Acquired vorinostat resistance shows partial cross-resistance to 'second-generation' HDAC inhibitors and correlates with loss of histone acetylation and apoptosis but not with altered HDAC and HAT activities

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Histone deacetylase (HDAC) inhibitors such as vorinostat (suberoylanilide hydroxamic acid), valproic acid, romidepsin (FK-228), and LBH589 comprise a relatively new class of potent anticancer agents. This study provides evidence for the potential of vorinostat to cause acquisition of multidrug resistance protein-independent resistance in HCT116 colon tumor cells. This acquired resistance is moderate (two-fold to three-fold), is nonreversible, and correlates with the loss of responses typically seen with HDAC inhibitors, that is the loss of acetylation of the histones H2A, H2B, H3, and H4, the loss of the G_2/M checkpoint activation, and the loss of caspase 3-dependent and caspase 7-dependent apoptosis. This acquired resistance also associates with cross-resistance to the hydroxamate-class (LBH589 and JNJ26481585) and to the aliphatic acid-class (valproic acid) HDAC inhibitors but not to the benzamide-class (MGCD0103) and the cyclic peptide-class (romidepsin) HDAC inhibitors. The acquired HDAC inhibitor resistance described here is not a result of altered HDAC and histone acetyltransferase activities and differs from that previously reported for romidepsin. *Anti-Cancer Drugs* 20:321–333

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Introduction

Vorinostat (suberoylanilide hydroxamic acid, SAHA) belongs to the continuously growing class of histone deacetylase (HDAC) inhibitors [1–3]. Preclinical studies with vorinostat have shown that its antiproliferative effects are associated with activation of the G_2/M cell cycle checkpoint and upregulation of p21, with downregulation of cyclin D1, and with acetylation of numerous transcription factors (e.g. p53) and other proteins (e.g. HSP90, tubulin) [1]. In general, HDAC inhibitors result in the accumulation of acetylated histones and of nonhistone proteins, and many of them exert strong antineoplastic activity. They also alter the gene expression pattern and thereby cause cell cycle arrest and apoptosis preferentially in tumor cells [4–8].

Resistance to an anticancer treatment, either present intrinsically in tumor cells or acquired during a treatment, is a frequently observed and persistent problem during cancer treatment. Acquired resistance is a particular problem, because tumors not only become resistant to the drugs originally used to treat them, but may also become cross-resistant to other drugs with different mechanisms of action. Mechanisms of resistance to HDAC inhibitors and their therapeutic implications have recently been reviewed [9]. In addition, the potential of HDAC inhibitors to cause drug resistance in tumor cells has recently become apparent. The HDAC inhibitor romidepsin (FK-228 or depsipeptide) has been shown to cause transient resistance by the reversible induction of multidrug resistance protein (MDR) expression in tumor cells and is to date the only HDAC inhibitor known to be substrate for multidrug resistance transporters [10,11]. Recently, we reported the generation of two MDR-independent, vorinostat-resistant sublines [12], these were the DNA mismatch repair (MMR)-proficient HCT116ch3 colorectal adenocarcinoma cell line (supplemented with chromosome 3 harboring the wild-type copy of the *MLH1* gene to compensate for the *MLH1* gene truncating mutation present in the parental MMR-deficient HCT116 cell line) and the MMR-deficient HCT116ch2 cell line (supplemented with the MLH1-irrelevant chromosome 2 for chromosome balance). Although a relationship between MLH1 expression and histone acetylation has been suggested [13,14], this vorinostat-induced resistance

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was independent of the presence or absence of MLH1 protein. Noteworthy, these vorinostat-resistant sublines were cross-resistant to the HDAC inhibitor trichostatin A, but retained sensitivity to non-HDAC inhibitor-type anticancer agents.

Using the parental HCT116 colorectal adenocarcinoma cell line, this study was designed to (i) elucidate in more detail the mechanism(s) behind resistance induction by vorinostat, (ii) investigate a possible cross-resistance to some 'second-generation' HDAC inhibitors, and (iii) exclude a possible effect of the presence of the extra chromosome in the respective cell lines on this type of acquired vorinosat resistance. Further evidence is provided that in HCT116 tumor cells vorinostat can lead to a multidrug resistance transporter-independent acquisition of resistance. This resistance correlates with the losses of histone acetylation, cell cycle attenuation, and apoptosis, but does not associate with altered HDAC and histone acetyltransferase (HAT) activities.

Materials and methods

Drugs and chemicals

Vorinostat (SAHA; Alexis Biochemicals, Lausen, Switzerland) and valproic acid (VPA; Sigma, Buchs, Switzerland) were purchased. LBH589 (Novartis Pharmaceutical Inc., Cambridge, Massachusetts, USA), MGCD0103 (ALTANA Pharma-Nycomed, Byk-Gulden Street 2, Konstanz, Germany), and JNJ26481585 (J&J Pharmaceutical Research & Development, Beerse, Belgium) were provided. Romidepsin (FK-228, depsipeptide) was provided by Gloucester Pharmaceuticals (Cambridge, Massachusetts, USA). Stock solutions (stored at −20°C) were prepared in dimethyl sulfoxide (vorinostat, LBH589, MGCD0103, JNJ26481585, romidepsin) or in H_2O (VPA).

Cell culture and generation of vorinostat-resistant sublines

An HCT116 human colorectal adenocarcinoma cell line (American Type Culture Collection, ATCC CCL 247; Rockville, Maryland, USA) and a HeLa cervical carcinoma cell line (provided by Dr G. Marra, Institute of Molecular Cancer Research, University of Zurich, Switzerland) were used. Both cell lines were cultured in IMDM-21980 (Invitrogen, Basel, Switzerland) containing 10% fetal calf serum (Oxoid, Basel, Switzerland) at 37°C and in an atmosphere with 5% CO_2 and 95% humidity.

The respective sublines (hereafter designated as HCT116/VOR or HeLa/VOR) were generated by stepwise exposures of the cell lines to increasing concentrations of vorinostat, starting with 2 μmol/l of vorinostat for both cell lines. Briefly, 100 000 cells seeded in cell culture flasks were treated with vorinostat on the next day. Forty-eight hours later, the vorinostat-containing medium was exchanged for vorinostat-free medium, followed by incubation of the cells for another 6 days to allow recovery of the surviving cells and by harvesting the cells by trypsinization. Cells were then transferred into new flasks, expanded to confluence, harvested, and reseeded (100 000) in flasks. On the next day, cells were treated with vorinostat and subjected to medium exchange, recovery, and harvesting as described. This protocol was repeated seven times, and for each cycle the concentration of vorinostat was increased, resulting in a 14-fold total increment for HCT116 (28 μmol/l) and a 35-fold for HeLa cells (70 μmol/l). A further increase in the selection pressure beyond these apparently maximal vorinostat concentrations failed to produce sufficient surviving cells for cell culture expansion.

The principle of selection was the clonal growth in the presence of increasing concentrations of vorinostat, on the basis that cells are altered by chronic vorinostat exposure in a way they acquire new features in an irreversible manner. The growth rates of the cell lines and the respective sublines were calculated from the doubling times from one passage to the subsequent, averaged for a period of 2 months, and compared with one another. The level of resistance was determined immediately after the cells have been expanded to confluency after the last cycle by the clonogenic assay [these are the half maximal inhibitory concentration (IC_{50}) values presented throughout the study] and was periodically monitored by the clonogenic assay against the parental cell line. The level of resistance was maintained over a period of at least 6 months even when cultured in the absence of the selection pressure of vorinostat. When seeded sparsely on culture plates, the cell lines and the sublines formed well-defined individual colonies.

