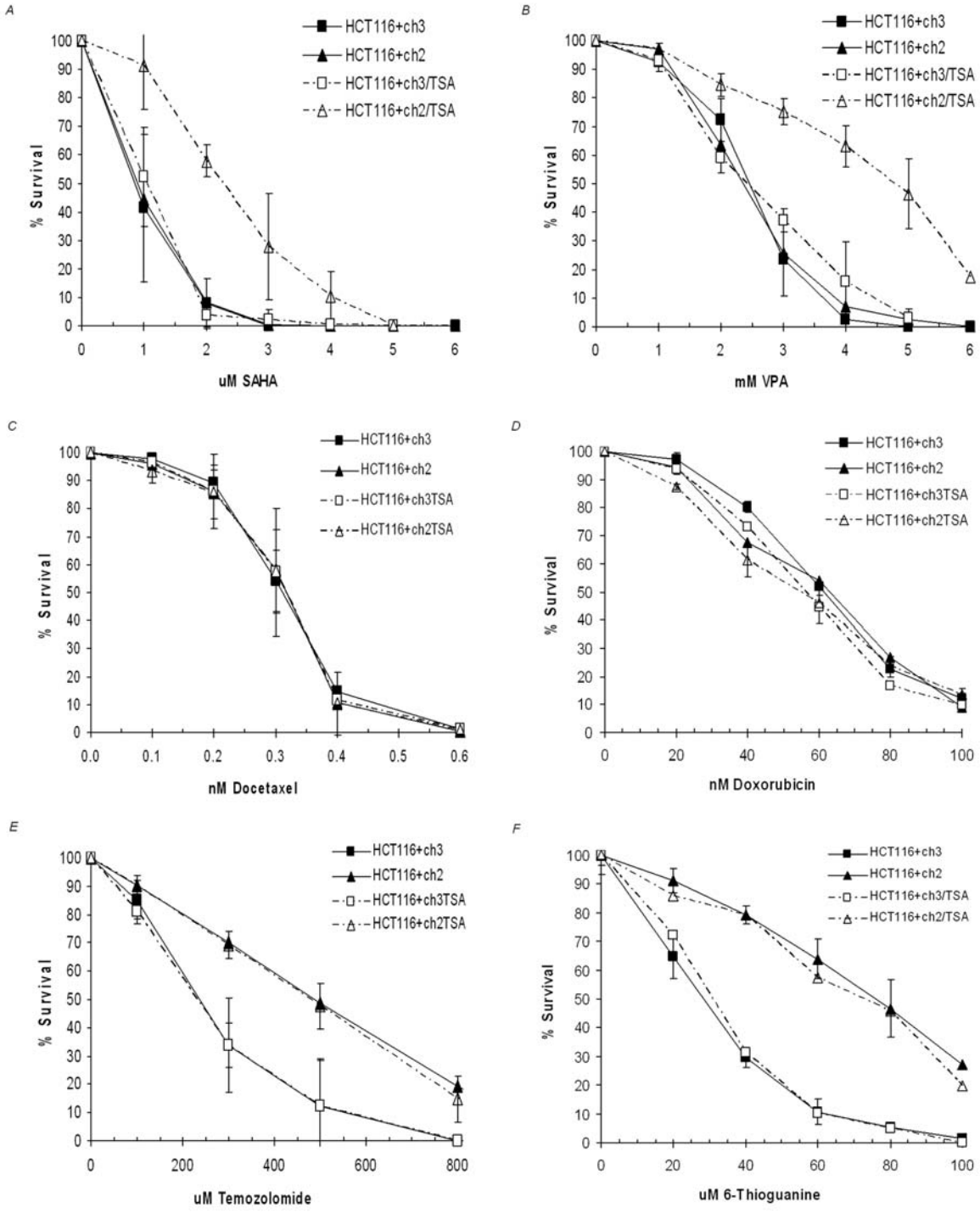

Figure 7. Clonogenic survival of the chromosome 3-complemented MLH1-proficient HCT116ch3 (black square) and the chromosome 2-complemented MLH1-deficient HCT116ch2 (black triangle) cell lines and the respective HCT116ch3/TSA (white square) and HCT116ch2/TSA (white triangle) sublines in response to treatment with the HDAC inhibitors SAHA (A) and VPA (B) and with the non-HDAC inhibitor-type agents docetaxel (C), doxorubicin (D), temozolomide (E), and 6-thioguanine (F). Each point is the mean ± SD of 3 independent experiments performed in triplicate cultures.

subline is cross-resistant to SAHA and VPA but retains sensitivity against 'classic' anticancer agents.