Drug sensitivity assays

Sensitivity of cells to the HDAC inhibitors tested here was assessed by clonogenic and growth inhibition assays. In a typical clonogenic assay setting, 600 cells in medium were plated onto 60-mm cell culture dishes, followed by drug addition on the next day. Cells were cultured for another 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 carried out at least three times in triplicate cultures. The relative colony formation (percentage of clonogenic survival) was plotted against the drug concentrations, and the IC_{50} concentrations were calculated by linear extrapolation. For growth inhibition, 50 000 cells were plated onto 35-mm culture dishes and treated with vorinostat (1, 2, 5, 10 μmol/l). Cells were harvested by trypsinization at multiples of 24 h after treatment and counted using a hematocytometer. In addition, Trypan blue-inclusion was used to monitor drug-induced necrosis. Cells were treated for 24 h with 5, 10, or 20 μmol/l of vorinostat, harvested by trypsinization after another 24-h

incubation, and resuspended in PBS containing 0.2% Trypan blue. Cells were inspected and categorized using a hematocytometer. Vital (Trypan blue-excluding) cells appear bright, and necrotic (Trypan blue-including) cells appear blue under the microscope.

Microscopy

Cells (200 000) were plated onto 35-mm cell culture dishes, grown to 70% confluence, and then grown for another 24 h without (controls) or with 10 µmol/l of vorinostat. Bright-field images were taken using a microscope (Leica DM-IL; Leica Microsystems, Heerbrugg, Switzerland) equipped with a photocamera (Leica DC-300F; Leica Microsystems).

Immunoblot analysis

All the experiments for immunoblot analysis and for cell cycle and apoptosis analyses (described below) were carried out the way that all the cultures were subconfluent at the time of analysis to avoid undesired effects because of, for example, contact inhibition. Immunoblot analysis was used to monitor protein expression and posttranslational modifications of proteins (phosphorylation, acetylation). After the cells have grown to 70% confluence in 60-mm dishes, they were treated with HDAC inhibitors and collected at various time points after treatment, washed in PBS, and lysed for immunoblot analysis performed following standard protocols. Briefly, 20-µg protein was separated using 10 or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by the blotting onto a polyvinylidene difluoride membrane (Amersham Biosciences, Otelfingen, Switzerland), and the detection by the specific primary antibodies and the respective secondary, horseradish peroxidase-conjugated anti-mouse (M15345; Transduction Laboratories, Lexington, Kentucky, USA) or anti-rabbit (7074; Cell Signaling; BioConcept, Allschwil, Switzerland) antibodies. The following primary antibodies were used (Cell Signaling, if not specified otherwise): acetyl-H2A (2576), acetyl-H2B (2575), acetyl-H3 (9671), acetyl-H4 (2594), acetyl-p53 (ab37318; Abcam, Cambridge, UK), acetyl-tubulin (T-6793; Sigma), acetyl-HSP90 (ABIN233817; antibodies-online, Aachen, Germany), MDR (sc-13131; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), multidrug resistance-associated protein 1 (MRP-1; sc-18835; Santa Cruz Biotechnology), HDAC1 (2062), HDAC2 (05-815; Upstate, Lake Placid, New York, USA), HDAC3 (2632), HDAC4 (2072), HDAC5 (2082), HDAC6 (2162), HDAC7 (2862), full-length and cleaved caspase-3 (9662, 9661), full-length and cleaved caspase-7 (9492, 9491), full-length and cleaved PARP-1 (9542, 9541), Bax (2772), Bak (3792), Bid (2002), Bim (4582), Bik (4592), Bok (4521), Bcl-2 (2872), Bcl-xL (2762), survivin (Pro-2233; ProSci Inc., Poway, California, USA), XIAP (2042), Mcl-1 (4572), p21 (2946), p27 (2552), p53 (sc-6243; Santa Cruz Biotechnology), cyclin B1 (4135), cyclin D1 (2926), cyclin D3 (2936), cyclin E2 (4132), thioredoxin (2285), thioredoxin-binding protein 2/VDUP-1 (sc-33099; Santa Cruz Biotechnology or 40-3700; ZYMED, Invitrogen, Carlsbad, California, USA), HSP90 (sc-7947; Santa Cruz Biotechnology), and phospho-HSP27 (2401). Anti-mouse β-actin (A5441; Sigma) or anti-rabbit β-tubulin (2148, Cell Signaling) was used as sample loading controls. Complexes were visualized by enhanced chemiluninescence (Amersham Biosciences) and autoradiography.

HDAC immunoprecipitation and determination of HDAC and HAT activities

Immunoprecipitation of HDAC1 (2062; Cell Signaling), HDAC2 (05–814; Upstate), HDAC3 (05–813; Upstate), and HDAC6 (07–732; Upstate) was done following standard protocols provided by the manufacturers from total cell extracts (lysates) of the vorinostat-sensitive HCT116 cell line and the vorinostat-resistant HCT116/VOR subline using Protein A agarose beads (16–266; Upstate) and the respective immunoprecipitation-qualified antibodies. Nuclear extracts of the sensitive HCT116 cell line and the resistant HCT116/VOR subline were produced using the TransFactor Extraction Kit and following the manufacturer's protocol (631921, Clontech, Takara Bio Europe, Saint-Germain-en-Laye, France). Protein concentration of nuclear and total cell extracts and the samples was determined by the BCA Protein Assay Kit (23227; Pierce, Perbio Science, Lausanne, Switzerland).

The HDAC and HAT enzymatic activities were determined in total or nuclear cell extracts using the colorimetric HDAC activity assay Kit (ab1432, Abcam), the fluorometric HDAC assay Kit (17–356; Upstate), and the fluorescent HAT activity assay kit (56100, Active Motif Europe, Rixensart, Belgium). Measurements were made with a SpectraFluor Plus Reader (Tecan AG, Hombrechtikon, Switzerland). The assays, including all standard assays, were performed according to the protocols provided by the manufacturers. All the activity assays were performed in two independent settings under conditions where neither the sample enzymatic activity, the substate, nor the assay incubation time were rate limiting. Enzymatic activities were standardized, that is, expressed as units or counts per amount of protein.

Cell cycle and apoptosis analyses by flow cytometry

Analyses of cell cycle profiles [propidium iodide (PI) incorporation in the DNA] and apoptosis (TUNEL DNA fragmentation) were performed by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences; Allschwil, Switzerland) with CELLQuest software (BD Biosciences). Data analyses for cell cycle distribution and apoptosis were performed on linear PI histograms using the mathematical software ModFit LT 2.0 (Verity Software House; Topsham, Maine, USA). For sample preparation, synchronized (2 mmol/l hydroxyurea for 14 h) cells were

grown to 70% confluence in 60-mm dishes and treated with 15 μmol/l of vorinostat. At different time points, adherent and floating cells were harvested, washed in PBS, and fixed with ice-cold 70% ethanol. For cell cycle analysis, cells were washed in PBS after removal of the ethanol by centrifugation, stained in 1 ml staining solution (50 μg/ml of PI and 100 U/ml RNAse A in PBS) by incubation at room temperature for 45 min in the dark, and then washed in PBS. For TUNEL apoptosis analysis, following ethanol removal the cells were washed in PBS, resuspended in the TUNEL reaction-mix, and incubated at 37°C for 90 min according to the manufacturer's protocol (In Situ Cell Death Detection Kit, Fluorescein; Roche Diagnostics, Rotkreuz, Switzerland).

Statistical analysis

The mean ± SD values were calculated. A P value of less than 0.05 is considered statistically significant (paired, two-tailed Student's t-test).

Results

Generation of the vorinostat-induced and stable vorinostat-resistant HCT116 sublines

A vorinostat-induced (resistant) subline (hereafter referred to as HCT116/VOR) was generated by stepwise exposures of the parental HCT116 human colorectal adenocarcinoma cell line to increasing concentrations of vorinostat. Clonogenic assay data showed that the vorinostat-induced subline was two-fold resistant ($P < 0.001$) to vorinostat as compared with the corresponding parental HCT116 cell line (Fig. 1a). The respective IC_{50} values were 1.32 ± 0.14 μmol/l for the resistant HCT116/VOR subline and 0.67 ± 0.08 μmol/l for the (sensitive) parental HCT116 cell line. The resistant subline exhibited a growth rate comparable with that of the parental HCT116 cell line, as the doubling times were 22.6 ± 0.9 h for HCT116 and 23.5 ± 1.2 h for HCT116/VOR. Similarly, vorinostat inhibited growth of the vorinostat-resistant HCT116/VOR subline less efficiently than the vorinostat-sensitive HCT116 cell line (Fig. 1b). For instance, at 96 h after treatment the respective values were five-fold (1 μmol/l vorinostat), eight-fold (2 μmol/l), four-fold (5 μmol/l), and 11-fold (10 μmol/l). This HCT116/VOR subline maintained resistance to vorinostat for over 30 passages (at least 6 months) even when cultured in medium without the presence of the selection pressure of vorinostat. This indicates that vorinostat can induce stable, that is nonreversible, resistance in HCT116 tumor cells.

Consistent with the clonogenic and growth inhibition assay data, bright-field microscopy for HCT116 cultures (Fig. 1c and d) showed that vorinostat treatment produced a larger reduction in the number of cells and more dramatic morphological changes (e.g. rounding up) in cultures with the parental cell line than in those with the vorinostat-resistant subline (a large fraction retains its fibroblast-like shape). No morphological differences between the parental and vorinostat-resistant (untreated) control cultures were apparent.

Reduced histone acetylation in the vorinostat-resistant subline

One result of the activity of HDAC inhibitors is the accumulation of acetylated histones. It was determined whether the vorinostat-induced resistance with the HCT116/VOR subline correlated with loss of histone acetylation. Immunoblot data showed that 15 μmol/l of vorinostat produced acetylation of the histones H2A, H2B, H3, and H4 in the parental cell line but not in the resistant subline (Fig. 2a). p53, tubulin, and HSP90 can also be substrates for HDACs. Vorinostat (15 μmol/l) produced increases in acetyl-p53 and acetyl-tubulin, but these increases were similar in the parental HCT116 and the resistant HCT116/VOR cells (Fig. 2a). The levels of acetylated HSP90 were also comparable in both cultures and were not affected by vorinostat. These results indicate that acquired vorinostat resistance correlates with loss of histone acetylation but not with alterations in the levels of acetylated p53, tubulin, and HSP90.

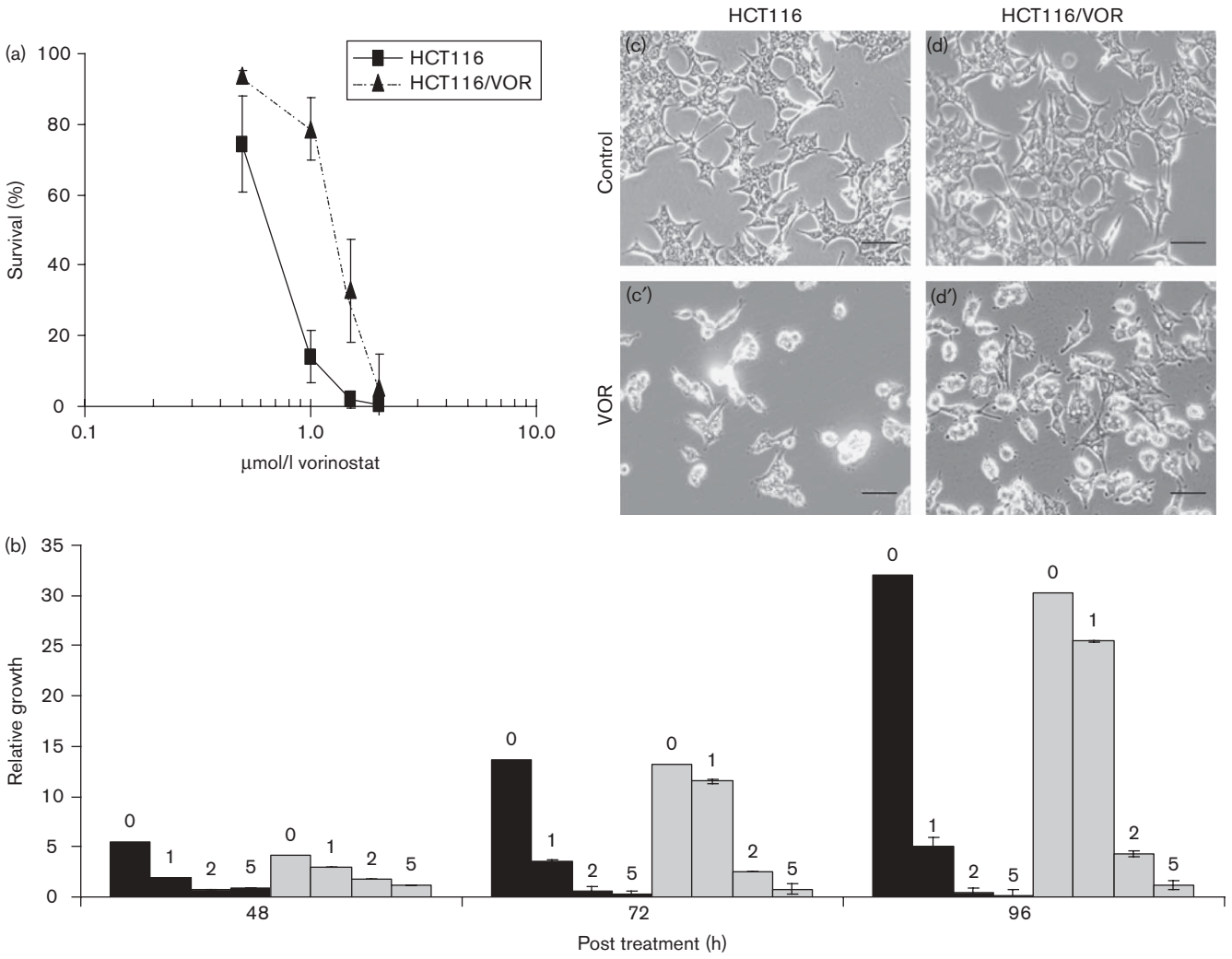
Lack of HDAC overexpression and of MDR expression in the vorinostat-resistant subline

There are several mechanisms that could give rise to reduced accumulation of acetylated histones seen with the vorinostat-resistant subline. These include the increased availability of HDAC enzymes because of the overexpression of one or more HDACs or the reduced availability of intracellular vorinostat because of the expression of multidrug resistance efflux transporters. However, immunoblot analysis showed that higher levels of HDAC1, HDAC2, HDAC3, HDAC5, or HDAC6, were not found in the vorinostat-resistant subline as compared with its sensitive counterpart (Fig. 2b). HDAC4 and HDAC7 were not detected in both cultures. Similarly, neither the MDR nor the MRP-1 transporters were expressed in the parental or resistant cell lines (Fig. 2c). These results indicate that acquired vorinostat resistance does neither correlate with expression of these multidrug resistance transporters nor with overexpression of HDACs.