Discussion

The antineoplastic activity of HDAC inhibitors is a well-known property of these compounds (3-7), but recent studies have revealed the potential of HDAC inhibitors such as depsipeptide (25,26), VPA and SAHA (1,2) to cause drug resistance acquisition in tumor cells.

The present study expands on the issue of resistance acquisition by HDAC inhibitors. First, TSA is identified as another HDAC inhibitor which can cause the acquisition of irreversible and multidrug resistance transporter-independent resistance in colon tumor cells. Second, the herein described resistance acquisition by TSA, in contrast to that by SAHA,

Table II. Sensitivity of the MLH1-proficient HCT116ch3 and the MLH1-deficient HCT116ch2 cell lines and the respective HCT116ch3/TSA and HCT116ch2/TSA sublines generated by stepwise exposures to TSA as expressed by the IC_{50} values determined by the clonogenic assay.

HDAC inhibitor	HCT116ch3	HCT116ch3/TSA	Resistance[a]	HCT116ch2	HCT116ch2/TSA	Resistance
SAHA (μM)	0.9±0.4[b]	1.0±0.4	1.11	0.9±0.4	2.4±0.3	2.67[c]
VPA (mM)	2.5±0.2	2.4±0.2	0.97	2.4±0.3	4.7±0.6	1.96[c]
Drug	HCT116ch3	HCT116ch3/TSA	Resistance	HCT116ch2	HCT116ch2/TSA	Resistance
Docetaxel (nM)	0.31±0.03	0.31±0.04	1.00	0.31±0.03	0.31±0.05	1.00
Doxorubicin (μM)	15.4±0.7	14.6±1.4	0.95	15.6±0.3	13.7±1.2	0.88
6-Thioguanine (μM)	28.0±1.0	25.3±4.0	0.90	80.0±4.0	70.3±2.9	0.88
Temozolomide (mM)	0.24±0.05	0.23±0.03	0.96	0.50±0.04	0.48±0.06	0.96

[a]Fold resistance, expressed as the ratio of the IC_{50} values of the sublines and the respective cell lines; [b]mean ± SD of at least 3 independent experiments; [c]$p<0.05$.

is dependent on the absence of MLH1 protein expression, i.e., it did only occur in MLH1-deficient colon tumor cells. Third, the TSA-mediated resistance acquisition in these cells strongly correlated with loss of accumulation of acetylated histones, loss of p21 induction, and loss of apoptosis activation accompanied by reduced Bax expression, three responses normally seen with HDAC inhibitors. Fourth, the acquired TSA resistance was associated with cross-resistance to other HDAC inhibitors such as SAHA and VPA, but not to non-HDAC inhibitor-type anticancer agents such as doxorubicin and docetaxel. From these results, it may be concluded that: i) TSA is a potential causative for HDAC inhibitor resistance acquisition; and ii) MLH1 exerts a protective function in colon tumor cells against HDAC inhibitor resistance acquisition by TSA.

The herein described findings raise some interesting questions. On the one hand, how can the presence of MLH1 protein exert its putative protective function against resistance acquisition by TSA, how can its absence allow for TSA-mediated resistance acquisition, and why does MLH1 expression not protect from resistance acquisition by the structurally related SAHA? On the other hand, what is the molecular nature that leads to the loss of accumulation of acetylated histones and how does this loss translate into cellular responses eventually leading to failure in activating cell death?

The finding that TSA-mediated resistance acquisition was seen only in MLH1-deficient but not in MLH1-proficient colon tumor cells and hence was dependent on the absence of MLH1 protein is with no doubt most notable. It may mean that MLH1, TSA, and HDACs are somehow functionally linked. MLH1 is one of at least 5 proteins of the MMR complex that functions in the post-replicative processing of base-base mismatches, in the prevention of recombination between non-identical DNA sequences, and in mediating the cytotoxic effect of a number of anticancer agents including cisplatin, 6-thioguanine, temozolomide, and doxorubicin (8-11). Defective MMR confers resistance to these agents (11) and can increase the mutation rate in tumor cells (27). TSA has recently been described to promote expression of the *MLH1* gene (12-14), to be potentially genotoxic due to hyperacetylation of centromers leading to aneuploidy, chromosome missegregation, and DNA strand breaks (19,20), and to slow radiation-induced DNA damage repair process in part by suppressing the *BRCA1* gene expression (18), a protein implicated in DNA strand break repair and in MMR.