Histone acetyltransferase and histone deacetylase activities

Resistance could arise through alterations in the enzymatic activities of HATs and HDACs. It was determined whether the resistant subline exhibits HAT and HDAC activities that differ from those of the sensitive cell line; that is whether the HAT activity was lower and/or the HDAC activity was higher in the resistant cells. Nuclear HAT activity was similar in the

Fig. 1



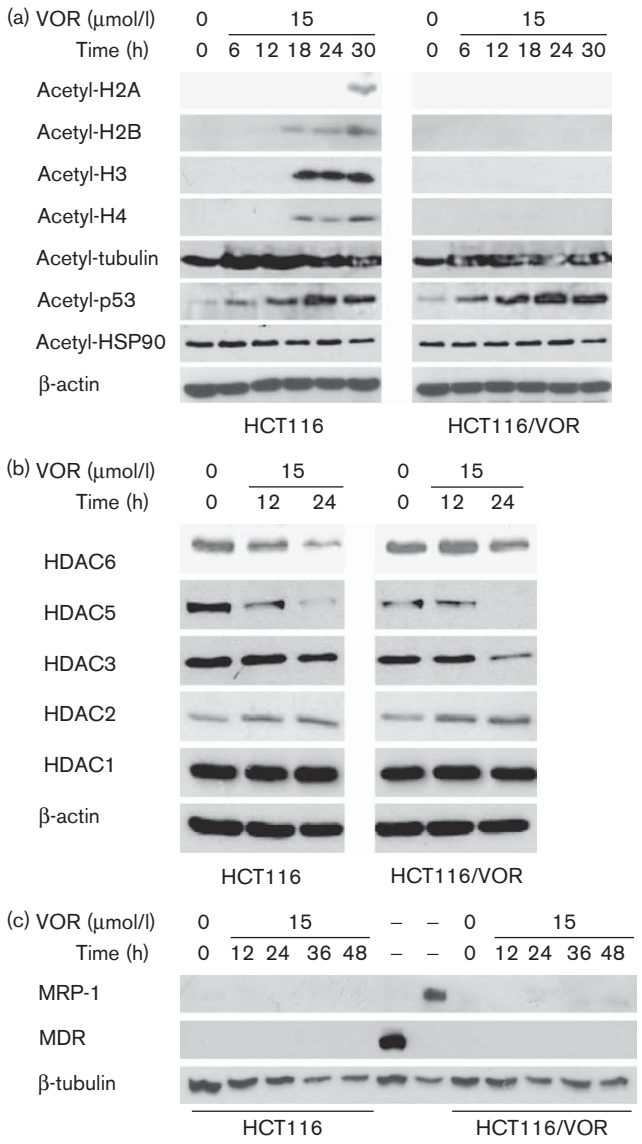
The effect of the histone deacetylase inhibitor vorinostat (VOR) on clonogenic survival (a) and growth inhibition (b) of the HCT116 colon tumor cell line and its respective subline (HCT116/VOR, dashed line) generated by stepwise exposures of the HCT116 cells to increasing concentrations of vorinostat. For the clonogenic assay, cultures were treated with vorinostat for 8 days, and colonies were fixed, stained with Giemsa, and counted (data points are the mean ± SD of at least three independent experiments). For the growth inhibition assay, vorinostat-sensitive cells (black columns) and vorinostat-resistant cells (grey columns) treated with various concentrations of vorinostat (numbers atop the columns, given in micromole/liter) were harvested and counted at multiples of 24-h after treatment. Data (mean ± SD of two independent experiments) are presented as relative growth (ratio of the number of cells at a given time point and number of cells initially plated). Representative bright-field images of vorinostat-sensitive HCT116 (c) and vorinostat-resistant HCT116/VOR (d) control cultures and the respective cultures captured 24 h after treatment with 10 µmol/l vorinostat (c′, d′), a large fraction of vorinostat-resistant cells retain their fibroblast-like shape, whereas the vorinostat-sensitive parental cells show a round-up shape and substantially decrease in number (magnification is × 20 and the scale bar equals 50 µmol/l).

resistant HCT116/VOR subline and in the sensitive HCT116 cell line (Fig. 3a). The enzymatic activities of HDAC1, HDAC2, HDAC3, and HDAC6 in the resistant HCT116/VOR subline and the sensitive HCT116 cell line were comparable and were comparably reduced by vorinostat (Fig. 3b). According to the activities of each individual HDAC tested, the overall HDAC activity was not different in both cultures (Fig. 3c) and was not differentially affected by vorinostat (Fig. 3d) and by VPA (data not shown). These results indicate that the sensitive and the resistant cells are not different with respect to the HAT and HDAC activities.

Loss of apoptosis and G_2/M cell cycle arrest in the vorinostat-resistant subline

The accumulation of acetylated histones in response to HDAC inhibitors causes the decondensing of chromatin, and this facilitates the expression of genes, leading to an arrest of the cell cycle at the G_2/M transition and to apoptosis. Accordingly, it was determined whether the loss of histone acetylation seen in the vorinostat-resistant subline is correlated with the reduced activation of this cell cycle checkpoint and of apoptosis. Quantitative analysis of the primary data derived from the flow cytometry analysis demonstrated that the fraction of cells

Fig. 2



Expression of acetylated histones, acetyl-tubulin, acetyl-p53, and acetyl-HSP90 (a), of histone deacetylase (HDAC) 1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, and HDAC7 (b), and of the multidrug resistance protein (MDR) and the multidrug resistance associated protein (MRP-1) (c) as a function of time after treatment with 15 μmol/l of vorinostat (VOR) in the vorinostat-sensitive (parental) HCT116 cell line and the vorinostat-resistant HCT116/VOR subline. Cells were treated with 15 μmol/l of vorinostat and lysed at the time points indicated. Proteins were separated by polyacrylamide gel electrophoresis analysis and blotted, and complexes were detected by chemiluminescence and autoradiography. Positive control lysates for MRP-1 (A549, sc-2413, Santa Cruz Biotechnology Inc.) and MDR (MES-SA/Dx5A549, sc-2284, Santa Cruz Biotechnology Inc.) were also loaded (center lanes in c). β-actin and β-tubulin served as sample loading controls. Representative of two independent data sets.

accumulated at the G_2/M checkpoint transition was 2.5-fold smaller in the HCT116/VOR subline than in the parental HCT116 cell line after treatment with 15 μmol/l of vorinostat (Fig. 4a).

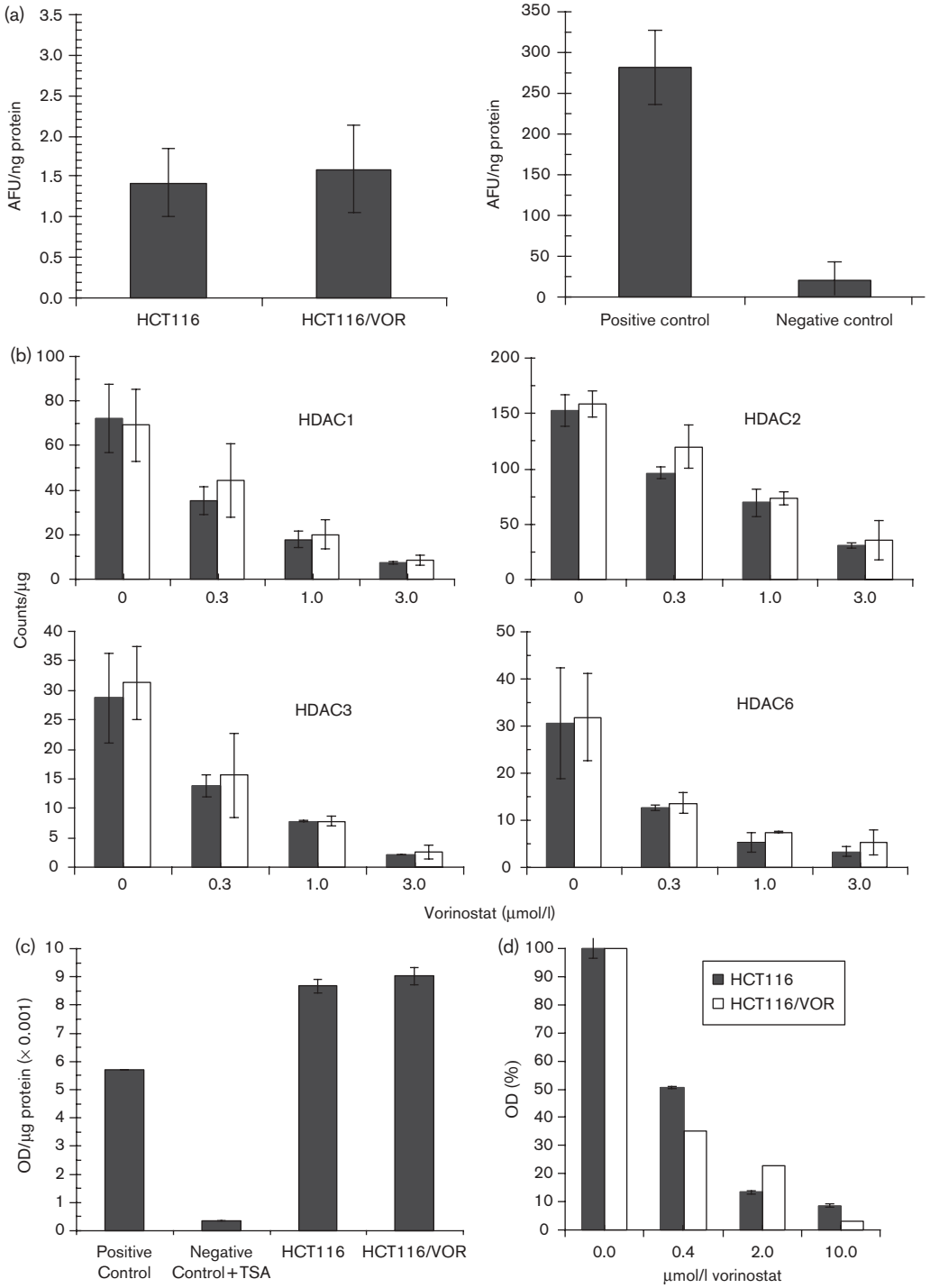
Immunoblot analysis (Fig. 4b) showed that 15 μmol/l of vorinostat failed to produce proteolytic cleavage of the precursors of caspase 3 and caspase 7 and of the PARP-1 precursor in the resistant subline as compared with its sensitive counterpart, and TUNEL analysis revealed that the resistant subline showed a four-fold lower DNA fragmentation (Fig. 4c). The fraction of Trypan blue-including cells was nearly the same in untreated cultures and in cultures treated with 5 or 10 μmol/l of vorinostat (slightly higher with 20 μmol/l); but there is no difference between the vorinostat-sensitive cell line and vorinostat-resistant subline (Fig. 4d), indicating that reduced susceptibility to necrosis does not account for vorinostat-induced resistance. These results indicate that acquired vorinostat resistance in HCT116 cells correlates with both loss of the G_2/M checkpoint and loss of caspase-dependent apoptosis.

Expression of HDAC inhibitor-responsive and cell cycle and apoptosis control proteins

HDAC inhibitors affect the expression of a variety of genes. Among those are proapoptotic Bax and anti-apoptotic Bcl-2, and the cell cycle regulators p21, p53, and the cyclins B1, D1, D3, and E. It was determined whether in the vorinostat-resistant cells the expression of Bax, p21, and p53 was downregulated and that of Bcl-2 and the cyclins was upregulated. Immunoblot data (Fig. 5) showed that expression levels of p21 (and to a lesser extent p27) were increased upon treatment with 15 μmol/l of vorinostat; but this was to a similar extent in the resistant and the sensitive cells. The protein levels of Bax, Bcl-2, and p53 were also similar, Bax and p53 did not change as a function of time after treatment with vorinostat, whereas that of Bcl-2 decreased. Similarly, the expression levels of the cyclins B1, D1, D3, and E2 in the resistant subline did not differ from that in the sensitive cell line; the expression of B1 and D1 was downregulated 24 h after vorinostat treatment, whereas the cyclins D3 and E2 were upregulated. Moreover, the basal expression level of anti-apoptotic survivin, XIAP, and Mcl-1 was not increased and that of proapoptotic Bid, Bim, Bik, and Bok was not decreased in the vorinostat-resistant subline as compared with the vorinostat-sensitive cell line. In both cultures, the protein level of XIAP was decreased, those of Mcl-1 and Bim were increased, and those of Bid, Bik, Bok, and survivin were unchanged in response to vorinostat.

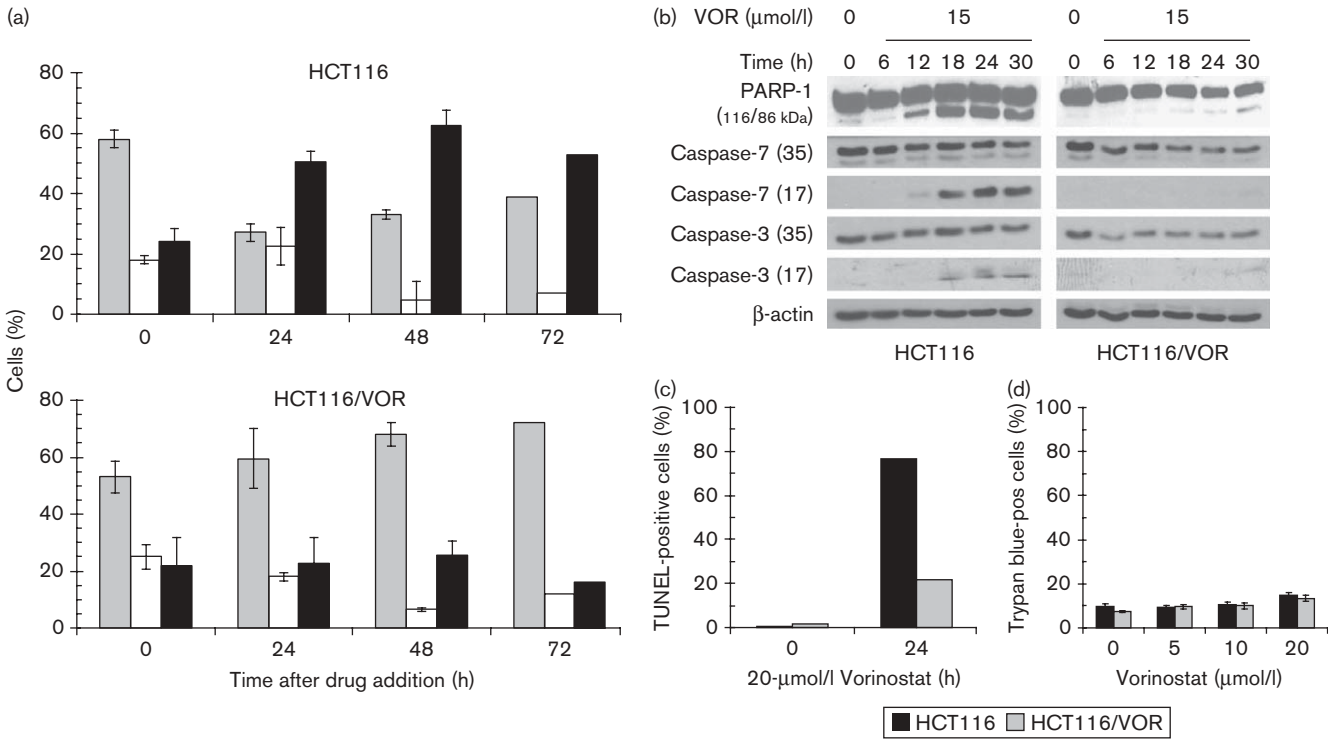
Anti-apoptotic thioredoxin, a protein that scavenges reactive oxygen species, which can be produced by HDAC inhibitors, was not overexpressed in the resistant subline, and thioredoxin-binding protein 2, which downregulates thioredoxin expression, was not detected. The levels of HSP90 and phosphorylated HSP27, two heat shock proteins with cytoprotective functions and reported to be down-regulated by HDAC inhibition, remained unaffected by