On this basis, one possible attempt at explaining the observed TSA resistance acquisition caused by stepwise exposures to increasing concentrations of TSA in cells with absent MLH1 protein might be as follows. Hyperacetylation of centromers by high concentrations of TSA may cause the formation of DNA strand breaks. The absence of MLH1 protein and hence of MMR function may result in insufficient and error-prone repair of these DNA strand breaks. While some of these may be tolerated without apparent consequences, some other 'severe' DNA strand breaks may cause genetic alterations in genes that normally mediate cytotoxic responses triggered by HDAC inhibitors. The finding that Bax expression is reduced in the MLH1-deficient, TSA-resistant cells seems in line with this idea. Conversely, the presence of MLH1 would allow for detection and proper repair of these DNA strand breaks introduced by high concentrations of TSA. This would, in turn, reduce the likelihood for genetic alteration in HDAC inhibitor-responsive genes and would ensure that the cell death program triggered by HDAC inhibitors is activated and executed. In this way the cells might, despite the selection pressure of TSA, maintain their ability to properly respond to HDAC inhibitors.

Despite its apparent appeal, this view is rather speculative, and a variety of obscurities remain. For instance, despite the putative effect of TSA on MLH1 expression, it is not completely understood, why MLH1 should protect from TSA-mediated but not from SAHA-mediated resistance acquisition as previously reported (1,2), in particular considering the close structural relationship between these two

pan-HDAC inhibitors. It also seems that the presence of any lesion or genetic alteration that would have been introduced by high concentrations of TSA does not affect the responsiveness to temozolomide and 6-thioguanine, two cytotoxic agents the effect of which depends on functional MMR.

Several attempts have been made to explain how loss of accumulation of acetylated histones is brought about and how this leads to (undesired) cell survival. One obvious reason is the expression of multidrug resistance efflux transporters that reduces the availability of the drug within the cell, and another obvious reason is the altered expression of HDACs and/or the altered HDAC enzymatic activity. However, expression of MDR or MRP-1, altered expression levels of HDAC-1, -2, -3, and -6, or altered overall HDAC activity were not found in the TSA-resistant subline. In particular, the enzymatic activity of individual HDAC-2, which can be abrogated by inactivating mutations in MSI-tumor cells and thus can confer apoptosis resistance (16,17), was comparable in the TSA-resistant and the TSA-sensitive cells, indicating that acquired TSA resistance cannot be explained by altered HDAC-2 activity. In addition, resistance acquisition cannot be explained by differential growth rates of the (original) cell lines from which the resistant variants were derived. It also cannot be explained by the presence of the extra chromosomes in the chromosome-complemented HCT116ch2 and HCT116ch3 cell lines, because resistance acquisition by TSA was also found with the MLH1-deficient HCT116 cell line (not chromosome-complemented) but not with the MLH1-proficient HT-29 colon tumor cell line.

One interesting finding is that the acquired TSA-resistant cells are cross-resistant to other HDAC inhibitors but not to 'classic' (non-HDAC inhibitor-type) anticancer agents. This suggests that this acquired HDAC inhibitor resistance is clearly an issue of defective HDAC inhibitor-responsive cytotoxic pathways, while cytotoxic pathways responsive to non-HDAC inhibitor-type drugs remain intact in these cells. In clinical terms, this may mean that cells that have become resistant during an HDAC inhibitor-based therapy might still be responsive to second-line therapy with 'classic' anticancer agents.

It is noteworthy that, except for the dependence on MLH1-absence, most of the described characteristics found with acquired TSA resistance were also found with HDAC inhibitor resistance acquisition by SAHA (2), including irreversibility, multidrug resistance transporter-independence, association with failure in accumulation of acetylated histones, loss of cell cycle check-point and apoptosis activation, cross-resistance to other HDAC inhibitors, responsiveness retention to 'classic' anticancer agents, and the absence of an association with altered HDAC expression and enzymatic activities.

In conclusion, the present study identifies TSA, besides SAHA and VPA, as another potential causative of HDAC inhibitor resistance acquisition in MLH1-deficient colon tumor cells, and it reveals a yet unknown function of MLH1 protein in protecting colon tumor cells from resistance acquisition by TSA.

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