Fig. 3



Histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities determined by in-vitro assay kits. (a) HAT activity expressed as arbitrary fluorescence unit (AFU) per nanogram nuclear extract protein from (sensitive) parental HCT116 and resistant HCT116/VOR cells; also shown are the positive (recombinant p300 catalytic domain) and the negative (recombinant p300 catalytic domain plus 15 µmol/l of the HAT activity quencher anacardic acid) assay controls. (b) HDAC activity of individual HDAC1, HDAC2, HDAC3, and HDAC6 expressed as counts per microgram HDAC from HCT116 (black columns) and HCT116/VOR (white columns) extracts. (c) Overall HDAC activity expressed as optical density (OD) per µg of nuclear extract protein from parental HCT116 and resistant HCT116/VOR cells; also shown are the positive (HeLa nuclear extract) and the negative (HeLa nuclear extract plus 20 µmol/l of trichostatin A) assay controls. (d) Overall HDAC activity expressed as the relative OD (percentage of untreated controls) of nuclear extracts from parental HCT116 and resistant HCT116/VOR cells as a function of treatment with vorinostat (VOR). Mean ± SD of two independent experiments. OD, optical density.

Fig. 4



(a) Cell cycle response of the vorinostat-sensitive parental HCT116 cell line and the vorinostat-resistant HCT116/VOR subline as a function of time after treatment with 15 μmol/l of vorinostat (VOR). Quantitative representation (mean ± SD of two independent data sets) of the percentage of cells accumulated in the different phases of the cell cycle: G_1 (gray bars), S-phase (white bars), and G_2/M (black bars). Hydroxyurea-synchronized cells were treated with 15 μmol/l of vorinostat, harvested, fixed, stained with propidium iodide, and analyzed by the flow cytometry. (b, c) Effect of vorinostat on apoptosis in the parental cell line HCT116 and the vorinostat-resistant subline HCT116/VOR. Cells were either treated with vorinostat or 15 μmol/l of vorinostat and analyzed for proteolytic cleavage of the full-length precursors of PARP-1 (116 kDa), of caspase-3 (35 kDa), and of caspase-7 (35 kDa) into their respective cleaved fragments (86, 17, 17 kDa) by immunoblotting (b), or treated with 20 μmol/l of vorinostat and analyzed for TUNEL-DNA fragmentation by flow cytometry (c). (d) Effect of 5, 10, or 20 μmol/l of vorinostat on necrosis in the parental HCT116 cell line and the vorinostat-resistant subline HCT116/VOR. Each data set is a representative data of two independent experiments.

vorinostat in both cultures. The base level of all these proteins was comparable in the vorinostat-sensitive and the vorinostat-resistant cells. These results indicate that resistance to apoptosis and to cell cycle attenuation in the vorinostat-induced subline is not reflected by detectable alterations in the expression of a large number of proteins usually affected by HDAC inhibitors and relevant to the control of these processes.

Cross-resistance to other histone deacetylase inhibitors

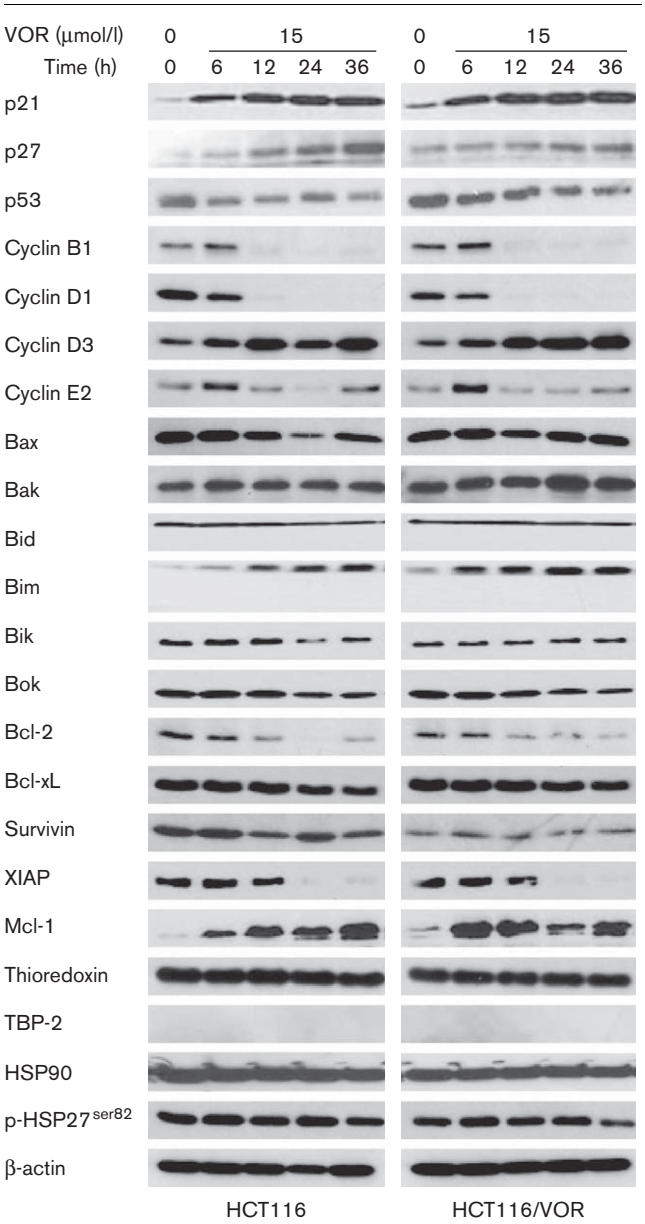
It was determined whether the HCT116/VOR subline was cross-resistant to other HDAC inhibitors. A statistically significant, two-fold to three-fold cross-resistance was found with VPA (Fig. 6a), LBH589 (Fig. 6b), JNJ26481585 (Fig. 6c), but not with MGCD0103 (Fig. 6d) and romidepsin (Fig. 6e). The respective IC_{50} values are presented (Table 1). Accordingly, treatment with VPA, LBH589 or JNJ26481585 did not result in accumulation of acetylated histones and cleaved PARP-1 in the vorinostat-resistant subline, whereas the ability to accumulate acetylated histones and to cleave PARP-1 was maintained in response to treatment with MGCD0103 and romidepsin.

Acetylated tubulin was essentially expressed to comparable levels in the vorinostat-resistant and the vorinostat-sensitive cells. It also looks as though LBH589 and JNJ26481585, in contrast to VPA, MGCD0103, and romidepsin produced an increase in acetyl-tubulin to some extent. Acetyl-HSP90 was present in both cultures to the same extent and was not affected by treatment with each one of the HDAC inhibitors. These results indicate that acquired resistance to vorinostat is accompanied by cross-resistance to at least some HDAC inhibitors, and that cross-resistance does not go along with alterations in the level of acetylated tubulin and acetylated HSP90.

No acquisition of resistance by vorinostat in HeLa cells

To determine whether resistance induction by vorinostat could also be seen with HeLa cells, the same protocol was

Fig. 5



Expression of cell cycle and apoptosis control proteins and other proteins possibly affected by histone deacetylase inhibitors as a function of time after treatment with 15 μmol/l of vorinostat (VOR) in vorinostat-sensitive parental (HCT116) and vorinostat-resistant (HCT116/VOR) cells. Cells were treated and lysed at the time points indicated. Proteins were separated by polyacrylamide gel electrophoresis analysis and immunoblotted, and complexes were detected by chemiluminescence and autoradiography. β-actin was used as the sample loading control. Data are representatives of at least two independent data sets. TBP, thioredoxin-binding protein 2.

applied to this tumor cell line. However, this protocol did not produce a vorinostat-resistant HeLa/VOR subline and neither loss of accumulation of acetylated histones nor loss of apoptosis were observed in the subline (data not shown). This indicates that HeLa cells are not susceptible to resistance acquisition by vorinostat.

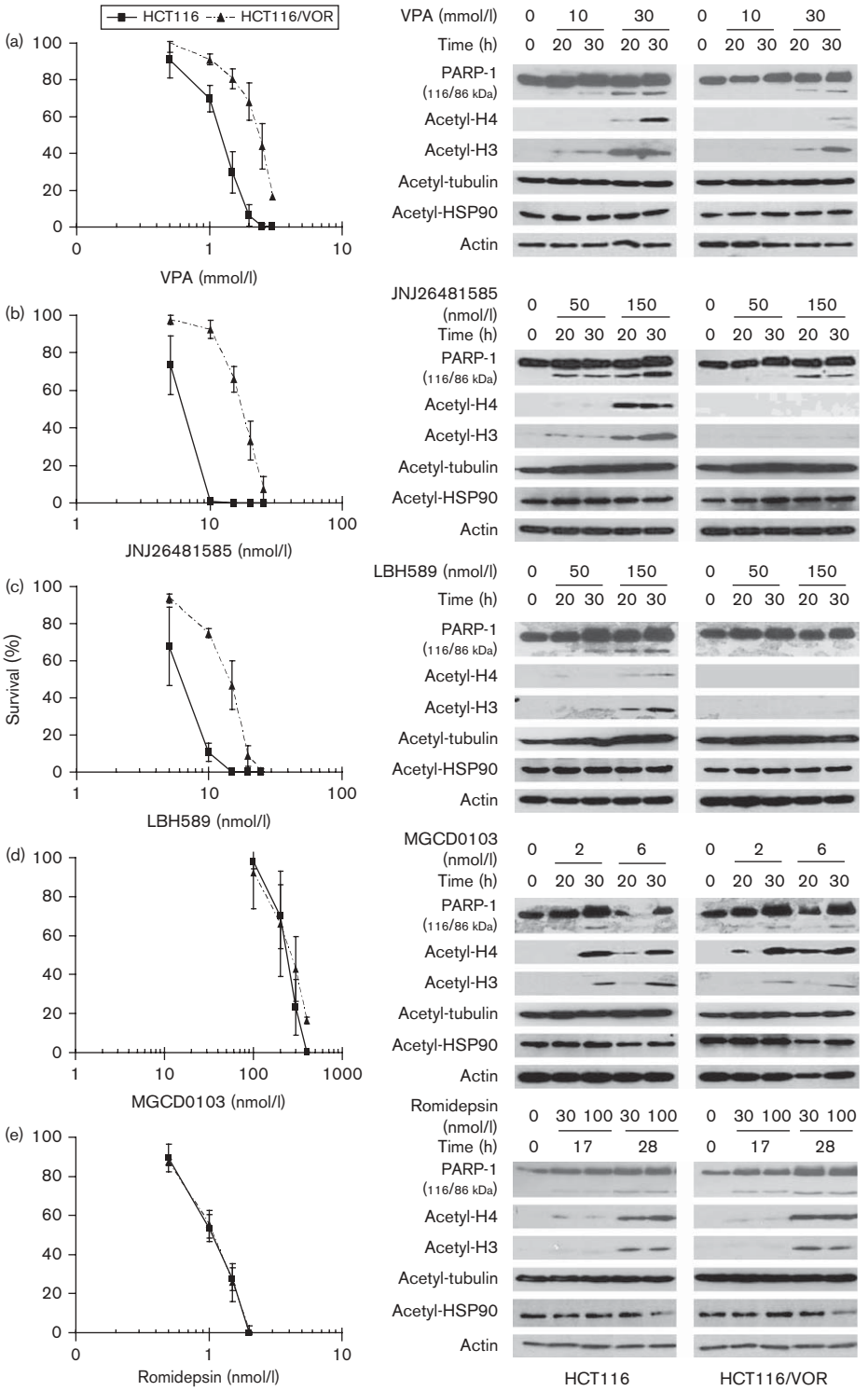
Discussion

The antineoplastic activity of HDAC inhibitors is an unquestionable property of these compounds; but recent studies have shed some light on another aspect of HDAC inhibitors, namely their association with resistance and their potential to cause resistance acquisition in tumor cells [9–12]. From this study with vorinostat the following conclusions may be drawn. First, expanding on a previous study [12], this one provides further evidence that vorinostat has the potential to cause stable and MDR-independent HDAC inhibitor resistance *in vitro*. Second, this acquired resistance clearly correlates with the losses of histone acetylation, cell cycle checkpoint activation, and apoptosis susceptibility. Third, this resistance cannot be explained by altered expression of selected HDACs, by altered HDAC and HAT activities, and by failure to induce p21 expression. Fourth, cross-resistance was found to VPA and hydroxamate-class HDAC inhibitors, but not to benzamide-class and cyclic peptide-class HDAC inhibitors. Fifth, using the parental HCT116 colorectal adenocarcinoma cell line, this study rules out the possibility that vorinostat resistance acquisition arises as a consequence of the presence of extra chromosomes in the chromosome-supplemented HCT116 cell lines that have been used in the previous study [12].

The potential of HDAC inhibitors to cause resistance has recently become apparent. Two studies have shown that the HDAC inhibitor romidepsin induced a reversible, up to 10 000-fold resistance in a variety of tumor cells because of the inducible and transient expressions of MDR and MRP-1 [10,11]. However, the vorinostat-induced resistance described here differs from that with romidepsin in several ways: it is nonreversible (i.e. was maintained even in the absence of vorinostat in the culture medium), moderate (two-fold), and cannot be explained by the efflux of vorinostat through the multidrug resistance transporters MDR and MRP-1. The latter is in line with the observations that MDR-mediated resistance is usually much greater than two-fold. Our results thus suggest that resistance acquisition by vorinostat and by romidepsin are based on the different mechanisms. Apparently, there is a selection pressure for vorinostat, beyond which cells are no longer vital. The observation that this virtually maximal vorinostat concentration (28 μmol/l) is substantially higher than the IC_{50} (1.3 μmol/l) for the vorinostat-resistant HCT116/VOR subline may mean that there is an at least partial reversal of vorinostat resistance after removal of the vorinostat selection pressure in HCT116 cells. A complete reversal (from 70 μmol/l selection pressure to IC_{50} 1.3 μmol/l) may be suggested for the HeLa cells.

Acetylation of histones is one hallmark of HDAC inhibitor-induced cellular responses. Accordingly, acquired

Fig. 6



The effect of the histone deacetylase inhibitors valproic acid (VPA) (a), JNJ26481585 (b), LBH589 (c), MGCD0103 (d), and romidepsin (e) on the clonogenic survival (left panel) and on the proteolytic cleavage of PARP-1, and on the acetylation of the histones H3 and H4 (right panel) and the nonhistone proteins tubulin and HSP90 in the (sensitive) parental HCT116 cell line and the resistant HCT116/VOR subline. Data points are the mean ± SD of at least three independent experiments (clonogenic assay). Representative of two independent data sets (immunoblotting).

Table 1 The IC_{50} values representing cross-resistance of HCT116 and HCT116/VOR cells determined by the clonogenic assay

	VPA (mmol/l)	JNJ26481585 (nmol/l)	LBH589 (nmol/l)	MGCD0103 (nmol/l)	Romidepsin (nmol/l)
HCT116	1.25 ± 0.11	6.00 ± 1.00	6.27 ± 1.32	241 ± 33	1.07 ± 0.10
HCT116/VOR	2.40 ± 0.13	17.30 ± 0.95	14.4 ± 1.10	252 ± 52	1.10 ± 0.10
Fold difference[a]	1.92	2.88	2.30	1.04	1.03
P values ($n=3$)	< 0.001	< 0.001	< 0.002	0.782	0.716

IC_{50}, half maximal inhibitory concentration; VPA, valproic acid.
[a]Ratio of IC_{50} values of HCT116/VOR and HCT116.

vorinostat resistance correlated with failure to acetylate the four histones. Reduced intracellular availability of vorinostat because of alterations in efflux or influx transporters is unlikely, as MDR transporters are not involved and there is no evidence that vorinostat is taken up by processes other than by diffusion. In addition, failure to acetylate histones may also arise from overexpressed or overactivated HDACs or from reduced HAT activity [15], but no lower HAT activity and no higher activity of individual HDACs (HDAC1, HDAC2, HDAC3, HDAC6) and of overall HDAC activity were found in the vorinostat-resistant subline. A previous study has shown that a truncating mutation in the *HDAC2* gene confers resistance to the HDAC inhibitor trichostatin A [16].

In addition to histones, nonhistone proteins such as tubulin, p53, and HSP90 are also acetylated as a result of HDAC inhibitors [17–19]. Acetylated tubulin associates with tumor growth inhibition and acetylated p53 promotes p53-dependent gene transcription [19,20]. Acetylated HSP90 is inactive and seems to promote apoptosis [21]. It was reasoned that the vorinostat-resistant cells have lower levels of acetylated tubulin, p53, or HSP90. However, vorinostat induced increases in acetylated p53 and tubulin in both the sensitive and resistant cells to a similar extent, and the levels of acetylated HSP90 remained unchanged. This indicates that vorinostat resistance was not accompanied by reduced acetylation of p53, tubulin, and HSP90.

The therapeutic effect of vorinostat is based on its ability to produce cell cycle arrest and apoptotic cell death. Vorinostat targets the G_2/M checkpoint in HCT116 tumor cells and the antitumor effect of vorinostat may be because of induction of polyploidy [22]. This study shows that the activation of the G_2/M checkpoint was run over and the induction of caspase-dependent apoptosis was markedly reduced in the vorinostat-resistant subline. In the vorinostat-resistant subline, the reduced susceptibility to apoptosis seems to be linked to the reduced levels of histone acetylation. It was occasionally observed that vorinostat concentrations, which induced (reduced with respect to the sensitive counterpart) apoptosis in the vorinostat-resistant subline, also showed (reduced) accumulation of acetylated histones, but accumulation of acetylated histones without apoptosis was never observed. It seems that, at least for vorinostat, apoptosis does not occur without histone acetylation, meaning that histone acetylation is required for vorinostat-induced apoptosis.

It was examined whether alterations in the expression of a number of cell cycle-relevant genes account for the observed loss of the G_2/M checkpoint activation. However, alterations in expression of cell cycle-relevant genes were not observed; p21 expression, which plays a key role in the cytostatic effect of vorinostat [22], and p27 expression were observed in both the resistant and the parental cell line. *Cyclin D1* is an HDAC inhibitor-responsive gene and is downregulated by vorinostat in tumor cells [23]. Indeed, vorinostat produced downregulation of the cyclins B1 and D1, but this was also seen in the resistant cells. Similarly, the HDAC inhibitor-responsive cyclins D3 and E2 were also upregulated in both cultures.

Reduced apoptosis in the vorinostat-resistant cells may be because of loss of proapoptotic Bax, Bak, Bid, Bim, Bik, and Bok; to upregulation of anti-apoptotic Bcl-2, Bcl-xL [24], Mcl-1, XIAP, and survivin; or to altered expression of HSP90 and phosphorylated HSP27, two heat shock proteins downregulated by HDAC inhibitors [25,26]. This was, however, not the case, as the expression of these proteins was similar in the vorinostat-resistant and in the parental (vorinostat-sensitive) cells. Similarly, acquired vorinostat resistance cannot be explained by the increased thioredoxin levels in these cells. Thioredoxin scavenges reactive oxygen species produced in response to vorinostat and MS-275 [7], and this results in the inhibition of oxidative stress-induced cell death [27].

An important finding from this study is that the vorinostat-resistant cells show cross-resistance to other 'first-generation' and 'second-generation' HDAC inhibitors, associated with failure to acetylate histones and to apoptose in response to these HDAC inhibitors. This is not only within a particular class of HDAC inhibitors (the hydroxamates LBH589, JNJ26481585, TSA) but also among members of different classes of HDAC inhibitors (e.g. the aliphatic acid, VPA). A particularly striking observation was that the vorinostat-resistant cells retained sensitivity and susceptibility to histone acetylation and apoptosis to the HDAC inhibitors MGCD0103 and romidepsin. The benzamide head group in MGCD0103 and the specific conversion of the cyclic

peptide romidepsin into its active form [28] seems to make the difference, as opposed to the hydroxamic acid (vorinostat, LBH589, JNJ26481585, TSA) or the acid (VPA) head groups. The absence of cross-resistance to these two HDAC inhibitors and the previously reported absence of cross-resistance to 'classic' (non-HDAC inhibitor-type) anticancer agents in the vorinostat-resistant cells may be of clinical interest [12]. Cross-resistance is not in accord with lower levels of acetylated tubulin nor with reduced levels of acetylated HSP90. The putative increase in acetylated tubulin in response to LBH589 and JNJ26481585 (in contrast to VPA, MGCD0103, and romidepsin) seems to be consistent with the notion that these two HDAC inhibitors are inhibitors of the tubulin deacetylase site of HDAC6. In addition, the finding that HDAC6 activity is comparable in the vorinostat-resistant and the vorinostat-sensitive cells is consistent with the comparable level of acetylated tubulin in both cultures.

It is of note that the vorinostat concentrations required to select for vorinostat resistance *in vitro* are in the range of those measured in the serum of patients treated with therapeutic doses in phase I/II studies [29]. This may mean that the generation of vorinostat-resistant cells might also occur in patients. It is somewhat surprising that in the experimental setting used in this study, the typical responses to vorinostat, that is, acetylation of histones and induction of cell cycle arrest and apoptosis, are observed at relatively high vorinostat concentrations (15 μmol/l), that is, higher than those required to abrogate clonogenicity (IC_{99} around 2 μmol/l). In addition, the acetylation of histones by vorinostat was detected at later time points than usual. It is of note that the vorinostat-induced responses were essentially also observed with 5 μmol/l but to a lesser extent.

Despite the clear-cut correlation between acquired vorinostat resistance and the loss of some molecular and cellular responses typically seen with HDAC inhibitors, the molecular basis of this resistance is still not understood. For instance, it is unclear why the effects of vorinostat on acetylation of nonhistone proteins (e.g. tubulin, p53, HSP90) and in particular on the expression of HDAC inhibitor-responsive genes assessed here (e.g. p21) are similar in the parental (sensitive) and the resistant cells. It is also unclear how failure to histone acetylation arises, whether this failure arise from other HDAC inhibitor sequestration or detoxification systems or from impaired transport of HDAC inhibitors into the nucleus, whether DNA methylation, a biochemical process cooperating with histone (de-)acetylation and involved in gene silencing [30], is altered at promoter sites, what may be the role of cellular polyamines [31], and why resistance acquisition was not observed with the HeLa cervical cancer cells. Appreciating the complexity of the molecular effects of HDAC inhibitors and the mechanisms of drug resistance, it is likely that not one particular mechanism but a multifactorial alteration of different cell-regulating pathways underlies vorinostat resistance. This mechanism probably arises because of the epigenetic targeting by the HDAC inhibitors.

Taken together, this study provides further evidence for the potential of vorinostat to cause acquisition of HDAC inhibitor resistance in HCT116 tumor cells. This acquired HDAC inhibitor resistance clearly correlates with the loss of important molecular responses typically seen with HDAC inhibitors and being responsible for the cytotoxic effect of these compounds.

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